Immunological and Metabolomics Tools for Health Assessment of Farmed New Zealand Chinook Salmon (*Oncorhynchus tshawytscha*)

Ronald Lulijwa

A thesis submitted to
Auckland University of Technology
in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

2021

Faculty of Health and Environmental Sciences

AQUACULTURE BIOTECHNOLOGY
RESEARCH GROUP

Thesis Abstract

New Zealand's Chinook salmon (*Oncorhynchus tshawytscha*) farming started in 1976 and has developed to become the number one farmed finfish in the country. New Zealand is the leading global producer and supplier of farmed *O. tshawytscha*. New Zealand's *O. tshawytscha* production remains free from diseases that have devastated global salmon farming (Diggles, 2016). However, the recent emergence of New Zealand rickettsia-like organism (NZ-RLO) (Brosnahan et al., 2017), continued industry growth, and climate change, necessitates the development of health assessment tools. The aim of this thesis was to develop immunological and metabolomic tools for farmed *O. tshawytscha* health assessment.

Farmed *O. tshawytscha* peripheral blood was characterised for cellular composition and a micro-volume blood technique was developed for isolation of fish peripheral blood mononuclear cells (PBMCs) using Lymphoprep. Differential cell counts identified five cell types including erythrocytes, lymphocytes, thrombocytes, monocytes and unquantifiable neutrophils, important in future health assessments. Isolated PBMCs enable field on-farm sampling for longitudinal studies and allow *in vitro* immunological assessments. Findings provided the possibility to make fish health assessments in the field without fish euthanisation.

The developed micro-volume blood technique was used to isolate *O. tshawytscha* PBMCs. These PBMCs were used to model the functional and targeted immune cytokine responses to Gram-negative bacterial lipopolysaccharide (LPS) from *Escherichia coli in vitro*. Bacterial LPS stimulated biphasic reactive oxygen species (ROS) production enhanced by interferon (*ifn*) inducible cytokines, and phagocytosis. LPS also upregulated pro-inflammatory interferon gamma (*ifnγ*), tumour necrosis factor alpha (*tnf-α*), and anti-inflammatory interleukin-10 (*il-10*) 24 h post-stimulus. This provided the first report of LPS induced immunomodulation in *O. tshawytscha in vitro.* The results have high application potential in modelling response mechanisms to emerging NZ-RLO pathogenesis.

The response mechanisms of *O. tshawytscha* to polyinosinic: polycytidylic acid [poly (I:C)] were investigated 24 h post-*in vivo* stimulation. The most striking results were observed at the metabolomic level. Poly (I:C) upregulated metabolites involved in branched-chain amino acid (BCAA)/glutathione and transsulphuration pathways and phospholipid metabolism, while those involved in energy metabolism were downregulated. At the molecular level, poly (I:C) enhanced antiviral *ifnγ* in head kidney (HK) and Mx1 protein in head kidney (HK), spleen (SP) and red blood cells (RBCs). Findings provide insights into poly (I:C) induced immune-related biomarkers at metabolic and molecular levels important in future investigations.

The effects of poly (I:C) in vivo on *O. tshawytscha* haematology, innate immunity, serum and liver metabolite profiles, HK, and SP cytokine transcript expression, over a 5-day period post-injection were studied. Important responses included enhanced neutrophil counts and PBMC ROS production. Metabolically, poly (I:C) upregulated liver and serum metabolites involved in BCAA

at day one and returned to normal by day five, while metabolites involved in glycolysis were persistently depleted. Metabolic results suggest that poly (I:C) induced response mechanisms similar to those observed in viral-infected fish, where the host metabolome is hijacked to favour viral replication. At the molecular level, poly (I:C) promoted antiviral *ifnγ* and Mx1, and anti-inflammatory *il-10* in fish lymphoid organs, depict *O. tshawytscha* immune defence against infection. Results may act as a primer for developing amelioration strategies against viral infections in aquaculture.

Finally, *O. tshawytscha* were subjected to a three-month thermal stress challenge (17°C vs 19°C-20°C) to identify blood biomarkers of thermal tolerance and growth performance (weight loss vs weight gain). Independent of growth performance, thermal stress induced leucocyte apoptosis, minor immune responses, and disturbed plasma osmoregulation via reduced Na^+/K^+-ATPase activity. Irrespective of culture temperature, fish that lost weight were characterised by several biomarker alterations in cellular haematology and plasma clinical chemistries suggestive of suppressed feed intake. Findings provide insights into physiological and growth effects of thermal stress on *O. tshawytscha*, useful in selective breeding strategies.

Overall, depending on resource availability, this thesis has demonstrated the usage of classical haematology, novel flow cytometry, molecular and metabolomic tools in farmed *O. tshawytscha* health assessment. The thesis thus recommends an integrated approach of classical assays with the more recent flow cytometry and metabolomics approaches to promote holistic farmed teleost health assessments.

Table of Contents

List of Tables

List of Figures

Attestation of Authorship

"I hereby declare that this thesis is my own work and that, to the best of my Knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements) nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or institution of higher learning".

Lulijwa Ronald

19th April 2021

Co-Authored Works

Chapter	Authors	Contribution	Sum	Signature
2 – Review 1	Ronald Lulijwa	▪Design and structure ▪Literature review ▪Writing	85%	
	Andrea C. Alfaro	▪Review/edit	5%	
	Tim Young	▪Structure ▪Review/edit	10%	
2 – Review 2	Ronald Lulijwa	▪Design and structure ▪Literature review ▪Writing	90%	
	Andrea C. Alfaro	▪Review/edit	3%	
	Fabrice Merien	▪Review/edit ▪Structure	2%	
	Jill Meyer	▪Review/edit	2%	JHMeyer
	Tim Young	▪Review/edit	3%	
4 – Case study 1	Ronald Lulijwa	▪Experimental design ▪Sampling ▪Sample analysis ▪Microscopy ▪Data processing and analysis ▪Writing	85%	
	Andrea C. Alfaro	▪Experimental design ▪Review/edit	4%	
	Fabrice Merien	▪Review/edit	1%	
	Mark Burdass	▪Sampling ▪Review/edit	2%	
	Tim young	▪Experimental design ▪Review/edit	4%	
	Jill Meyer	▪Microscopy ▪Review/edit	2%	JHMeyer
	Van Thao Nguyen	▪Review/edit	1%	
	Caroline Trembath	▪Review/edit	1%	
5 – Case study 2	Ronald Lulijwa	▪Experimental design ▪Sampling ▪Sample analysis ▪Data processing and analysis Writing	85%	
	Andrea C. Alfaro	▪Experimental design Review/edit	4%	
	Fabrice Merien	Review/edit	2%	
	Mark Burdass	Review/edit	2%	
	Jill Meyer	Review/edit	2%	JHMeyer
	Leonie Venter	▪Experimental design ▪Sampling Review/edit	2%	
	Tim Young	▪Experimental design Review/edit	3%	
6 – Case study 3	Ronald Lulijwa	▪Experimental design ▪Sampling	85%	

		▪Sample analysis ▪Data processing and analysis ▪Writing		
	Andrea C. Alfaro	▪Experimental design ▪Review/edit	4%	
	Fabrice Merien	▪Review/edit ▪Sample analysis	2%	
	Mark Burdass	▪Review/edit	2%	
	Jill Meyer	▪Microscopy ▪Review/edit	2%	JHMeyer
	Leonie Venter	▪Experimental design ▪Sampling ▪Review/edit	2%	
	Tim Young	▪Experimental design ▪Review/edit	3%	
7 – Case study 4	Ronald Lulijwa	▪Experimental design ▪Sampling ▪Sample analysis ▪Data processing and analysis ▪Writing	85%	
	Andrea C. Alfaro	▪Experimental design ▪Review/edit	4%	
	Fabrice Merien	▪Review/edit ▪Sample analysis	2%	
	Mark Burdass	▪Review/edit	2%	
	Jill Meyer	▪Microscopy ▪Review/edit	2%	JHMeyer
	Leonie Venter	▪Experimental design ▪Sampling ▪Review/edit	2%	
	Tim Young	▪Experimental design ▪Review/edit	3%	
8 – Case study 5	Ronald Lulijwa	▪Experimental design ▪Sampling ▪Sample analysis ▪Data processing and analysis ▪Writing	80%	
	Tim Young	▪Experimental design ▪Sampling ▪Sample analysis ▪Data processing and analysis ▪Writing	10%	
	Nataly J. Delorme	▪Sampling ▪Review/edit	2%	
	Seumas P. Walker	▪Experimental design ▪Review/edit	2%	
	Jane E. Symonds	▪Experimental design ▪Review/edit	4%	
	Andrea C. Alfaro	▪Review/edit	2%	

Acknowledgements

The work presented in this thesis was done at the Aquaculture Biotechnology Research Group (ABRG), School of Science, Faculty of Health and Environmental Sciences, Auckland University of Technology (AUT), New Zealand, in collaboration with Cawthron Aquaculture Park (CAP), Nelson Marlborough Institute of Technology (NMIT), Glenduan based Aquaculture facility in Nelson, New Zealand, under the supervision of Professor Andrea C. Alfaro and Professor Fabrice Merien. The study was approved by NMIT Animal Ethics Committee (NMIT-AEC-2017-NMIT-01), (NMIT-AEC-2018-NMIT-03) and frozen samples provided by CAP, required no ethics. No live fish were held at AUT for this work.

Firstly, this work was made possible by the New Zealand AID doctoral scholarships to Ronald Lulijwa through the Ministry of Foreign Affairs and Trade (MFAT), thank you very much for this life changing opportunity. In addition, the scholarship team at AUT played a crucial role in facilitating success during this study and I remain highly indebted to Margaret Leniston, Petrina Hibben, Sandelyn Agnes Su, Ruth O'Sullivan and Sacha Pointon. Thank you very much for everything you offered.

Secondly, I am thankful to my primary supervisor Professor Andrea C. Alfaro for giving me the opportunity to undertake a PhD under her guidance. I greatly appreciate the opportunity you offered, the technical support and skilful guidance during this PhD journey, you are such an amazing supervisor. In addition, my supervisor also offered financial support that greatly contributed to successful completion of laboratory work.

I also thank Leanne D Jones of NMIT for her generous support in maintaining the study animals during laboratory sessions at the Glenduan Aquaculture Park in Nelson, New Zealand, Kathryn Hattersley at NMIT medical laboratory and the Cawthron Institute's Dr. Seumas Walker for the technical support during this study. In addition, I am thankful to the technical support at AUT, particularly Meena Patel, the laboratory manager, and logical staff at the School of Applied Sciences.

Special thanks also go to the ABRG's research family specifically Drs. Tim Young, Leonie Venter and Ujwal Bastakonti for the numerous discussions and meetings, through which this work was greatly improved. Thank you very much for also being there for me every time I had questions following on my head. You have been very important for me during this PhD, and I will miss having you as a part of my daily life.

I am grateful to my closest family (Mum, brothers, sisters, and in-laws), all friends for support, love, and help through good and difficult times. Thank you all. Thank you my dear Steadia Kyosiimire, for being there for me with your support, patience, and love, not forgetting my sons Elisha Lulijwa, David Lulijwa and Darius Willy Lulijwa for braving the sacrifices, that enabled me to achieve this PhD.

Ronald Lulijwa

April 2021

1 Chapter 1: Introduction and Thesis Structure

The researcher preparing to withdraw blood samples from New Zealand farmed Chinook salmon (*Oncorhynchus tshawytscha*), the model species used in this thesis.

1.1 Introduction

1.2 Aquaculture and salmonid farming

Aquaculture is the controlled production of aquatic plants and animals, and it is one of the fastest growing food production sectors worldwide (FAO, 2014). Global aquaculture production has grown at an average annual rate of 5.3% in the last 17 years, employed over 20 million people in 2018, and drove per capita consumption to a record of 20.3 kg in 2016. Having supplied 46% of fish worldwide in 2018, aquaculture will probably become the primary source of fish by 2050 (FAO, 2018c, 2020b). In 2018, farmed aquatic animal production stood at 82 million tons, of which finfish contributed 66%, molluscs 22%, crustaceans 11%, and 1% composed of minor species. The FAO 2018 figures estimated 622 aquatic species to be farmed in inland, coastal, and marine aquaculture systems worldwide (FAO, 2020b).

Atlantic salmon (*Salmo salar*) and rainbow trout (*Onchorhycus mykiss*) are the most farmed salmonids. Of the 54 million tons of finfish produced in 2018, *S. salar* and *O. mykiss* contributed a combined 6.1% by volume (FAO, 2020b). Despite the small contribution by volume, salmonids are the second most valuable species after shrimp (Asche et al., 2018). But because of geographical, physicochemical, and biological differences, commercial farming only takes place in Norway, Chile, Scotland, Canada, Faroe Islands, Ireland, Iceland, USA, South Island of New Zealand, and Tasmania. Given its favourable farming traits, *S. salar*, is one of the most profitable, innovated, and successful salmonid species with Norway, Chile, Scotland, Canada, Faroe Island, and Ireland leading production (Bachmann-Vargas et al., 2021; Iversen et al., 2020; MOWI, 2020). The major *O. mykiss* producers are Norway, Chile, Scotland, and Faroe Islands (MOWI, 2020; Scotland, 2019). New Zealand leads in global Chinook salmon (*Oncorhynchus tshawytscha*) production, with the USA's Alaska, Canada, and Chile as its challengers (FAO, 2010; MOWI, 2020). Due to the growing demand, limited sea space, diseases, environmental issues and stringent regulatory requirements, commercial investments in land-based *S. salar* and *O. mykiss* farming are increasing in the USA, Norway, Denmark, Canada, Iceland, Switzerland, Poland, Japan, China, UAE, South Korea, and South Africa (Bjorndal & Tusvik, 2019; Gibson, 2021; Owen, 2019; The Fish Site, 2021). New Zealand supplies almost 90% of farmed *O. tshawytscha* , while Chile produces 95% of coho salmon (*Oncorhynchus kisutch*) (Bachmann-Vargas et al., 2021; Casanovas et al., 2021).

As the aquaculture industry strives to expand to fill the gap in capture fishery, many challenges are plaguing the industry. These include climate change driven marine heatwaves, rising seawater temperatures, stressful extreme weather conditions, ocean events and pathogens (Cheung & Frölicher, 2020; Hvas et al., 2020; Wade et al., 2019). For example, in Chile, the infectious salmon anaemia virus (ISAV) decimated the industry over a decade ago and the algal bloom kills in 2016 reduced production (Bachmann-Vargas et al., 2021; MOWI, 2020). In Europe, the Norwegian *S. salar* industry registered 16%, 19% and 14% mortalities in 2015, 2016

and 2017 respectively due to sea lice and stressful weather events (Moe et al., 2017; Norwegian Veterinary Institute, 2019). Due to increased sea lice challenges in Norway, production costs have increased while successful ISAV management in Chile has improved production efficiency, thanks to better biosecurity and ecosystem approach (Bachmann-Vargas et al., 2021; Iversen et al., 2019). In New Zealand, rickettsia-like organism (NZ-RLO2) and increased water temperature caused summer mortalities in 2015 in the Marlborough sounds (Brosnahan et al., 2017; Brosnahan et al., 2019c). This situation has been exacerbated by climate change influenced rise in sea surface temperatures (SSTs) (Salinger et al., 2020; Salinger et al., 2019). These challenges necessitate the development of industry health assessment tools.

1.3 The New Zealand aquaculture industry

New Zealand's commercial aquaculture began over 60 years ago with intertidal production of endemic rock oysters (*Saccostrea commercialis*) which was later replaced by the fast growing exotic pacific oysters (*Crassostrea gigas*) in the 1970s in the Northland (FAO, 2007). Commercial farming of New Zealand's key aquaculture product, the Greenshell™ mussel (*Perna canaliculus*), began in the 1960s after the collapse of dredge fisheries in the Hauraki Gulf in the Northland and the South Island Marlborough Sounds (Dawber, 2004). Despite the lack of endemic salmonids in New Zealand, *O. tshawytscha* populations were established in the South Island and commercial farming began in the 1970s (Unwin et al., 1989). Because of its wild success, *O. tshawytscha* is the only commercially farmed finfish and the second most important aquaculture product in New Zealand. Although *O. tshawytscha* is produced on a small scale, it is highly valued and thus the biggest challenger to *S. salar* production (MOWI, 2020). This is due to *O. tshawytscha*'s characteristic large fillets of premium texture, taste and nutritional value (Stenton-Dozey et al., 2020).

Although still small, the New Zealand aquaculture sector provides rural employment, business opportunities, and export revenues. For example, aquaculture employed 3000 people in 2018 (Stenton-Dozey et al., 2020). Due to the economic importance of aquaculture, the government's target is to reach NZ$ 3 billion by 2035 in annual sales (MPI, 2019). Thus, given the economic importance of *O. tshawytscha*, it was chosen for the development of health assessment tools to safeguard against future pathogens.

1.3.1 The genesis of *O. tshawytscha* production in New Zealand

Early attempts to transplant several salmonids before the 1930s were futile in Brazil, Mexico, Hawaii and Nicaragua (McDowall, 1988). For instance, establishment failed for pink salmon (*Oncorhynchus gorbuscha*) and *O. kisutch* in North America (Lear, 1980), *O.gorbuscha* in Russia (Bakshtansky, 1980) and Norway (Berg, 1977), and various *Oncorhynchus* strains in Chile (Zamorano, 1991). The New Zealand *O. tshawytscha* is one of the very few successful commercial introductions (Childerhose & Trim, 1979).

The New Zealand contingent of exotic salmonids includes *O. tshawytscha*, Brown trout (*Salmo trutta*) and *O. mykiss*, along with the less common *S. salar*, Brook trout (*Salvelinus fontinalis*), Lake trout (*Salvelinus namaycush*) and sockeye salmon (*Oncorhynchus nerka*) (McDowall, 1990). European settlers introduced salmonids to New Zealand between the late 1800 and early 1900 (McDowall, 1994). There are two established trout species (*S. trutta* and *O. mykiss*) in the country's freshwater systems. Introduced from Tasmania as ova from 1867-1875 in the South Island and 1870 in the North Island, *S. trutta* is distributed in New Zealand's sweet waters and south of the Coromandel Peninsula (Townsend, 1996). Imported from California in the 1880s, *O. mykiss* was established, and it is present on the North and the South Islands (Scott et al., 1978; Scott & Poynter, 1991). Both *S. trutta* and *O. mykiss* are not farmed and are banned for commercial sales across New Zealand. Their stocks are enhanced by private hatcheries and acclimatisation societies (Brosnahan, 2020).

Of the less common char species, *S. fontinalis* ova arrived in the 1880s from the Atlantic Coast of North America while *S. namaycush* arrived once, from Canada 115 years ago (McDowall, 1990). The two species have a small wild population, with *S. fontinalis* established in the North and South Islands, while *S. namaycush* only occurs in the South Island (McDowall, 1994). Both char species are not farmed or recreationally exploited in New Zealand (Brosnahan, 2020).

Besides trout and char, one member of the genus Salmo (*S. salar*) and two of the genus *Oncorhynchus* (*O. nerka* and *O. tshawytscha*) were introduced between the 1800s and 1900s. Introductions for *S. salar* were made between 1864 and 1911 from Britain, Germany and Canada, with small populations in the South Island's upper Waiau river, and are maintained through stock enhancements (McDowall, 1994). The consignment for *S. nerka* ova were donated by Canadians in 1902 (Brosnahan, 2020; Scott, 1984). However, *O. nerka* and *S. salar* failed to establish large wild populations (McDowall, 1990).

New Zealand *O. tshawytscha* was introduced from California and adapted to local waters in the South Island (McDowall, 1990). Initial introductions took place in the 1870s (McDowall, 1994) with the first ova consignment from the United States Fishery Commission, arriving at the Hawkes Bay Acclimatisation Society, in 1875 (Thomson, 1922). Supplied by the Baird Hatchery in California, more ova arrived in 1876, 1877 and 1878. However, the 1870s introductions failed to establish river spawning stocks due to small batch releases in several unsuitable rivers, and L. F. Ayson pushed for more importations in the early 1900s (McDowall, 1994). The New Zealand government received five consignments (Thomson, 1922). These included 500,000 in 1901; 300,000 in 1904; 300,000 in 1905; 500,000 in 1906 and 1907 (New Zealand Marine Department, 1885-1927). The consignment in 1901 contained fall-run smolts from Battle Creek, while deliveries from 1904 to 1907 came from various hatcheries along the Sacramento River basin. It was probably the 1904 to 1907 delivery that established the current stock of New Zealand *O.*

tshawytscha (McDowall, 1994). By the 1950s, wild *O. tshawytscha* populations had established in rivers along the east coast of South Island (McDowall, 1990).

Actual *O. tshawytscha* farming began with government interests in hatchery based re-stocking of wild stocks and commercial sea ranching in the mid-1970s. The Silverstream hatchery was built on the Kaiapoi River by the Council of South Island Acclimatisation Societies (McDowall, 1991) and was handed over to the Ministry of Agriculture and Fisheries (Hardy, 1989). Subsequent plans for commercial sea ranching in the Waitaki River, at the Coleridge Power Station, and on the Waikoropupu springs, and several other sites failed for economic and technical reasons (Anon, 1976; McDowall, 1991; Todd, 1987).

Failed commercial ranching, stimulated research into optimising the size and number of *O. tshawytscha* returns to freshwaters. Between the late 1970s to early 1980s, the Glenariffe salmon research station successfully optimised smolt sizes and period of release (Unwin et al., 1989). Demand for *O. tshawytscha* ova grew quickly to satisfy commercial sea ranching, freshwater pond production and later, sea cage farming (McDowall, 1991). Thus, Glenariffe developed all female *O. tshawytscha* stocks (Hopkins & Todd, 1991), and the government called for commercial production which positioned Glenariffe as a major supplier of ova (McDowall, 1991). Farmed in the South Island, *O. tshawytscha* is a New Zealand niche premium supply to the world. Recent New Zealand *O. tshawytscha* production estimates are illustrated (Figure 1.1). The New Zealand *O. tshawytscha* farming generated over 16,000 tons in 2018 worth over NZ$ 350 million (FAO, 2020a), and continued growth is anticipated.

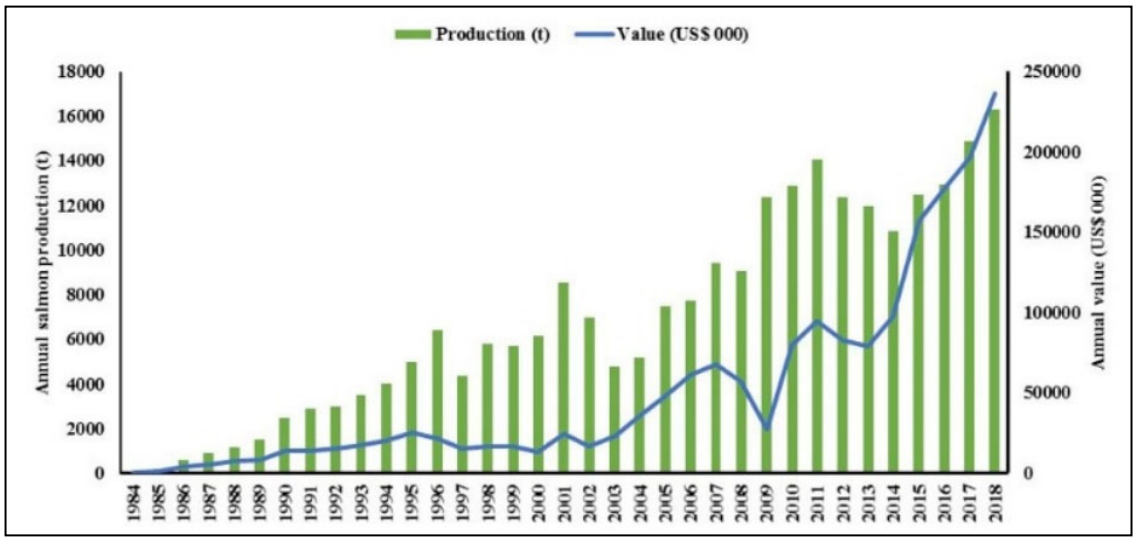


Figure 1.1. New Zealand chinook salmon (*Oncorhynchus tshawytscha*) production and value estimates 1984 to 2018 (FAO, 2020a).

New Zealand *O. tshawytscha* seed is produced in freshwater hatcheries and transferred as smolts (at about 200 days old) to freshwater or sea pens for rearing to a market size of 2 to 5 kg at 3-years of age (Brosnahan, 2020). Seawater production occurs in the Marlborough Sounds, Stewart Island and Akaroa Harbour, while freshwater farms in ponds, raceways and hydro-power

canals take place in Canterbury (NZ Salmon Farmers Association INC, n.d.-b) (Figure 1.2). Farms in the Marlborough Sounds produce 56% of salmon (NZ Salmon Farmers Association INC, n.d.-b), and are operated by the New Zealand King Salmon (NZKS) company at Otanerau Bay, Ngamahau, Clay point, Te Pangu Bay, Ruakaka Bay, Kopāua and Waitata (NZKS., 2021).

The other 29% of salmon production comes from two Sanford farms at Big Glory Bay in Stewart Island (NZ Salmon Farmers Association INC, n.d.-b). Fifteen percent is combined production from Canterbury and includes Akaroa Sea farm; freshwater Mt Cook Alpine Salmon in Tekapo and High Country Salmon in Twizel (NZ Salmon Farmers Association INC, n.d.-b). Other minor freshwater operations are pond-based and include South Westland Salmon farm, West Coast, Paringa and Ladybird Salmon farm at Omarama, Otago region (Figure 1.2).

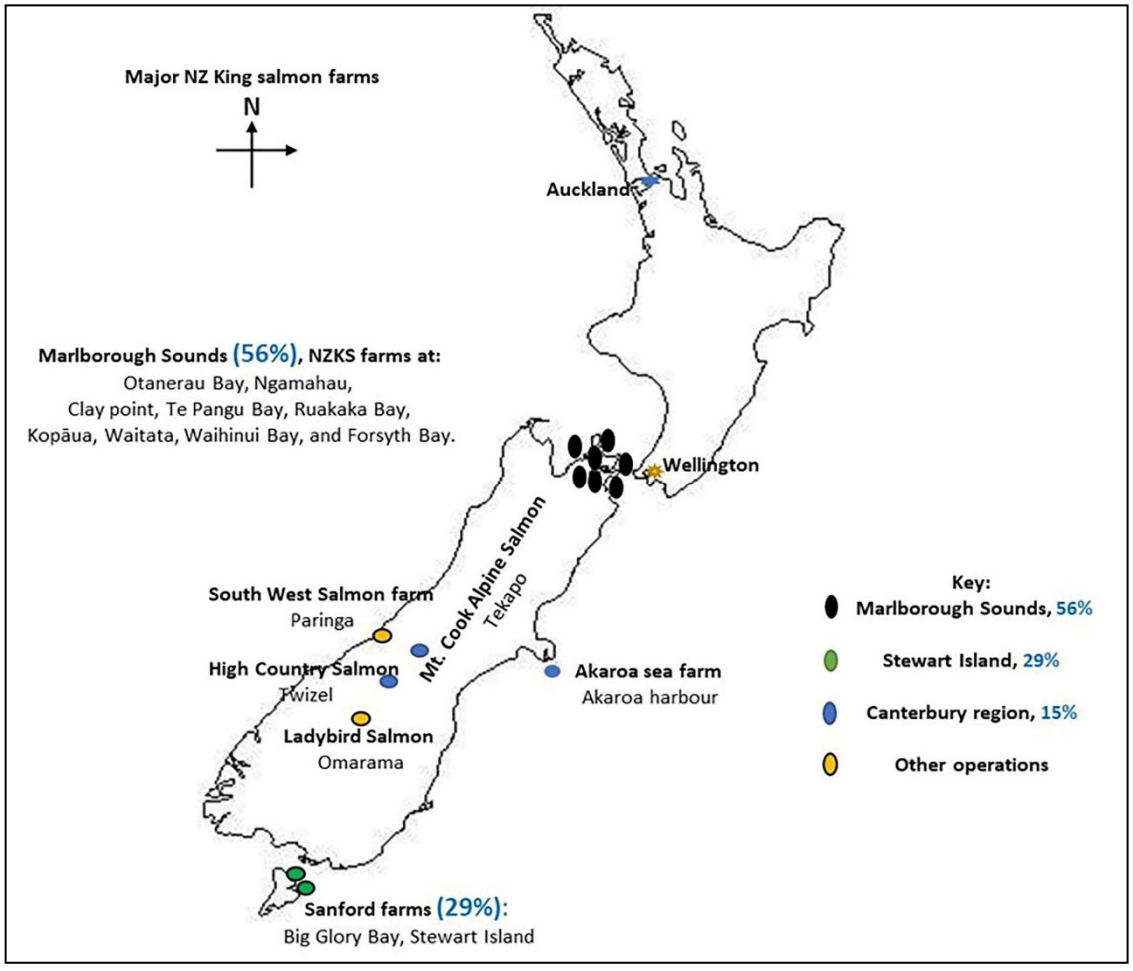


Figure 1.2. Major New Zealand chinook salmon (*Oncorhynchus tshawytscha*) production sites in the South Island, New Zealand (NZKS, 2021; NZ Salmon Farmers Association INC, n.d.-b).

1.4 Rationale and significance of the study

Farmed *O. tshawytscha* is New Zealand's flagship finfish species, making the country a leading global supplier. Due to New Zealand's geographical location and excellent biosecurity protocols, its *O. tshawytscha* industry is one of the most sustainable (Unwin et al., 1989). The New Zealand's *O. tshawytscha* aquaculture has had minor disease outbreaks, compared to major

bacterial, parasitic, and viral pathogens that have distorted production elsewhere (Anderson, 1996; Diggles, 2016). These include ISAV, viral haemorrhagic septicaemia virus (VHS), infectious haematopoietic necrosis virus (IPNV), salmonid alphavirus and *Renibacterium salmoninarum*, Pilchard orthomyxovirus (POMV) and Tasmanian salmonid reovirus (TSRV) (Diggles, 2016). Diseases caused by viruses, bacteria, and parasites have led to significant aquaculture losses (Fazio, 2019). For instance, under intensive aquaculture, viruses are inevitable since they coexist with fish at high densities (10^7 mL^{-1} of seawater), replicate fast and persist in the aquatic environment (Oidtmann et al., 2018; Semple et al., 2018). However, intensification and husbandry stresses provide the opportunity for outbreak of viral diseases (Kibenge, 2019). For example, piscine orthoreovirus (PRV) infected *S. salar* juveniles were destroyed in 2018 in the USA (Kibenge, 2019), ISAV first caused losses in Norway in 1984 and continued to ravage salmon farming in Canada and Scotland in 1997, USA in 2000 (Xiao et al., 2018), and in Chile between 2007 and 2009 (Bachmann-Vargas et al., 2021). In the past, pathogenic bacteria of *Yersinia ruckeri*, *Flavobacterium psychrophilum, Nocardia* species, *Vibrio ordalii* and the parasitic *Myxobolus cerebralis* caused elevated *O. tshawytscha* mortalities. However, disease incidences associated with these pathogens remain uncommon in New Zealand and none is listed by the World Organisation for Animal Health (OIE) (Brosnahan, 2020). Thus, the major threat to New Zealand's *O. tshawytscha* is disease emergence.

However, higher summer mortalities have occurred at sea-based farms in the Marlborough Sounds since 2012 with a peak in 2015 (Brosnahan et al., 2019a; Norman et al., 2013). Three strains of rickettsia-like organism (NZ-RLO: NZ-RLO1, NZ-RLO2, and NZ-RLO3) and *Tenacibaculum maritimum* were detected in farmed fish (Brosnahan et al., 2019b; Brosnahan et al., 2017; Brosnahan et al., 2019a). Later investigations confirmed NZ-RLO2 as the pathogen associated with *O. tshawytscha* summer mortalities (Brosnahan et al., 2019c). Furthermore, *O. tshawytscha* farmers in New Zealand registered up to 20% industry losses during the summer months in 2017, 2018 and 2019 following elevated sea surface temperatures (SSTs) in the range of 2-5°C above the species thermal limits (NZKS., 2019; Salinger et al., 2020; Salinger et al., 2019). Continued industry growth, amidst climate change influenced rising seawater temperatures and ocean acidification, make fish immunology understanding an indispensable asset to allow routine health assessment (Uribe et al., 2011).

Despite the economic importance of *O. tshawytscha* to New Zealanders, we found limited literature on haematology, immunology, and metabolomics. This knowledge gap presents an immense challenge to *O. tshawytscha* health management, overall industry performance and sustainability. We designed this thesis to feed into two Ministry of Business, Innovation and Employment (MBIE) funded programmes aimed at improving *O. tshawytscha* production efficiency and profitability and develop health diagnostic tools. The thesis contributed to these projects by developing immunological and metabolomics tools for the health assessment of farmed *O. tshawytscha*. The study was designed with the aim to:

i. Review the use of metabolomics approaches in farmed salmonid research.
ii. Review the methods used to isolate salmonid leucocytes, and their application in fish immunological assessments.
iii. Characterise *O. tshawytscha* blood and validate a flow cytometry cell count and viability assay kit.
iv. Profile *in vitro* immune response of *O. tshawytscha* peripheral blood mononuclear cells (PBMCs) stimulated by bacterial lipopolysaccharides (LPS).
v. Determine the immune and metabolic responses of *O. tshawytscha* smolts to a short-term polyinosinic: polycytidylic acid [poly (I:C)] challenge.
vi. Assess the immune and metabolic responses of *O. tshawytscha* smolts to prolonged poly (I: C) exposure.
vii. Uncouple the haemato-biochemical biomarkers associated with thermal stress and growth performance in *O. tshawytscha*.

1.5 Thesis structure

The thesis comprised two literature review sections (**Chapters 2** and **3**), five experimental sections (**Chapters 4, 5, 6, 7** and **8**), a thesis discussion and a conclusion section (**Chapter 9**). **Chapter 2** summarised research findings and identified gaps from studies that applied metabolomics in salmonid aquaculture research while **Chapter 3** established the immunological techniques applied in salmonids. **Chapters 2** and **3** can be read as standalone units that were formatted for specific journals. The experimental aspects of the thesis were developed through a series of discussions by the Auckland University of Technology (AUT) Aquaculture Biotechnology Research Group (ABRG) in Auckland, our collaborators at the Nelson Marlborough Institute of Technology (NMIT) and the Cawthron Institute Aquaculture Park (CAP) in Nelson. Findings from these studies generated information that formed part of a bigger MBIE funded program aimed at improving farmed *O. tshawytscha* production and aquatic health. Sections of this thesis in **Chapters 4, 5, 6, 7** and **8** were published in specific peer reviewed journals. Experimental work and sample collection under these chapters were conducted at the CAP and NMIT aquaculture facilities (Glenduan, Nelson, New Zealand) where the study fish were availed, and subsequent sample analysis was conducted at AUT. In the following sections, we highlighted actual work done under each of the experimental chapters.

Chapter 1: General introduction

This chapter aimed to introduce the thesis subject by highlighting the global aquaculture production scenario, and the contribution of salmonids to fish farming. The chapter then mapped out the New Zealand aquaculture industry, the history of salmonids in New Zealand, *O. tshawytscha* aquaculture and economic importance. It states the rationale, aims for the thesis and its structure.

Chapter 2: Literature review 1

This review chapter has been submitted for publication in *Reviews in Aquaculture*. It analysed the use of metabolomics in farmed salmonid research, and highlighted popular analytical platforms, studied salmon species and sample types. The review also outlined the latest research themes and summarised response mechanisms. The review identified research gaps under each research theme, provided future research perspectives and recommended actions for improving teleost metabolomics research.

Chapter 3: Literature review 2

This review chapter was published in *Fish & Shellfish Immunolog*y. It reviewed the methods used to isolate salmonid leucocytes, and their use to unravel fish immunocompetence following exposure to physical, chemical, and biological manipulations. The chapter surveyed for developed fish immune cell lines and their potential uses in salmonid immunology. The review provided a shopping list of immunological techniques that researchers of other fish species can consult.

Chapter 4: Characterisation of Chinook salmon (*Oncorhynchus tshawytscha*) blood and validation of flow cytometry cell count and viability assay kit.

This chapter was published in *Fish & Shellfish Immunology*. This chapter characterised the cellular composition of *O. tshawytscha* peripheral blood, and developed a micro-volume method to isolate fish PBMCs. The study used light microscopy and flow cytometry to characterise isolated PBMCs, and applied isolated cells to validate the Muse® cell count and viability assay kit.

Chapter 5: *In vitro* immune response of Chinook salmon (*Oncorhynchus tshawytscha*) peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharides.

This chapter was published in *Fish & Shellfish Immunology*. The objective of this chapter was to characterise the cellular functional and molecular immunomodulatory responses of farmed *O. tshawytscha* PBMCs *in vitro* using LPS from *Escherichia coli* (*E. coli*) serotypes O111: B4 and O55: B5 plus a phorbol ester PMA. Findings from this study contributed towards the understanding of immunocompetence in *O. tshawytscha* stocks under bacterial infections.

Chapter 6: Metabolic and immune responses of Chinook salmon (*Oncorhynchus tshawytscha*) smolts to a short-term Poly (I:C) challenge.

This chapter was published in the *Journal of Fish Biology*. The objective was to illustrate the mechanisms by which *O. tshawytscha* responds to *in vivo* poly (I: C) 24 h post-exposure. A multidisciplinary approach was used to measure fish responses, including classical haematology, PBMC latex bead phagocytosis, the Muse® flow cytometry to assess PBMC viability, reactive oxygen species (ROS) and nitric oxide (NO) production; liver and serum gas chromatography mass spectrometry (GC-MS) metabolomics; and targeted immune cytokine expression in fish red blood cells (RBCs), spleen (SP) and head kidney (HK). These results provided insights into short-term effects of poly (I:C) at haematological, innate immunity and metabolic levels.

Chapter 7: Polyinosinic: polycytidylic acid (poly I:C) *in vivo* enhances Chinook salmon (*Oncorhynchus tshawytscha*) immunity and alters the fish metabolome.

This chapter was published in the Journal of *Aquaculture International*. The goal of this chapter was to characterise response mechanisms of *O. tshawytscha* to prolonged *in vivo* poly (I:C) exposure via integrated assessment of effects on haematological parameter, PBMC functional parameters, serum and liver metabolic profiles, and targeted cytokine expression in HK and SP. Findings provided potential for design of amelioration strategies against future viral pathogens via metabolome reprogramming.

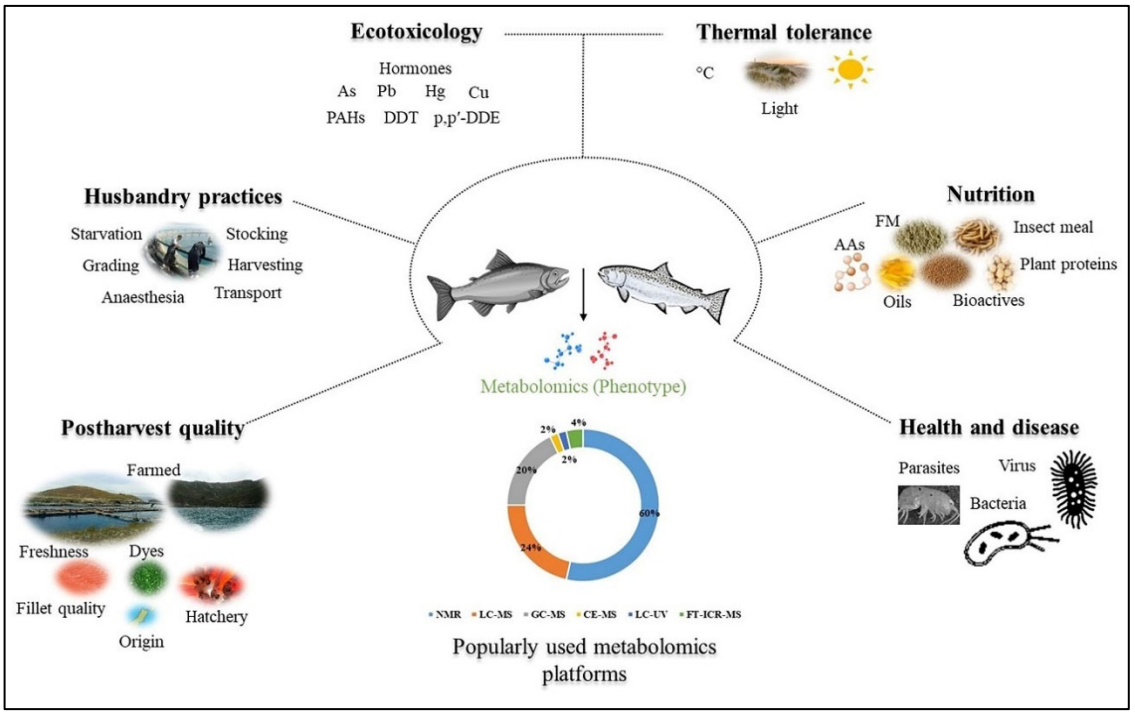
Chapter 8: Uncoupling interdependence of thermal stress and growth performance on Chinook salmon, *Oncorhynchus tshawytscha* blood biochemistry and immune capacity.

This chapter has been formatted for submission to the *Journal Metabolites*. The purpose was to study the effects of thermal stress and growth performance on *O. tshawytscha* cellular haematology and serum biochemistry. A multidisciplinary strategy was adopted to assess fish responses, including haematology, PBMC viability, ROS production and caspase activities, and serum biochemistry. Results from this study informed a selective breeding program for enhanced thermal tolerance.

Chapter 9: Thesis summary and conclusion

This section is a summary of the experimental findings in this study. It also provides information on limitations encountered and outlines recommendations for future research.

2 Chapter 2. Literature Review 1: Metabolomics in Salmonid Aquaculture Research: Applications and Future Perspectives.



Graphical representation of the metabolomics applications in salmonid aquaculture research.

This chapter has been submitted for publication as:

Lulijwa, R., Alfaro, A.C., &Young, T. (Undated). Metabolomics in salmonid aquaculture research: application and future perspectives. Submitted to *Reviews in Aquaculture*.

2.1 Prelude to literature review 1

Compared to traditional terrestrial animal production, aquaculture is a recently developed technology. Aquaculture has rapidly grown to complement capture fishery and guarantee the supply of food fish. For salmonids, the rapid growth has come with challenges with the potential to derail progress. Traditional assessments such as biometrics, feed digestibility, biochemical assays and omics including genomics, transcriptomics, and proteomics, have been successfully applied in several fields of aquaculture. However, many of the traditional approaches are insufficient since they do not depict the downstream metabolic effects. Metabolomics offers the opportunity to characterise physiological changes in lower molecular weight compounds in farmed salmonids exposed to exogenous and endogenous challenges. Previous reviews on aquaculture metabolomics synthesised data on developmental physiology, nutrition, health risks and disease, ecotoxicology (pollution), hatchery production, and postharvest quality management, authentication, and strategies for use in aquatic sciences. This review summarised findings and identified gaps from studies that applied metabolomics to solve farmed salmonids challenges. The review highlighted commonly used analytical platforms, studied species, and used samples. The chapter identified the latest research themes and points out the mechanistic findings. Based on identified research gaps, insights for future metabolomics studies were provided.

2.2 Introduction

Salmonids are the third-largest group of farmed finfish species after cyprinids and tilapines (FAO, 2019). Salmon farming contributes to the growing aquaculture sector (FAO, 2014). Major farmed salmonids include Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), and contributed 6.1% of the 54 million tons in 2018 (FAO, 2018c, 2020b). As aquaculture continues to expand, various challenges have emerged. These include but are not limited to broad categories under ecotoxicology (Chen et al., 2020), thermal tolerance (Cheung & Frölicher, 2020; Wade et al., 2019), nutrition (Roques et al., 2018c), postharvest quality (Ma et al., 2020), health and disease (Brosnahan et al., 2019b; Brosnahan et al., 2017; Brosnahan et al., 2019a) and husbandry practices (Raposo de Magalhães et al., 2020b).

Novel omics-based approaches can contribute to improving various aspects of the aquaculture value chain. They provide holistic characterisation and quantification of bio-molecules that reflect organismal responses to intrinsic and extrinsic factors (Coughlin, 2014). Of these, genomics (the study of genomes) (Snyder & Gerstein, 2003) has been used in genetic improvement (Jun et al., 2020), nutrition (Skugor et al., 2011), health, and pathogen characterisation (Tekedar et al., 2020), including vaccination and therapeutic development (Munang'andu et al., 2018). Other sub-disciplines include transcriptomics (the profiling of gene expression in a cell, organ, or organisms) (Whitfield et al., 2004), and proteomics (the study of changes in protein expression, dynamics, and post-translational alterations) (Graves & Haystead, 2002; Whitfield et al., 2004). The use of transcriptomics and proteomics in aquaculture research is gaining traction (Raposo de Magalhães et al., 2020a). Metabolomics involves the characterisation of changes in metabolites, the end products of gene expression. Metabolomics is increasingly being applied in aquaculture research (Alfaro & Young, 2018; Roques et al., 2018c; Samuelsson & Larsson, 2008).

Unlike the species differences observed at genomic, transcriptomic, and proteomic levels due to unique sequences of DNA, metabolite structure and function are conserved across taxa with their roles in specific metabolic processes being shared (Peng et al., 2015b). Metabolomics gives a physiological snapshot and allows mapping the differences among cells, tissues, organs, or organisms due to an underlying trait or condition or following exposure to a stimulus. These differences represent metabolic signatures which can elucidate functional relationships between genetic variations and biochemical phenotypes, understand the role/s of key biochemical pathways, and identify metabolite biomarkers for a range of purposes (Alfaro & Young, 2018). Increasingly being applied in aquaculture, metabolomics provides opportunities to assess several factors or key issues along the salmon value chain. We anticipate that metabolomics will contribute to the future improvement of farmed fish performance and productivity.

Past reviews in farmed finfish metabolomics synthesised broad contributions to developmental physiology, nutrition, health and immunology, ecotoxicology, hatchery

production, and postharvest quality management (Alfaro & Young, 2018; Roques et al., 2018c; Samuelsson & Larsson, 2008). Further reviews outlined applications in seafood traceability and authentication (Ghidini et al., 2019a), and provided an important overview of the various methodologies and processes involved in aquatic metabolomics (Young & Alfaro, 2018). This review took a specific focus and assessed the growing body of literature on metabolomics research in farmed salmonids. We have provided a database of key articles, mapped out popular analytical platforms, highlighted the most studied species and sample types, identified key research themes, and have outlined some mechanistic findings that metabolomics has unraveled. We also identified research gaps and provided insights into future applications of metabolomics in salmonid aquaculture research.

To fulfill the aim for this review, peer reviewed literature published between 2003 and 2020 was sought for using Google Scholar, Scopus, and Web of Science databases. We retrieved articles based on their relevance to the search strings including keyword search (salmon* OR '*genus names*') AND (metabolom* OR metabonom*). We included a range of salmonid genus names for extension. In addition, articles were checked for relevance and grouped into general categories for discussion, comprising research foci on ecotoxicology, thermal tolerance, nutrition, postharvest quality, health risks and disease, and husbandry practices.

2.3 Metabolomics analytical platforms

Metabolomics uses a variety of analytical platforms comprising mass spectrometry (MS), nuclear magnetic resonance (NMR), and vibrational-based techniques via Raman and infrared spectroscopy. While detailed descriptions of these platforms are outside the scope for this review, we briefly summarised their capabilities and limitations. We encourage interested readers to find detailed information elsewhere (Villas-Boas et al., 2007; Young & Alfaro, 2018).

NMR is an efficient and popular platform for structural assessment and absolute quantification of metabolites. Although NMR is highly reproducible, requires minimal sample preparation, and is quick to perform (5-10 min per sample), it typically detects in the micromolar (μM) to millimolar (mM) concentration range and is traditionally less sensitive than MS-based platforms (Goldansaz et al., 2017). However, innovative advances in NMR technology are improving sensitivity and selectivity (Giraudeau, 2020). In this review, NMR was the most used analytical platform in salmon aquaculture scanning across research themes (Figure 2.1 and Table 2.1). Although NMR dominated studies, the use of MS-based approaches is becoming more popular (Table 2.1).

MS-based platforms characterise the molecular weights of metabolites and can quantify their concentrations in the nanomolar (nM) to picomolar (pM) range. This allows the detection of additional metabolites compared to NMR (Goldansaz et al., 2017). MS platforms are coupled to a sample pre-separation device prior to being introduced into the mass spectrometer. Common MS platforms include liquid chromatography-MS (LC-MS), gas chromatography-MS (GC-MS)

and capillary electrophoresis-MS (CE-MS). These tools may be applied as stand-alone or in tandem to detect a wider range of metabolites (Lei et al., 2011).

LC-MS is used to separate and analyse heat-labile and non-volatile metabolites, and is more sensitive than GC-MS (Goldansaz et al., 2017), particularly when used in tandem with MS. A major limitation of LC-MS is low spectral reproducibility which complicates the matching of metabolites within existing spectral libraries (Young & Alfaro, 2018). Note that ultra-performance liquid chromatography-MS (UPLC-MS), and high-performance liquid chromatography-MS (HPLC-MS) are LC-MS (Goldansaz et al., 2017). LC-MS was the second most used analytical platform for salmonid research (Figure 2.1). The platform was popularly applied in ecotoxicological studies, although nutritional investigators also used it (Table 2.1). For a practical example, see how LC-MS was employed to profile the effects of contaminants in salmon (Meador et al., 2020).

GC-MS was the third most used analytical platform in salmon metabolomics (Figure 2.1) across industry research themes (Table 2.1). GC-MS analysis separates heat stable and volatile metabolites. GC-MS is reproducible, robust, sensitive, and achieves excellent metabolite separation. However, GC-MS is unsuitable for heat-labile or high molecular weight metabolites, and is destructive. Sample extracts destined for GC-MS analysis may also require derivatisation (Young & Alfaro, 2018). For an example, see how Young et al. (2019), used GC-MS to demonstrate metabolic effects of acute handling practices and anaesthesia in salmon.

CE-MS sensitivity is comparable to GC-MS and LC-MS, does not require sample pre-treatment, can use small samples, and can detect certain metabolites that are challenging for other MS platforms. However, CE suffers from poor reproducibility and lack of separation for nonpolar metabolites (Goldansaz et al., 2017). This could potentially explain its limited use in salmon metabolomics (Table 2.1). Nevertheless, CE-MS may complement other MS-based tools.

Some infrequently used modifications exist to NMR and MS-based platforms. Interested readers are encouraged to consult the indicated sources below. However, these modifications offer flexibility to aquaculture metabolomics. For instance, high-resolution magic angle spinning (^{1}H HR-MAS-NMR) can directly be used on intact tissue and microprobes have been developed for microgram samples (Goodacre et al., 2004; Jarak et al., 2018; Lucas-Torres et al., 2018). Multidimensional 2D NMR such as total correlated spectroscopy (1D ^{1}H, 2D ^{1}H-^{1}H TOCSY-NMR), heteronuclear single quantum coherence spectroscopy (^{1}H-^{13}C-HSQC-NMR) (Shumilina et al., 2015), one-dimensional ^{1}H and two-dimensional ^{1}H-^{1}H *J*-resolved nuclear magnetic resonance spectroscopy (^{1}H ^{1}H*J*RES-NMR) (Lin et al., 2009) are used to reduce peak overlap and provide extra structural information (Marchand et al., 2017). Nevertheless, they suffer from low speed, and most modifications being used to fast track industry adoption are still at infancy stage, limiting use in large scale studies (Giraudeau, 2020).

Variations to MS-based platforms include comprehensive two-dimensional gas chromatography mass spectrometry (GCxGC-MS) which utilises two sequential pre-separation columns to enhance metabolite separation. This can reduce co-elution of metabolites in complex biological samples, increase sensitivity, and substantially expand the range of detectable metabolites (Cipriano et al., 2015; Stilo et al., 2021). Direct analysis in real time-high resolution mass spectrometry (DART-HR-MS) is convenient, sensitive, reproducible, requires minimal sample preparation and is ideal for product authentication (Fiorino et al., 2019; Fiorino et al., 2018). Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) is quantitative and offers wide metabolite coverage and high sensitivity (Gaoping et al., 2020; Softeland et al., 2014). A fast (15 s per sample) and sensitive direct flow injection-MS (DFI-MS) method which does not require pre-separation can process many samples and detect thousands of features (Sarvin et al., 2020). This has wide use potential across the salmon value chain.

Infrared (IR) and Raman spectroscopy are vibrational-based platforms that do not allow metabolite identification but give information on broader metabolite functional groups. This complicates the interpretation of spectra derived from complex metabolite mixtures and provides lower resolution data. However, spectroscopy is fast (10-60 s per sample), cheap, nondestructive, and reproducible. These techniques offer wide predictive applications in aquaculture across research themes identified in this review. Technical details about these platforms are available elsewhere (Morais et al., 2020; Young & Alfaro, 2018). For industry application, see how IR was used to authenticate fish and predict nutritional value (Gonçalves et al., 2020), and Raman to select *O. mykiss* with high polyunsaturated fatty acid (PUFA) deposition (Prado et al., 2020).

Future salmonid studies are likely to benefit from employing analytical platforms with higher sensitivities to increase the coverage of metabolite detection. It is however important to point out that no single platform can detect all metabolites and the use of multiple tools will be required to comprehensively map out the salmon metabolome. This review demonstrated that the majority (68%) of studies used single platforms, 16 (32%) used two platforms, and none used more than two platforms (Table 2.1). For example, two papers used LC-MS and GC-MS in combination to detect the highest number of metabolites (n=496) in *S. salar* and characterised the physiological effects of toxin exposures (Olsvik et al., 2017b; Olsvik & Søfteland, 2018). Assimilation of multiplatform-based metabolomics in aquaculture would be desirable, as in medicine and animal sciences (Foroutan et al., 2020; Suhre et al., 2010).

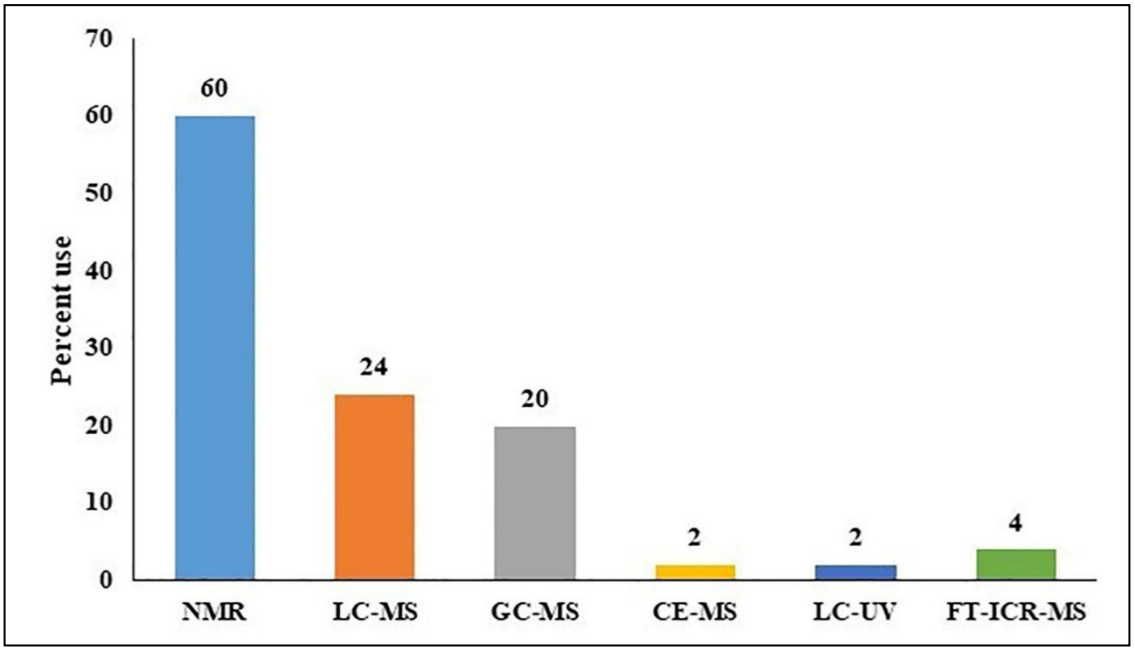


Figure 2.1 Common metabolomics analytical platforms used in salmon research. NMR: nuclear magnetic resonance; LC-MS: liquid chromatography mass spectrometry; GC-MS: gas chromatography mass spectrometry; CE-MS: capillary electrophoresis mass spectrometry; LC-UV: liquid chromatography ultraviolet; and FT-ICR-MS: fourier-transform ion cyclotron resonance mass spectrometry. Data comes from 50 reviewed salmonid metabolomics studies from 2003 to October 2020. Percentage values exceed 100 because papers used multiple methods.

2.4 Salmonid species and sample types

The most investigated salmonid is *S. salar*, followed by *O. mykiss,* Chinook salmon (*Oncorhynchus tshawytscha*), and Arctic char (*Salvelinus alpinus*). Coho salmon (*Oncorhynchus kisutch*) is the least studied (Figure 2.2a). Interest in research activities reflects the dominance of *S. salar* and *O. mykiss* in aquaculture production.

Studies used eleven different sample types (Figure 2.2b). Across fish species, muscle, liver, and plasma were the most used samples. Muscle samples were the most used in *S. salar* while plasma dominated *O. mykiss* studies (Figure 2.2b and Table 2.1). Fillets are the primary food product from farmed salmon for human consumption, and this explains the prime interest in this tissue for research in *S. salar*. Indeed, muscle metabolite profiling was common in diet-based studies to inform fish nutritional content and postharvest quality assessment. Muscle lipid profiles reflect aquafeed composition, making it popular in nutritional studies (Kwasek et al., 2020). Muscle and liver can provide a snapshot of metabolic processes induced by nutritional manipulations, hence their popular use (Figure 2.2b and Table 2.1) (Roques et al., 2018c). Fish skeletal muscle comprises the deep white mass and the thin red component (Rabah, 2005). White muscle is rich in glycogen and is anaerobic, while the red oxidative muscle along the lateral line region, is rich in blood capillaries, myoglobin, mitochondria, glycogen and lipids (George & Don, 1978; Kiessling et al., 2006). In farmed salmon, white muscle is pink because of the high level of pigmented carotenoids provided in the feed. Red muscle forms a thin superficial lateral layer

underneath the skin. Because of differences in composition, white and red muscle possess different metabolic profiles. Thus, the entire skeletal muscle is suitable for quality assessments, while the white muscle is used in nutrition and metabolism (Roques et al., 2018c).

Fish liver provides global morphological, molecular and functional sexual differences, making this organ important in reproductive biology (Qiao et al., 2016). While liver samples can provide important metabolic information, consistency must be established regarding the exact lobe and location due to variation in metabolites from different sections (Abro et al., 2014; Cheng et al., 2016b). However, several studies employed isolated liver hepatocytes, which may solve issues associated with sample heterogeneity (Figure 2.2b and Table 2.1). Blood plasma or serum was the third most used sample type since they can depict systemic metabolic processes (Roques et al., 2018c) making this biofluid and liver important in stress studies, while kidney was used for immune assessment (Liu et al., 2016). While no study used lymphoid isolated leucocytes or peripheral blood mononuclear cells (PBMC), (likely due to biomass limitations), single-cell or microfluidic NMR devices suggest future use (Finch et al., 2016; Giraudeau, 2020; Lucas-Torres et al., 2018).

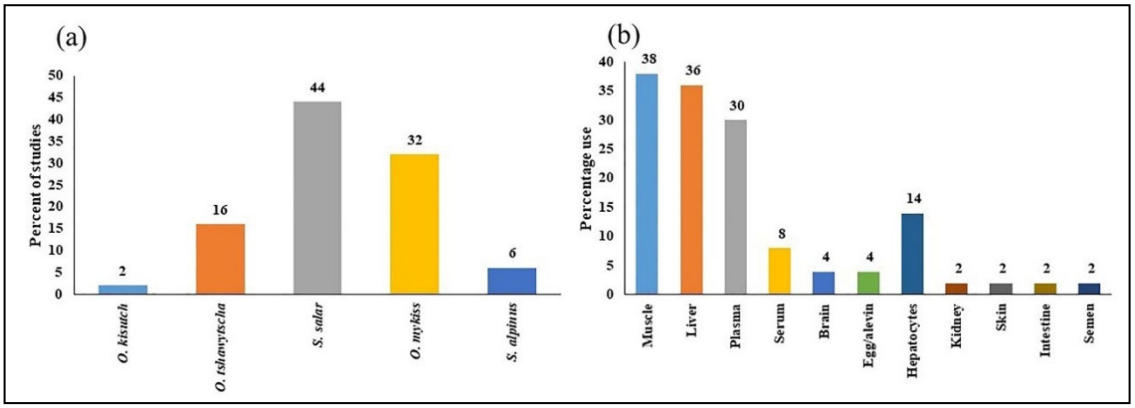


Figure 2.2 The most studied salmonid species by metabolomics (a), and the most used tissues and organs to study salmonid metabolomics (b). Coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and Arctic char (*Salvelinus alpinus*). Data come from 50 reviewed salmonid metabolomics studies from 2003 to October 2020. Percentage values exceed 100 because papers used multiple sample types in Figure (b).

Kidney, skin, intestine and semen were the least analysed sample types found in this review (Figure 2.2b and Table 2.1). For instance, fish intestinal sections were used once in a nutritional study (Coccia et al., 2019), and the same applied to semen to assess quality (Dietrich et al., 2019), skin in dye detection (Dubreil et al., 2019) while the brain, eyed egg or embryos were applied in toxicity assessment (Magnuson et al., 2020b). This review did not record the use of gills and non-invasive skin mucus as previously demonstrated in other teleosts (Ekman et al., 2015; Sun et al., 2020). Studies on PBMCs and fecal matter are also missing in salmon metabolomics (Hano et al., 2021; Liu et al., 2014). However, growing interest in the gut microbiome and its influence on fish health and physiology (Steiner et al., 2020) makes stool

metabolomics interesting in farmed teleosts (Hano et al., 2021). The development of metabolomics methods along these lines will enable the use of nonlethal sampling.

2.5 Chemometric methods

Data resulting from metabolomics analysis present as large spectra with complex information. Since primary metabolomics data handling methods vary and depend on the type of data and the analytical platform, it is difficult to give general advice (Young & Alfaro, 2018). Processing of raw data (chromatograms and spectra) typically involves data conversion, noise filtering, retention time correction, peak detection, chromatogram alignment, and metabolite identification using customised in-house libraries, freely available repositories, or proprietary software. Prior to statistical analysis, data are often taken through a series of integrity checks and normalization processes to minimize technical errors and detection of false significances (Cambiaghi et al., 2016; Young & Alfaro, 2018). Indeed, recent developments in data processing, open-source metabolite libraries, and bioinformatics strategies have enabled broad feature identifications and have enhanced secondary data analysis to inform metabolic perturbations (Chong et al., 2018).

Data analysis is achieved via univariate and multivariate approaches. This review analysed papers for the most used chemometric methods (Figure 2.3). Several studies combined methods (Table 2.1) in agreement with Sugimoto et al. (2012) who noted complementary use of univariate and multivariate approaches. Univariate methods assess single metabolites at a time, and often incorporate corrections for multiple hypothesis testing. Popular univariate tests included parametric *t*-tests and analysis of variance (ANOVA) or their non-parametric equivalents (Young & Alfaro, 2018). Standard *t*-tests and ANOVA were the most applied univariate approaches (Figure 2.3). For instance, Shumilina et al. (2020) used one-way ANOVA to discern between frozen and fresh salmon fillets, and Jasour et al. (2017) employed the independent *t*-test to determine inclusion levels of feather meal in *O. mykiss* diets. In addition, two-way ANOVA was used to reveal how dietary vitamin A interacts with hydrocarbon in contaminated feed to reduce toxicity (Berntssen et al., 2016).

Multivariate approaches analyse all metabolites at the same time. They are used for data reduction and often utilized to understand global between-sample similarity/dissimilarity. They also aid the search for metabolic changes to explain an underlying phenotype or a response to a treatment (Young & Alfaro, 2018). Unsupervised multivariate methods require no knowledge of sample class. They include principal component analysis (PCA), hierarchical cluster analysis (HCA), and K-means clustering (KMC) (Andreopoulos et al., 2009; Ghidini et al., 2019a; Young & Alfaro, 2018). This review identified PCA (n =30) as the most used statistical method in salmonid metabolomics research (Figure 2.3). For instance, PCA was used by Magnuson et al. (2020a) to demonstrate the toxic effects of pesticide exposure in *O. tshawytscha* prior to subsequent pathway analysis. In agreement, PCA (Figure 2.3) was popularly used to identify

sample outliers and groupings prior to supervised modelling, just as previous reviews noted (Ghidini et al., 2019a; Young & Alfaro, 2018). Cluster-based HCA and KMC are excellent grouping tools for samples with similar metabolite profiles using dendrograms and cluster means. For details on these methods, we refer readers elsewhere (Sugimoto et al., 2012; Young & Alfaro, 2018).

Supervised multivariate methods use sample group information to solve classification problems. Common supervised techniques are the family of partial least squares (PLS) regression models, used to analyse the interrelationship between the independent (X) groups and the measured or response (Y) variables to achieve maximum separation. These methods include partial least squares discriminant analysis (PLS-DA), orthogonal partial least square-discriminant analysis (OPLS-DA), sparse partial least squares discriminant analysis (sPLS-DA) (Meador et al., 2020), and orthogonal signal corrected-PLS-DA (OSC-PLS-DA) (Roques et al., 2020a). They are used to enhance group separation by rotating principal components to identify metabolites that contribute most to groups (Sugimoto et al., 2012). See specific details about these methods in recent reviews (Ghidini et al., 2019a; Young & Alfaro, 2018).

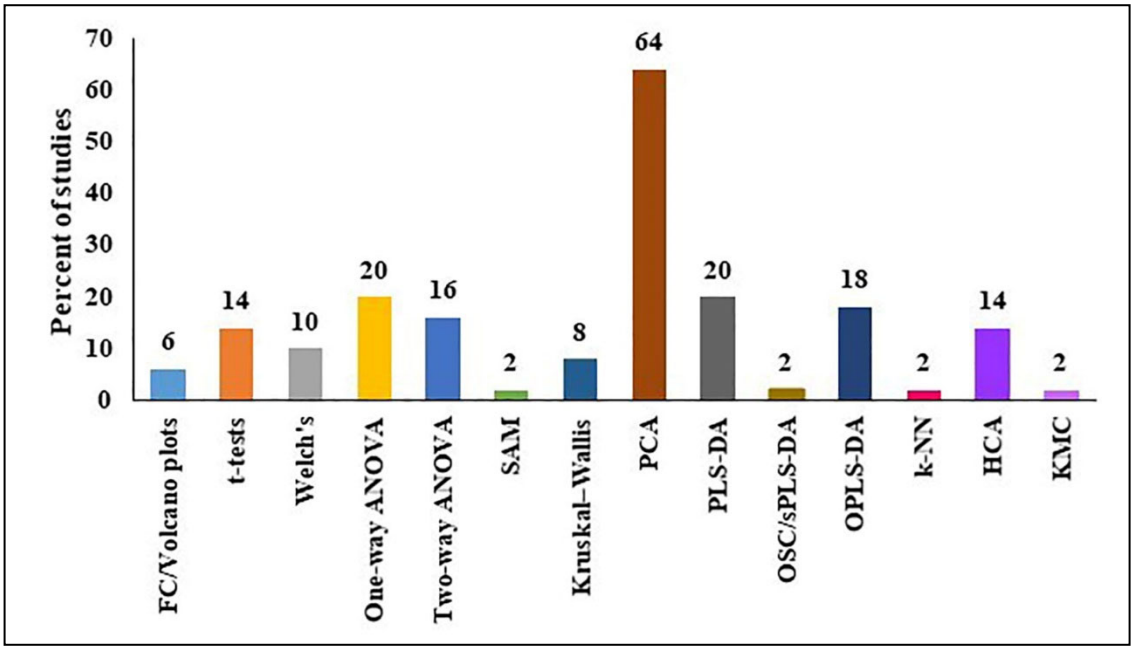


Figure 2.3 The most used chemometric techniques in salmonid metabolomics data analysis and presentation. FC: fold change, *t*-tests: includes student, pair and independent sample tests, Welch's: Welch's two-sample *t*-tests, ANOVA: analysis of variance, SAM: significant analysis of microarrays/and metabolites, PCA: principal component analysis, sPLS-DA: sparse partial least squares discriminant analysis, PLS-DA: partial least squares discriminant analysis, OPLS-DA: orthogonal projections to latent structures discriminant analysis, OSC-PLS-DA: orthogonal signal corrected PLS-DA, k-NN: k-nearest neighbors, HCA: hierarchical cluster analysis and KMC: K-means clustering. Data come from 50 reviewed salmonid metabolomics studies from 2003 to October 2020. Percentage values exceed 100 because papers used multiple techniques.

Supervised PLS-DA and OPLS-DA were used in ten and nine studies in this review (Figure 2.3). For recent PLS modelling examples in salmon metabolomics, see how PLS-DA and

sPLS-DA were used to segregate fish exposed to anaesthetics and contaminants (Meador et al., 2020; Rahimi et al., 2020). In addition, OSC-PLS-DA and OPLS-DA models were used to classify fish by diet and to identify responsible metabolites in nutritional studies (Roques et al., 2020a; Wagner et al., 2019). The vulnerability of PLS-DA to high orthogonal variation (uncorrelatedness) is eased by OPLS-DA modelling, which enhances separation by distinguishing between predictive and orthogonal variation (Ghidini, et al., 2019a). This may explain the increasing use of OPLS-DA modelling. Both techniques can be used for sample classification and/or to highlight potential metabolite biomarkers, but care must be taken to validate the PLS models since they are prone to overfitting and false discoveries.

There are other data analysis methods that did not feature in this review, but might be useful for specific cases in salmon metabolomics research. For example, unsupervised multivariate analysis of variance (MANOVA) is used to segregate main effects and interactions when several continuous dependent variables are investigated (Deborde et al., 2021). Complemented with PCA, variance-based linear or quadratic discriminant analysis (LDA, QDA) methods can be applied in several areas such as product authentication (Zeng et al., 2021). In addition, data classification and regression-based methods such as K-nearest neighbors (k-NN), random forests (RF), and support vector machines (SVM) can be used to impute missing data and predict specific traits (Cipriano et al., 2015; Rajab, 2020; Rangel-Huerta et al., 2020; Raposo de Magalhães et al., 2020). Artificial neural networks (ANN) is useful in classification, parameter separation and quality control (Sugimoto et al., 2012). Finally, pattern recognition by soft independent modelling of class analogy (SIMCA) can be used to determine product origin for authentication (Adenan et al., 2020). We direct interested readers to review details on these chemometric methods elsewhere (Boccard et al., 2010; Ghidini et al., 2019a; Sugimoto et al., 2012; Young & Alfaro, 2018).

Table 2.1 Metabolomics applications in salmonid aquaculture research

Species	Treatments	Sample type	Sample size (n/treatment)	Analytical platform	Extraction	Metabolites	Statistics	Reference
O. kisutch	Effect of growth hormone over expression in transgenic fish	Liver and muscle	3-4	CE-MS	Polar/nonpolar	361	Excel Statistics. One-way ANOVA.	(Nakano et al., 2019)
O. tshawytscha	Effect of short-term poly (I:C) in vivo	Serum	5	GC-MS	Polar	141	MetaboAnalyst 4.0. SAM, HCA, and FC.	(Lulijwa et al., 2020a)
	Monitoring poly (I:C) effects in vivo	Serum and liver	3	GC-MS	Polar	298	MetaboAnalyst 4.0. One-way ANOVA, PCA, HCA and KEGG pathway analysis.	(Lulijwa et al., 2020b)
	Effect of 16 contaminants of emerging concern	Liver	7	LC-MS	Polar	1688 ions	MetaboAnalyst 4.0 FC, HCA, and sPLS-DA	(Meador et al., 2020)
	Bifenthrin toxicity	Brain	15	LC-MS	Polar	80	SPSS 24: PCA, HCA and pathway analysis	(Magnuson et al., 2020a)
	Effect of acute handling, anesthesia and euthanasia	Plasma	5	GC-MS	Polar	105	MetaboAnalyst 4.0. PLS-DA	(Young et al., 2019)
	Crude oil toxicity	Muscle	8	^{1}H-NMR	Polar	12	PLS-Toolbox 3.5. PCA and one-way ANOVA.	(Van Scoy et al., 2010)
	Crude oil toxicity	Liver and muscle	6	^{1}H-NMR and ^{1}HLRES NMR	Polar	44	Matlab and SAS 9.0. PCA and two-way ANOVA.	(Lin et al., 2009)
	Pesticide toxicity	Eyed eggs and alevins	6	^{1}H-NMR and LC-UV	Polar	20	Number Cruncher Statistical System 2001. PCA and one-way ANOVA.	(Viant et al., 2006)
S. salar	Effect of peracetic acid	Plasma	10	LC-MS	Polar	39	MS-Omics ApS. PCA followed by FC and *t*-test	(Lazado et al., 2020)
	Muscle spiked with dioxon and furans	Muscle		GCxGC-MS	Polar	11	LECO software ChromaTOF BT v5.40.12.0. Data were processed for basic peak finding, spectral deconvolution and peak area integration.	(Stultz et al., 2020)
	Distinguish fresh and thawed fish	Loin fillet	3	^{1}H-NMR	Polar	3	SPSS 25. PCA one-way ANOVA.	(Shumilina et al., 2020)
	Spoilage of packed fish	Fillet	2kg	^{1}H-NMR	Polar	33	Matlab. Normalization.	(Jääskeläinen et al., 2019)
	In vitro effects of p,p'-DDE on hepatocytes	Hepatocytes	5	LC-MS/MS and GC-MS	Polar/nonpolar	496	GraphPad Prism 6.0. Welch's two-sample *t*-tests and KEGG pathway analysis.	(Olsvik & Søfteland, 2018)
	In vitro toxicity of pirimiphos-methyl	Hepatocytes	6	LC-MS and GC-MS	Polar/nonpolar	379	Program R. Two-way ANOVA and Welch's two-sample *t*-tests, HCA and KEGG pathway analysis.	(Olsvik et al., 2017a)
	In vitro toxicity of bisphenol A and genistein	Hepatocytes	5	LC-MS and GC-MS	Polar/nonpolar	496	Program R. Two-way ANOVA and Welch's two-sample *t*-tests and KEGG pathway analysis.	(Olsvik et al., 2017b)
	Effect of dietary vitamin A	Liver and muscle	6	LC-MS/MS and GC-MS	Polar/nonpolar	329	Qlucore omics explorer 2.3. Two-way ANOVA.	(Berntssen et al., 2016)
	Effect of dietary omega-3 and vitamin E	Hepatocytes	6	^{1}H-NMR and FT-ICR-MS	Polar/nonpolar	13	GraphPad Prism 6.0. PCA, PLS-DA and Kruskal–Wallis.	(Softeland et al., 2016)
	Response to *Aeromonas salmonicida* infection	Kidney	8	^{1}H-NMR	Polar	18	SIMCA-P. 11.0. PCA, OPLS-DA.	(Liu et al., 2016)
	Effects of months-long inappetence during spawning	Serum	20	GCxGC-MS	Polar	57	MetaboAnalyst 2. k-NN, PCA, PLS-DA, *t*-test and KEGG pathway enrichment analysis.	(Cipriano et al., 2015)
	Monitor post-mortem changes	Fillet	2	^{1}H -TOCSY-NMR, ^{1}H-^{1}H-TOCSY-NMR and ^{1}H-^{13}C HSQC-NMR	Polar	49	MestReC 4.9.8.0. Spectral processing.	(Shumilina et al., 2015)
	Effects of vitamin E on chlorpyrifos toxicity	Hepatocytes	6	LC-MS/MS and GC-MS	Polar/nonpolar	329	Program R. Two-way ANOVA, HCA, and Welch's two-sample *t*-tests.	(Olsvik et al., 2015)
	Dietary sesamin	Liver and white muscle	6	^{1}H-NMR	Polar/nonpolar	48+	SIMCA-P + 12.0.1. PCA, PLS-DA and one-way ANOVA.	(Wagner et al., 2014)
	Toxicological effect of contaminants	Hepatocytes	-	^{1}H-NMR and FT-ICR-MS	Polar/nonpolar	149+ ions	Prometab 3.3. PCA, PLS-DA.	(Softeland et al., 2014)
	Argenine over supplementation	Plasma, liver	12	LC-MS	Polar	10+	R. 3.02. *t*-test and PCA.	(Andersen et al., 2015)

	Argenine supplementation	Hepatocytes	6	^{1}H-NMR	Polar/nonpolar	17	Statistica11.0. PCA, OPLS-DA and two-way ANOVA.	(Andersen et al., 2014)
	Effects of elevated temperature	Plasma	12	^{1}H-NMR	Polar/nonpolar	45	SAS 9.2. PCA, OPLS-DA.	(Kullgren et al., 2013)
	Effect of short-term feeding	Liver and muscle	6	^{1}H-NMR and ^{1}H HR-MAS-NMR	Polar/nonpolar	61+	Matlab. PCA visualisation.	(Bankefors et al., 2011)
	Long-term handling stress	Plasma	118	^{1}H-NMR	Polar/nonpolar	9	Matlab. PCA and PLS-DA.	(Karakach et al., 2009)
	Quality assessment	Muscle	-	^{1}H-NMR	Polar/nonpolar	76+	No statistical analysis.	(Gribbestad et al., 2005)
	Response to *A. salmonicida* infection	Plasma	9	^{1}H-NMR	Polar	22	SIMCA-P 10.0. PCA visualisation.	(Solanky et al., 2005)
O. mykiss	Bifenthrin toxicity	Brain	3,4 and 5	LC-MS	Polar	181	SPSS 24. PCA and KEGG pathway.	(Magnuson et al., 2020b)
	Effect of anesthesia	Plasma	10	^{1}H-NMR	Polar	37	SPSS 19.0. PLS-DA, independent sample *t*-test and KEGG pathway.	(Rahimi et al., 2020)
	Detection of victoria pure blue	Muscle and skin	6	LC-MS	Polar/nonpolar	15	*t*-test	(Dubreil et al., 2020)
	Effect of protein-rich yeast fraction	Plasma, liver, and muscle	9	^{1}H-NMR	Polar	35+	BioStatFlow tool based on R 2.9. PCA, KMC, Kruskal–Wallis.	(Roques et al., 2020b)
	Effect of black soldier fly protein extracts	Plasma, liver, and muscle	9	^{1}H-NMR	Polar	84	BioStatFlow tool based on R 2.9. NIPALS, OSC-PLS-DA, Kruskal–Wallis and one-way ANOVA and HCA.	(Roques et al., 2020a)
	Effect of dietary glycerol	Liver and muscle	12	^{1}H-NMR	Polar/nonpolar	36	MetaboAnalyst 4.0. PCA, PLS-DA.	(Palma et al., 2019)
	Uptake of bioactive extract	Intestinal sections	3	^{1}H-NMR	Polar/nonpolar	17	Statistica 7.0. OPLS-DA analysed.	(Coccia et al., 2019)
	Effect of diazinon	Serum/plasma and liver	60	^{1}H-NMR	Polar	45	SPSS 19.0. PCA, PLS-DA, two-way ANOVA.	(Hajirezaee et al., 2019)
	Characterisation of seminal plasma	Seminal plasma	7	LC-MS	Polar	48	Mass Profiler Professional 12.6.1 and SIMCA-P+ 13.0.3. PCA and Welch's	(Dietrich et al., 2019)
	Effect of feathermeal as a protein alternative	Liver and plasma	21	^{1}H-NMR	Polar/nonpolar	43	SIMCA-P 14.0. PCA, one-way ANOVA, two-way ANOVA, and independent sample t-test.	(Jasour et al., 2017)
	Effect of starvation	Serum, liver and muscle	6	GCxGC-MS	Polar/nonpolar	143	MSort. Paired *t*-test.	(Baumgarner & Cooper, 2012)
	Effect of different sewage effluents	Plasma	25	^{1}H-NMR	Polar/nonpolar	39	SIMCA-P.11.0 PCA, OPLS-DA.	(Samuelsson et al., 2011)
	Effect of food deprivation	Plasma, liver and muscle	15	^{1}H-NMR	Polar/nonpolar	31	SIMCA-P.11.0 PCA, OPLS-DA.	(Kullgren et al., 2010)
	Temperature stress	Developing eggs	4	^{1}H-NMR	Polar	11+	Number Cruncher Statistical System 2001. PCA, one-way ANOVA.	(Turner et al., 2007)
	Effect of synthetic estrogen ethinylestradiol (EE_2)	Plasma	21	^{1}H-NMR	Polar/nonpolar	23+	SIMCA-P.10.5. PCA, PLS-DA.	(Samuelsson et al., 2006)
	Effect of chronic exposure to elevated temperature (15°C vs 20°C)	Muscle, liver	3	^{1}H-NMR	Polar	16	PLS−Toolbox. 3.0. PCA and one-way ANOVA.	(Viant et al., 2003)
S.alpinus	Effect of fishmeal (FM) replacement	Liver and muscle	9	^{1}H-NMR	Polar/nonpolar	118+	SIMCA-P 13.0. PCA, OPLS-DA and Kruskal–Wallis test.	(Wagner et al., 2019)
	Evaluation of fish feed raw materials	Liver and white muscle	10	^{1}H-NMR	Polar/nonpolar	94	SIMCA-P 13.0. PCA and OPLS-DA.	(Cheng et al., 2016a)
	Effect of zygomycetes and FM diets	Liver	12	^{1}H-NMR	Polar/nonpolar	15	SIMCA-P+ 12.0.1. PCA and OPLS-DA.	(Abro et al., 2014)

CE-MS: capillary electrophoresis mass spectrometry, GC-MS: gas chromatography mass spectrometry, LC-MS: liquid chromatography mass spectrometry, ^{1}H-NMR: proton nuclear magnetic resonance, ^{1}H*J*RES NMR: two-dimensional *J*-resolved nuclear magnetic resonance spectroscopy, HPLC-UV: high-performance liquid chromatography-ultraviolet, GCxGC-MS: comprehensive two-dimensional gas chromatography mass spectrometry, LC-MS-MS: liquid chromatography tandem mass spectrometry, FT-ICR-MS: fourier-transform ion cyclotron resonance mass spectrometry, ^{1}H-TOCSY-NMR: total correlation spectroscopy NMR, ^{1}H-^{13}C HSQC NMR: heteronuclear single quantum coherence spectroscopy, and ^{1}H HR-MAS-NMR: ^{1}H high-resolution magic angle spinning NMR. Poly (I:C): polyinosinic: polycytidylic acid, p, p'-DDE: dichlorodiphenyldichloroethylene, FM: fishmeal, ANOVA: analysis of variance, SAM: significant analysis of microarray/and metabolites, FC: fold change, KEGG: Kyoto encyclopaedia of genomes and genes, PCA: principal component analysis, sPLS-DA: sparse partial least squares discriminant analysis, PLS-DA: partial least squares discriminant analysis, k-NN: k-nearest neighbors, OPLS-DA: orthogonal projections to latent structures discriminant analysis, OSC-PLS-DA: orthogonal signal corrected PLS-DA, NIPALS: nonlinear iterative partial least squares, kg: kilogram HCA: hierarchical *cluster* analysis and KMC: K-means clustering. Data comes from 50 reviewed salmonid metabolomics studies from 2003 to October 2020.

2.6 Research themes in salmon metabolomics

Six research themes emerged from this review. Research primarily concentrated on ecotoxicology (n=15) and nutrition (n=14) (Figure 2.4). The rapid rise in nutrition-based metabolomics is likely due to recent interest in the search for sustainable aquafeed ingredients (Roques et al., 2018c). Other research themes including husbandry practices, postharvest quality, health risks and disease, and thermal tolerance are emerging. This could be because of rising concerns for animal welfare, farmed fish nutritional value, and climate change. There is limited integration of metabolomics in salmon breeding programmes (Gribbestad et al., 2005; Nakano et al., 2019). The use of metabolomics in breeding programmes will contribute to the accurate prediction of important production and health traits (Goldansaz et al., 2017).

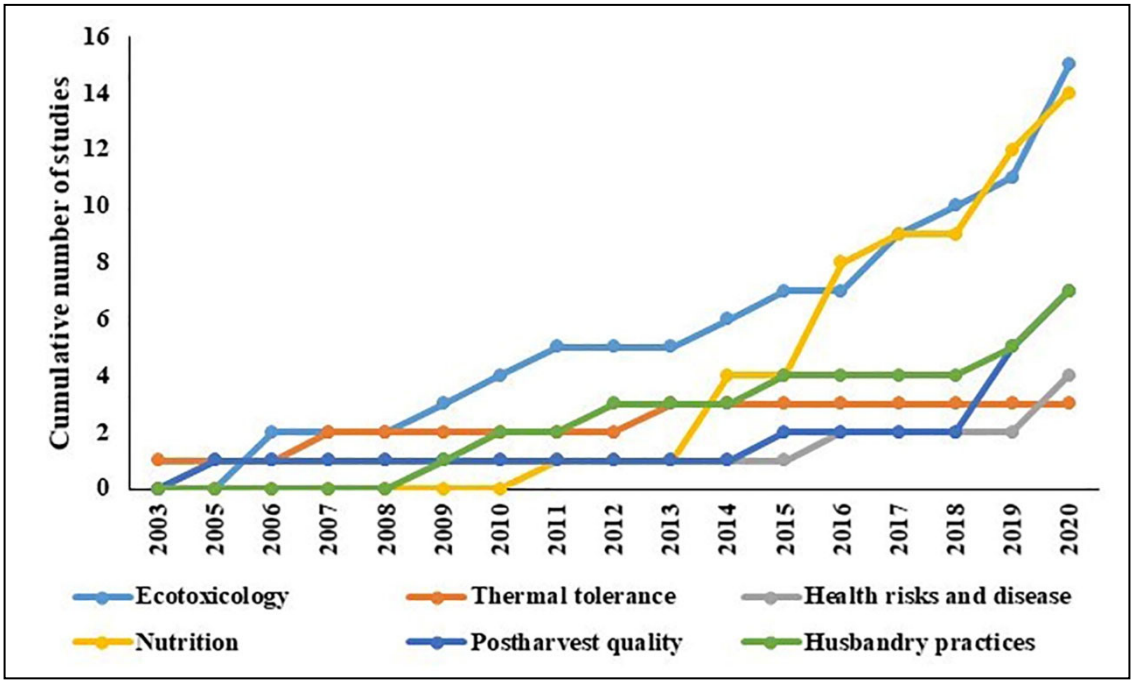


Figure 2.4 Evolution in metabolomics research themes in farmed salmonid aquaculture research. Data comes from 50 reviewed salmonid metabolomics studies from 2003 to October 2020.

In the subsequent sections, we summarise under each research theme, the use of metabolomics to unravel salmonid physiological response mechanisms. A synthesis section will provide key gaps under each theme and point out future research opportunities.

2.6.1 Ecotoxicology

Ecotoxicology is the study of the harmful effects of synthetic chemicals, their by-products, and breakdown substances on organisms at population, community and ecosystem levels (Zaidi & Imam, 2008). The application of metabolomics approaches in ecotoxicology is called environmental metabolomics (Samuelsson et al., 2006). The approach has been used to investigate effects of solvents and endocrine-disrupting chemicals in salmonids. Metabolomics can enhance visualisation of dose-responses, enable characterisation of mechanisms for unknown contaminants, allow biomarker identification and can detect specific changes in metabolomes.

Since the initial use of metabolomics in salmon research in the early 2000s (Samuelsson et al., 2006; Viant et al., 2006), over a dozen studies have been accomplished (Table 2.1).

Under this theme, metabolomics has been used to assess efficacy of sewage treatment systems (Samuelsson et al., 2011), and study toxicity of emerging contaminants (Meador et al., 2020), heavy oil spills (Lin et al., 2009; Van Scoy et al., 2010), synthetic hormones (Samuelsson et al., 2006; Samuelsson & Larsson, 2008) and pesticides (Magnuson et al., 2020b; Magnuson et al., 2020a; Olsvik et al., 2017a; Olsvik et al., 2017b; Olsvik & Søfteland, 2018; Softeland et al., 2014; Viant et al., 2006). Metabolomics has also been used to develop toxicity control strategies using bioactives (Hajirezaee et al., 2019; Lin et al., 2009; Olsvik et al., 2015; Van Scoy et al., 2010). We list in the following sections, mechanistic insights of toxicity and give some industry-specific metabolomics applications.

Samuelsson et al. (2011), used ^{1}H-NMR to determine the effects of different sewage effluents on the *O. mykiss* plasma metabolome, against targeted biological and chemical assays. Results revealed that sewage effluents treated by combined sand filter and high ozonation or moving bed biofilm reactor upregulated levels of high-density lipoproteins (HDL) and low-density lipoproteins (LDL), phosphatidylcholine, cholesterol, glucose, glutamine, and alanine. Plasma levels for very-low-density lipoprotein (VLDL) and glycerol lipids were downregulated. Findings suggested that advanced sewage treatment technologies disturbed fish energy, amino acid (AA), lipid metabolism, and induced stress hence a precaution for their use. Meanwhile, activated carbon did not cause modifications in the fish metabolome (Samuelsson et al., 2011). This study demonstrated the robustness of metabolomics against traditional assays in physiological assessments. A recent LC-MS investigation of juvenile *O. tshawytscha* exposed to varying concentrations of contaminants of emerging concern found in effluents revealed disruption to various metabolic processes in the liver. Medium and high doses affected metabolic pathways involved in the biosynthesis and metabolism of AA, lipids, purines, pyrimidines, and sugars. Results suggested adverse health implications with potential population-wide effects (Meador et al., 2020), further demonstrating the use in ecotoxicology monitoring.

Polycyclic aromatic hydrocarbons (PAHs) are abundant in environmental waters and are detected in fish and benthic invertebrates (Honda & Suzuki, 2020; Snyder et al., 2015). Being lipophilic, PAHs bio-accumulate in the fish liver (Honda & Suzuki, 2020). PAHs are immuno- and cardio-toxic, and fish exposure disrupts the endocrine system, disturbs liver lipid and bone metabolism, and causes embryo abnormalities (Honda & Suzuki, 2020; Snyder et al., 2015; Softeland et al., 2014). Some PAHs are genotoxic and induce cancerous mutations (Honda & Suzuki, 2020). In two studies, ^{1}H-NMR profiling was used to examine the toxic effects of crude oil and dispersed oil exposure in liver and muscle of *O. tshawytscha* pre-smolts and smolts (Lin et al., 2009; Van Scoy et al., 2010). Tissue metabolite analysis revealed enhanced stress via disturbance to citric acid cycle intermediates (succinate, glutamate,) AA metabolism (histidine, glutamine, glycine, proline, and methionine), energy metabolism (valine, leucine, isoleucine,

lactate, and ATP), kidney function (creatinine), and phospholipid metabolism (glycerophosphorylcholine). Authors resolved that identified metabolites served as toxic or stress biomarkers of crude oil exposure and informed response strategies to environmental oil spills (Lin et al., 2009; Van Scoy et al., 2010).

Endocrine disrupting chemicals (EDCs) include synthetic hormones, pesticides, plasticisers, dioxins, and fungicides (Samuelsson & Larsson, 2008). Samuelsson et al. (2006) assessed the physiological effects of environmental estrogen ethinylestradiol (EE) via ^{1}H-NMR analysis of *O. mykiss* plasma. High dose EE enhanced levels of plasma vitellogenin and phospholipids, while alanine and cholesterol were downregulated compared to non-exposed control fish. Results suggested that EE induced perturbations to reproductive, phospholipid, and energy-related metabolic processes, and thus the need for safe use and disposal (Samuelsson et al., 2006).

Agricultural pesticides are used to protect crops from disease, pests, rodents, and to ensure high crop quality and yield (Wang et al., 2020). Due to increased pesticide production and use in industrial farming, there is a growing threat to aquatic health (Chen et al., 2020). Pesticides are often based on organophosphate, pyrethroid, carbamate, and organochlorine chemistries (Yao et al., 2020). For review, see detailed descriptions of organophosphate and pyrethroid (Yao et al., 2020), carbamate (Oliveira et al., 2020), and organochlorine (Martyniuk et al., 2020; Pattnaik, 2020). Overall, high-dose organophosphate pesticides lead to neurological, respiratory, and cardiac disorders (Yao et al., 2020). Carbamates bio accumulate in tissues and disrupt the endocrine system (Oliveira et al., 2020). Organochlorines are lipophilic, toxic, carcinogenic, and can impair fish reproduction (Kumari, 2020; Martyniuk et al., 2020; Pattnaik, 2020). Organochlorine pesticides are banned in many countries (Samuelsson & Larsson, 2008) and are being replaced with biodegradable and less toxic pesticides. Some current use pesticides include mevinphos, ethoprop, dichlorvos, prometryn, terbutryn, and triadimefon, and are classified as emerging pollutants (Chen et al., 2020). Physiological exposure assessments of aquatic organisms are therefore important to determine for these compounds (Stultz et al., 2020).

The first study of metabolic effects of pesticides used LC-MS and ^{1}H-NMR to analyse toxicities of dinoseb, diazinon and esfenvalerate on *O. tshawytscha* eyed eggs and alevins (Viant et al., 2006). Dinoseb and esfenvalerate down-regulated adenosine triphosphate (ATP) and phosphocreatine (PCr), which suggested PCr utilisation to regenerate ATP lost in energy metabolism due to sub lethal stress. Diazinon was toxic to eyed eggs and alevins. Diazinon also disturbed several processes involved in eyed egg ATP production, the citric acid cycle and succinic acid production. Diazinon also induced anaerobic metabolism, lactic acid production, protein catabolism, and ammonia release. Thus, the pesticides induced major metabolic disturbances to early life stages in *O. tshawytscha* (Viant et al., 2006). This study demonstrated that metabolomics may therefore be used to guide the choice of agricultural and urban pesticides, due to high sensitivity when compared to traditional toxicity assays. Similarly, Softeland et al.

(2014) used ^{1}H-NMR and FT-ICR to investigate singular and mixed toxicities of endosulfan, phenanthrene and benzo (a) pyrene, organophosphate chlorpyrifos on *S. salar* hepatocytes *in vitro*. While NMR metabolomics data did not reveal changes caused by pesticides, FT-ICR data revealed toxicant specific effects as chlorpyrifos downregulated unsaturated fatty acid synthesis. Endosulfan and benzo (a) pyrene disturbed steroid hormone synthesis and vitamin D3 metabolism respectively (Softeland et al., 2014).

To showcase the application of metabolomics in solving toxicity problems in salmonid aquaculture, we highlighted a study by Olsvik and Søfteland (2018). Authors aimed to identify biomarkers of exposure to environmental levels of a legacy pesticide found in *S. salar* aquafeed. Researchers employed combined GC-MS and LC-MS to produce the metabolite profile of *S. salar* hepatocytes following 48 h exposure to 100 µM of the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT) derivative, dichlorodiphenyldichloroethylene (p,p′-DDE). Hepatocyte global metabolome assessment revealed that p, p′-DDE elevated energy molecules involved in bile acid metabolism and branched chain amino acids (BCAA) and phospholipid metabolites of diacylglyceral and sphingolipids. Results suggested that p,p′-DDE disturbed energy homeostasis, phospholipid metabolism, and antioxidant capacity. The authors pointed out that p,p′-DDE disturbed methionine metabolism and endocrine functioning (Olsvik & Søfteland, 2018). In other toxicity studies of lipid-soluble pesticides found in salmon feed, similar findings were reported. For instance, Olsvik et al. (2017b), profiled *S. salar* hepatocytes responses to bisphenol A (BPA) and plant-derived genistein. Genistein and BPA perturbed glutamate metabolism, energy balance, and production. While BPA affected methionine metabolism, genistein disturbed biosynthesis of nucleotides uridine and pyrimidine, important in liver detoxification (Olsvik et al., 2017b). Similarly, the organophosphate pesticide pirimiphos-methyl perturbed vitamins, phospholipid, energy and tryptophan metabolism and downregulated glutathione metabolism in *S. salar* hepatocytes (Olsvik et al., 2017a). These papers revealed the application of metabolomics to unravel previously unknown adverse effects of pesticides in salmon feed.

Recently, the toxicity of the pyrethroid insecticide bifenthrin was investigated in the brain of *O. tshawytscha* by Magnuson et al. (2020a) using LC-MS. Sub-lethal bifenthrin exposure downregulated brain levels of inosine, hypoxanthine, and guanosine. A similar study on brains of *O. mykiss* found that bifenthrin decreases levels for acetyl-l-carnitine, docosahexaenoic acid (DHA), and betaine (Magnuson et al., 2020b). Observed metabolic changes suggested that bifenthrin induced neurotoxicity via disturbance to brain regulatory processes involved in neuro functioning, apoptosis, necrosis, and oxidative stress (Magnuson et al., 2020b; Magnuson et al., 2020a). Both findings demonstrate that metabolomics may be used to unravel brain-specific biomarkers of pyrethroid insecticide exposure to salmonids or vertebrates in receiving aquatic ecosystems.

Metabolomics has also been used to design toxicity amelioration strategies. For instance, ^{1}H-NMR revealed that crude oil treatment with dispersant reduced toxicity in *O. tshawytscha* (Lin et al., 2009; Van Scoy et al., 2010). In addition, GC-MS and LC-MS approaches demonstrated that supplementary vitamin E eased chlorpyrifos toxicity in *S. salar* via blockade of long chain fatty acids (LC-FA), dipeptides and AA elevation, and enhanced carbohydrate use (Olsvik et al., 2015). Relatedly, ^{1}H-NMR showed that supplementary omega-3 eicosapentaenoic acid (EPA) or vitamin E lowered the toxicity of mixed PAHs (benzo (a) pyrene and phenanthrene) and pesticides (chlorpyrifos and endosulfan) by antagonising effects on phospholipid membrane function and vitamin D metabolism (Softeland et al., 2016). Finally, ^{1}H-NMR revealed that Ginkgo biloba herbal extract mid-range dose of 2 g kg^{-1} significantly reduced diazonin toxicity by upregulating metabolic processes involved in AA, energy, and osmotic regulation (Hajirezaee et al., 2019).

Overall, findings demonstrated that metabolomics strategies can allow the discovery of pollutant-specific exposure biomarkers associated with disturbances to energy, protein, phospholipid, redox, and vitamin metabolism, and pathways relevant to reproductive and neuronal functioning. Studies also showed that metabolomics may aid the design of amelioration strategies against aquatic pollutants.

2.6.2 Thermal tolerance

Fish have narrow thermal tolerance ranges, and deviations from the optimum temperature can have profound physiological effects (Ibarz et al., 2010). Low temperatures can induce changes in cellular structure, function, metabolism, protein folding, assembly, activity, and stability (Pan et al., 2008; Simon et al., 1995). Elevated temperatures can also depress metabolism (Aurélio et al., 2013), alter oxidative regulation, and affect immunity (Madeira et al., 2013; Vinagre et al., 2012). Therefore, fish mortalities occur due to cold snap and heat wave events (Younes et al., 2020). Despite its relevance in aquaculture, metabolomics studies on this theme are the least among salmonids. With global ocean temperatures expected to rise 1–4°C by the end of the century (Alfonso et al., 2020), the number of thermal-related experiments is likely to grow, driven by the need to develop resilient breeds for aquaculture, and better manage fisheries. For instance, recent incidences of marine heatwaves have resulted in salmon mortalities during summer months in New Zealand (Broekhuizen et al., 2021; Salinger et al., 2020; Salinger et al., 2019). In the sections below, we highlight the contribution of metabolomics towards the understanding of the physiological effects of thermal stress.

The effects of chronic elevated temperatures on *O. mykiss* were investigated through ^{1}H-NMR profiling (Viant et al., 2003). Metabolomics showed that a higher temperature (20°C vs. 15°C) induced thermal stress and downregulated levels of PCr and ATP in muscle, and glycogen levels in the liver. This result demonstrated the increased energy cost associated with maintenance at higher temperatures, with requirements for production of heat shock proteins (hsp) to regulate chronic heat stress (Viant et al., 2003). In a comparable study, Kullgren et al. (2013) assessed metabolic effects of elevated temperature in *S. salar*. ^{1}H-NMR plasma metabolomics revealed

that *S. salar* kept at elevated temperature (12 Vs 18°C) for three-months had lower levels of glutamine, tyrosine, and phenylalanine involved in energy metabolism. Higher temperatures also induced a shift in lipid metabolism. These findings suggested protein catabolism in *S. salar* exposed to elevated temperatures (Kullgren et al., 2013), to regulate thermal stress via enhanced hsp production. ^{1}H-NMR-metabolomics was used to understand response mechanisms to thermal stress using *O. mykiss* eggs incubated at 8°C or 18°C. Egg metabolomics established high variations in batches from different females but exhibited downregulation of tyrosine and phenylalanine, glycine, betaine, and choline levels at elevated temperatures. The same metabolites were elevated in eggs incubated at 8°C. Findings depicted catabolic AA and lipid metabolism and disturbance to oxidative regulation at elevated temperature (Turner et al., 2007).

These investigations highlight metabolomics application towards our understanding of physiological effects induced by thermal stress on energy, protein, non-essential AA, and lipid metabolism, oxidative stress, and thermal regulation in salmonids. This will enhance our knowledge of individual or population differences in physiological and fitness responses to climate change-associated effects on farmed salmonids (Alfonso et al., 2020).

2.6.3 Nutrition

Traditional biometric, proximate composition and digestibility techniques for conducting fish nutritional studies are insufficient to understand feed effects on fish metabolism (Roques et al., 2018c). Metabolomics has been used to characterise fish feed (Roques et al., 2018b) and is positioned to reveal the physiological effects of dietary manipulations (Roques et al., 2018c). In salmonids, several investigations have emerged with a specific focus on dietary supplements and the search for sustainable aquafeed ingredients. We present these contributions in the subsequent sections.

2.6.3.1 Effects of dietary or functional feed supplements

As global captured fisheries continue to decline, aquaculture has increased in importance (FAO, 2020b). Farmed fish in comparison with wild counterparts may vary in nutritional quality and health state, depending on diets (Li et al., 2020; Tewary & Patra, 2008). Besides satisfying nutrition, farmers administer functional feeds to improve feed digestibility and absorption, support fast growth, stimulate the gut and general fish health (Encarnação, 2016; Martinez-Rubio et al., 2013).

A paper by Coccia et al. (2019) used ^{1}H-NMR to assess the efficacy of hydroxytyrosol in an *ex vivo O. mykiss* intestinal model explant. Tissue ^{1}H-NMR profiles revealed that hydroxytyrosol enhanced essential amino acid (EAA) and essential fatty acid (EFA) profiles in the pyloric caeca and mid intestinal sections. Findings indicated that hydroxytyrosol prevented AA degradation and activated lipolysis. The study also suggested a model system for conducting preliminary studies for dietary supplements prior to actual large-scale works (Coccia et al., 2019).

Through NMR metabolomics, the effect of dietary glycerol on *O. mykiss* and European seabass (*Dicentrarchus labrax*) muscle and liver were studied (Palma et al., 2019). Liver and

muscle metabolic profiles showed that supplementary glycerol did not affect *O. mykiss* energy metabolism and choline involved in phospholipid metabolism, when compared to *D. labrax*. Results suggested species-specific response mechanisms to glycerol supplementation (Palma et al., 2019).

Wagner et al. (2014), investigated the physiological effects of supplementary sesamin using ^{1}H-NMR on *S. salar* liver and white muscle metabolomes. Liver and muscle data showed that high sesamin increased *S. salar* glucose, glycerine, leucine, valine, creatine, carnitine, and muscle creatine, PCr, lactate, and nucleotide levels. Results suggested elevated energy metabolism commensurate with the low growth rates and final body weights in tested fish. Elevated liver levels for BCAA and glucose in fish fed high dietary sesamin proposed disturbance to the citric acid cycle, which yielded less energy to support fish growth and metabolism. Phenotypic variables also exhibited reduced growth and condition factor at high inclusion levels. Under the observed physiological state, elevated creatine helped to transport energy to cells while increased levels of muscle metabolites suggested energy stress (Wagner et al., 2014). Findings reveal the complementary power of metabolomics to biometrics in developing novel aquafeed.

Proteins included in diets provide AAs to support growth, reproduction, maintenance, and to fuel normal metabolism, in carnivorous species (Ballantyne, 2001; Morin et al., 2020; Wilson, 2003). Using ^{1}H-NMR, Andersen et al. (2014), investigated the effects of long-term arginine supplementation on *S. salar* energy metabolism *in vivo* and *in vitro* via polyamine activation. In both cases, results showed that high arginine enhanced fish energy metabolism, revealed by decreased glucose, lactate, and fumarate; and increased lysine levels and survival in *S. salar* hepatocytes, independent of polyamine activation (Andersen et al., 2014). A follow-up plasma and liver LC-MS study demonstrated that supplementary arginine downregulated plasma valine due to use in energy metabolism. Downregulated plasma vitamin D3 suggested use in activated nitric oxide production or insulin production. Elevated liver biliverdin indicated oxidative stress as revealed by increased oxidised glutathione and prostaglandin F2 alpha methyl ether. Results suggested the link between dietary arginine, vitamin D and haemoglobin degradation to biliverdin to abate oxidative damage (Andersen et al., 2015).

Metabolomics has gained use in the search for alternatives to plant-based aquafeed ingredients. With FM-based protein sources being a major issue in aquaculture, a recent study by Roques et al. (2020a) highlights how metabolomics can be used to contribute to solving this challenge. Investigators used ^{1}H-NMR to assess the effect of increasing insect protein hydrolysates in a plant-based diet on *O. mykiss* feed utilisation using muscle, liver and plasma samples. Results revealed a dose-dependent increase in plasma, muscle, and liver metabolites involved in protein biosynthesis and energy metabolism. In agreement, there were observable differences in body weight, feed intake and utilisation with increasing inclusion levels of insect protein hydrolyte. Researchers concluded that insect protein hydrolysate can replace plant-based

protein sources in fish diets due to superiority in free dietary AAs and peptides required in protein biosynthesis and energy metabolism (Roques et al., 2020a).

In a similar contribution, Roques et al. (2020b) studied the metabolic effects of increased supplementary protein-rich baker's yeast (*Saccharomyces cerevisiae*) fractions (5, 10 and 15%) in a plant-based diet via the liver, muscle, and plasma ^{1}H-NMR analysis. Results showed increased muscle aspartate and glucose, while asparagine decreased in fish supplemented with 10% yeast protein. Both metabolites are precursors of oxaloacetate, used in the citric acid cycle where glucose is a major energy molecule. Thus, 10% yeast enhanced muscle energy production. Investigators achieved maximal glutamate in the liver at 5% yeast inclusion. Glutamate being a precursor for α-ketoglutarate used in the citric acid cycle, suggested optimal liver energy metabolism at lower yeast inclusion levels. Increased liver betaine with yeast supplementation suggested restoration of osmotic balance. Reduced plasma and muscle histidine with increased yeast inclusion proposed that a plant-based diet disturbed histidine metabolism, which is required to regulate buffering in plant-based diets. They concluded that, yeast provides EAA required in protein biosynthesis and energy metabolism, which corresponded with substantial increases in body weight and feed utilisation at higher yeast inclusion compared to controls (Roques et al., 2020b).

In summary, these studies demonstrate the potential of discovery-based metabolomics towards elucidating the beneficial effects of dietary supplements and plant-based protein alternatives (e.g., on energy production and protein biosynthesis to support somatic growth and fish health). Observed metabolic effects corresponded with phenotypic changes. These approaches establish the potential of metabolomics as a complementary tool to optimise aquaculture nutrition to boost production and health parameters.

2.6.3.2 Effects of fishmeal (FM) and fish oil (FO) replacement

The continued decline in global capture fishery, the high cost, and limited availability of FM and FO are likely to affect aquaculture production (Malcorps et al., 2019; Shepherd & Jackson, 2013). Over the past twenty years, research into alternative plant-based ingredients has reduced over-reliance on seafood ingredients. Plant-based protein sources alone have antinutritional factors and cannot be standalone substitutes for carnivorous fish (Roques et al., 2018c). This has driven the need to search for nutritional, low-cost and sustainable ingredients for complete or partial FM and FO replacement (Camargo et al., 2020). Metabolomics can reveal physiological influences of new and novel fish feed ingredients aimed at substituting FO and FM (Roques et al., 2018c).

Abro et al. (2014) used ^{1}H-NMR spectroscopy to explore the effect of complete FM replacement with zygomycetes (*Rhizopus oryzae*) biomass or commercial diet of unknown composition in *S. alpinus* liver. There were no differences in metabolic profiles between fish fed FM, and zygomycetes biomass. Fish fed FM and zygomycetes biomass had elevated asparagine, creatine, SN-glycero-3-phosphocholine, and formate than those fed on the commercial diet. There

was increased acetate and choline in fish fed FM than zygomycetes, and lysine, beta-alanine, glucose, and inosine in the zygomycetes than the commercial diet group. Results showed that FM and zygomycets have sufficient free AAs, such as choline to support protein biosynthesis and creatinine to enhance muscle ATP energy supply. Beta-alanine regulates oxidative stress and its metabolic involvement in the malonate semialdehyde pathway explains a higher abundance of acetate in zygomycetes fed fish. Metabolomics proved that zygomycetes biomass provided comparable physiological responses with FM in *S. alpinus* liver, while the commercial diet was inferior (Abro et al., 2014). Muscle NMR showed that 20% *R. oryzae* zit 102 FM replacement diet upregulated lactate and downregulated creatine and anserine in *S. salar* (Bankefors et al., 2011).

Researchers have also investigated partial FM replacement metabolic effects with several protein sources. In a model *O. mykiss*, liver ^{1}H-NMR metabolomics showed that FM replacement with higher inclusion levels of feather meal elevated glucogenic BCAA, tyrosine, methionine, arginine, and phenylalanine, oxidative products, and induced AA racemisation. Metabolomics established that elevated feather meal inclusion disturbed fish energy metabolism and ATP production via the citric acid cycle, while lower inclusion levels may be acceptable (Jasour et al., 2017). Likewise, the physiological effects of 40% FM replacement with either mussel meal, zygomycetes fungi, extracted baker's yeast, or non-extracted baker's yeast on liver and muscle lipid and metabolite profiles were studied using ^{1}H-NMR (Wagner et al., 2019). Results showed that non-extracted baker's yeast elevated muscle and liver betaine involved in energy metabolism and promoted N, N-dimethylglycine, and methionine metabolism. Zygomycetes enhanced liver levels of phosphocholine and phosphatidylcholine involved in phospholipid metabolism. Extracted baker's yeast elevated muscle levels of proline involved in oxidative regulation, while increase in BCAA suggested high protein energy catabolism. In muscle of fish fed mussel meal, higher 3-aminoisobutyric acid is important in BCAA metabolism, while elevated malonic acid is involved in pyrimidine and FA metabolism. Pyrimidine is a substrate for 3-aminoisobutyrate. The results demonstrated ingredient specific metabolic effects in liver and muscle with the least effect observed with mussel meal, a suitable ingredient to replace FM in *S. alpinus* (Wagner et al., 2019).

Physiological effects of combined FO and FM (Cheng et al., 2016a) or singular FO (Ruyter et al., 2019) replacement in salmon diets have also been investigated. The use of decontaminated Baltic sea-sourced alternative FO and FM were explored in *S. alpinus* liver and muscle using ^{1}H-NMR (Cheng et al., 2016a). Findings showed that, liver levels for BCAA, alanine, beta-alanine, glucose, glycine, methionine, phenylalanine, and tyrosine were elevated in fish fed contaminated FM and FO. These data suggested the presence of contaminants in Baltic sea-sourced raw ingredients as evidenced by disturbance to protein and energy intermediates of the citric acid cycle. Elevated liver choline indicate disturbance to phospholipid metabolism and possible oxidative damage. They observed further evidence for disturbance to energy metabolism as decreased and increased glucose and pyruvate in the muscle of fish that received contaminated

FO and FM. Decontaminated Baltic Sea-sourced alternative FO and FM reduce disturbances in normal energy homeostasis and hepatic toxicity. Findings showed the novelty of metabolomics approaches in the search for alternative fish feed ingredients (Cheng et al., 2016a).

Ruyter et al. (2019), investigated the potential use of engineered omega-3 Canola oil, rich in DHA and alpha linoleic acid (ALA) as a FO replacement in diets for *S.salar* fingerlings. Muscle GC-MS fatty acid analysis and liver lipidomics established that omega-3 rich Canola oil (DHA-CA) fed fish exhibited similar muscle EPA+DHA content as those that received FO. Metabolomics guided researchers to the conclusion that DHA-CA can replace FO in juvenile *S. salar* diets as a source for DHA. In the liver, the DHA-CA diet enhanced levels for omega-3 ALA and a higher DHA: EPA ratio. Results demonstrated the potential use of metabolomics strategies in the innovative global search for FO alternatives (Ruyter et al., 2019).

To summarise, metabolomics can provide valuable information on physiological effects following complete or partial FM replacement with novel nutritional protein ingredients. Effects of replacing FO with different FA sources in salmon feed further demonstrate the potential metabolomics may hold in the search for sustainable alternatives to FM and FO ingredients.

2.6.4 Postharvest quality

Quality assurance is an important aspect in aquaculture and is likely to gain attention because of the long-held belief that wild fish is of superior quality to farmed counterparts (Ma et al., 2020). Aquaculture farms must guarantee quality along various points of the value chain. Metabolomics strategies can be employed for postharvest quality management of fish (Samuelsson & Larsson, 2008; Shumilina et al., 2018; Shumilina et al., 2016).

Studies on fish quality build on fundamental targeted metabolomics profiling performed using GC-MS, LC-MS, HR-MAS-NMR, and DART-HR-MS for hypothesis testing purposes. The techniques have been used in salmonids to: quantity muscle total lipids, omega-3, DHA, and EPA content (Aursand et al., 2008; Aursand et al., 1993; Cai et al., 2014; Nestor et al., 2010), authenticate farmed fish and assign origin (Aursand et al., 2009; Axelson et al., 2009; Fiorino et al., 2019; Ghidini et al., 2019b; Ghidini et al., 2019a; Totland et al., 2017), identify freshness and quality indicators of K-value and trimethylamine nitrogen (TMA-N) (Heude et al., 2015), lipid species during chilled storage (Fiorino et al., 2018), and detect dye residues (Dubreil et al., 2019). Practical applications of metabolomics strategies to authenticate, monitor, and regulate the quality of farmed salmonids follow below.

In their study, Gribbestad et al. (2005) developed an ^{1}H-NMR protocol for the classification and authentication of *S. salar*. The platform enabled identification of individual AAs, hypoxanthine, FAs, anserine, and lactate, indicative of nutritional composition and quality of whole *S. salar* and muscle tissue, with high potential to validate fish (Gribbestad et al., 2005). This ^{1}H-NMR protocol is of particular importance as it can allow farmers and processors to classify whole fish or fillets and consumers to make choices based on nutritional value. The method also allows easy traceability and authentication of salmon products to prevent

mislabelling of farmed as wild in the market. Based on targeted methods used to distinguish farmed and wild salmon, ^{1}H-NMR was used to examine the effect of salmon feed on wild saithe (*Pollachius virens*), (Maruhenda Egea et al., 2015). Liver and muscle metabolomics revealed higher lactate, glutamine, glutamate, and alanine in wild *P. virens* caught around salmon farms. Cage foraging wild *P. virens* also had reduced choline and glucose levels compared to control wild fish, because of limitations in the salmon feed they consumed. Results suggested that wild *P. virens* caught near farms obtained most of their food from lost pellets and/or salmon faeces. This reflected the difference in tissue metabolic profile between the two groups. Elevated lactate and AAs indicated lactic acid anaerobic fermentation due to insufficient oxygen transport to fish tissues in cage foraging wild *P. virens* (Maruhenda Egea et al., 2015). Findings showed the novelty of metabolomics approaches to our understanding of the potential physiological effects of aquaculture on wild fish stocks.

In addition, a ^{1}H-^{13}C-NMR approach was used to monitor post-mortem spoilage in *S. salar* fillets stored at 0°C and 4°C, at day 0, 2, 4, 7, 10 and 14 (Shumilina et al., 2015). Fillet NMR profiling found 49 metabolites, suggestive of autolytic and microbial spoilage under chilled storage. Fillets stored at 4°C showed faster reduction in glutamate and glycine over the storage period, which signified microbial degradation. Histidine, beta-alanine, and 1-methyl-histidine breakdown were higher at 4°C than at 0°C due to autolytic spoilage. Meanwhile, increased acetic acid and succinic acid, ethanol, and 2, 3-butanediol at 4°C indicated microbial sugar fermentation. In addition, fillets stored at 4°C showed higher ATP breakdown K-value, and increased conversion of trimethylamine oxide (TMAO) to trimethylamine (TMA) than fillets stored at 0°C. Breakdown of tyrosine and lysine resulted in a specific increase of odorous tyramine and cadaverine in fillets stored at 4°C (Shumilina et al., 2015). LC-MS unravelled spoilage of farmed *S. salar* fillet and tuna during chilled storage. Metabolomics identified TMA as a spoilage biomarker, while a decrease in glucose followed by an increase in organic acids pointed to bacterial sugar fermentation. Thus, dominant bacterial genus *Photobacterium* converted tissue TMAO to elevated TMA and utilised glucose as a carbon source to enhance spoilage during chilled storage of vacuum-packed *S. salar* (Jääskeläinen et al., 2019).

Recently, ^{1}H-NMR metabolomics distinguished fresh from frozen-thawed *S. salar* fillets. Metabolomics identified aspartate as a biomarker that discerned frozen/thawed from fresh *S. salar* fillets. Increased aspartate in frozen/thawed fish was due to enhanced leakage and activity of aspartate aminotransferase (AAT) which transfers an amine group from 2-oxoglutarate to L-aspartate. Results suggested the potential use of metabolomics in routine fish quality management and monitoring (Shumilina et al., 2020).

Dubreil et al. (2019) developed an LC-MS protocol to identify biomarkers for illegal dye exposure in model *O. mykiss*. Tissue metabolite analysis detected triarylmethane like leucomalachite green (LMG) or deethyl-leuco-victoria pure blue BO (DLVBPO) in *O. mykiss* muscle and liver as dye residues. While victoria pure blue BO enhanced glychodeoxycholate,

malachite green reduced taurodeoxycholic acid, bile acids, which were identified as biomarkers for dye exposures (Dubreil et al., 2019). For a typical industry example, a study by Dubreil et al. (2020) demonstrates how metabolomics can help to regulate illegal chemicals in aquaculture. In this study, LC-MS was used to investigate the metabolism and tissue distribution of victoria pure blue BO over a 24 h exposure in *O. mykiss* and identify residue markers for illegal use. Results showed elevated VPBO in muscle, liver, skin, and plasma, with maximum levels in plasma and liver 2 h post-exposure. Following 64 days of depuration, elevated VPBO levels were detected in muscle and skin, and the compound DLVPBO was identified as a residue biomarker for exposure (Dubreil et al., 2020). Results further showed that metabolomics can be used by regulators to detect biomarkers suggestive of illegal chemical treatments in aquaculture products.

Overall, it is likely that metabolomics will be embraced as an effective management tool along the various points of the value chain to control chemical residues and product adulteration.

2.6.5 Health risks and disease

With the need to increase aquaculture to complement capture fishery landings, fish pathogens have emerged and caused heavy industry losses (Fazio, 2019). Alongside traditional diagnostic tools, metabolomics has enabled the discovery of pathogen-associated biomarkers (Liu et al., 2016) and amelioration strategies devised (Gong et al., 2020; Yang et al., 2020). Metabolomics has also enabled discovery of drug resistance biomarkers (Xu et al., 2019) and is used to explore underlying mechanisms (Jiang et al., 2020). Among salmonids, we found a handful of papers on this theme. In subsequent sections, we showcase the effectiveness of metabolomics in unraveling host-pathogen responses in farmed salmonids.

The first study employed ^{1}H-NMR to investigate the effect of *A. salmonicida* on *S. salar*, plasma metabolome 21 days post-infection (Solanky et al., 2005). Plasma spectra revealed that fish that survived *A. salmonicida* had reduced levels of triglycerides, LDL, phosphocholine, increased VLDL, cholesterol, and choline-based residues, with minor changes to AAs, and carbohydrates. *A. salmonicida* disturbed phospholipid metabolism and induced background effects on energy and protein metabolism and muscle activity. Thus, metabolomics may aid early disease diagnosis and enhance our understanding of poorly studied host-pathogen interfaces (Solanky et al., 2005). ^{1}H-NMR was used to examine the pathogenesis of *A. salmonicida* in *S. salar* kidney (Liu et al., 2016). Authors found significant deviations in levels for fumarate, alanine, valine, glycine, aspartate, choline, glycerophosphocholine, and betaine in infected fish at seven and 14-days post-infection. Detected metabolites are involved in pathways for citrate, glycolysis, tryptophan, and urea metabolism. Thus, *A. salmonicida* induced inflammation, inhibited AA absorption, disturbed protein biosynthesis and normal cell metabolism to favour own growth (Liu et al., 2016).

Viral pathogens are a major problem to salmon farming in many countries (Anderson, 1996; Diggles, 2016). Despite the scale of the problem, there are no metabolomics studies into mechanisms of viral pathogenesis in salmonids. However, research on salmonids may benefit

from preliminary observations made in other teleosts (Cho et al., 2017). Thus, we adapted a study that applied metabolomics to investigate viral effects in farmed crucian carp (*Carassius auratus*). Here, Tang et al. (2019) used LC-MS to study the mechanism of Cyprinid herpesvirus 2 (CyHV-2) in *C. auratus* serum. Investigators compared the metabolic profiles of infected dying fish and healthy *C. auratus* within seven days. Serum metabolite analysis revealed 75 differentially altered molecules between the dying and healthy animals. Serum metabolomics revealed elevation in levels of carbohydrates, FAs, AAs, and AA-associated metabolites in infected *C. auratus*. Results suggested that viral infection altered energy production by upregulating glycolytic, AA and phospholipid metabolism, to serve own replication (Tang et al., 2019). Findings demonstrate the *in vivo* infection mechanism of a viral agent which may be important in developing disease control and prevention strategies.

Among salmonids, recent studies with *O. tshawytscha* used GC-MS to explain physiological effects following *in vivo* administration of polyinosinic: polycytidylic acid [poly (I:C)] (Lulijwa et al., 2020a, 2020b). The first study investigated the response mechanisms to poly (I:C) 24 h post-stimulation. Serum metabolite analysis identified the downregulation of glycolytic carbohydrates and elevated levels for metabolites involved in BCAA/glutathione and phospholipid metabolism (Lulijwa et al., 2020a). The second study profiled liver and serum responses to poly (I:C) *in vivo*, 0-5 days post-administration (Lulijwa et al., 2020b). Metabolomics identified elevated liver and serum compounds involved in BCAA metabolism at day 1 and return to normal by day 5. Liver and serum metabolites involved in glycolysis were downregulated throughout the post-exposure period, while liver phospholipid metabolism fluctuated over the post-exposure period (Lulijwa et al., 2020b). Results revealed that poly (I:C) *in vivo* induced viral mimics in *O. tshawytscha* aimed at host-metabolome reprogramming to favour viral replication as noted in fish (Schiøtz et al., 2009).

In summary, metabolomics approaches may be an important tool in early disease diagnosis and will contribute to our understanding of host-pathogen relationships for poorly known viral, bacterial, fungal, and parasitic pathogens, common in the salmon farming industry.

2.6.6 Husbandry practices

With increasing consumer interest in farmed animal welfare, better knowledge of husbandry practices on fish welfare is gaining attention (Raposo de Magalhães et al., 2020b). Aquaculture farms aim to keep animals under optimal physiological conditions to achieve faster growth and meet production targets. Using metabolomics to unravel welfare biomarkers is a promising tool with great potential for optimising zootechnics and production.

2.6.6.1 Hatchery and broodstock management

Metabolomics holds the potential for improving hatchery husbandry practices. The approach has already been used in teleosts to identify biomarkers for sexual maturity (Zhou et al., 2017), segregate fish sexes (Ekman et al., 2015), assess gamete quality and maturation (Dietrich et al., 2019; Gribbestad et al., 2005; Xu et al., 2016; Yi et al., 2020), monitor growth and

developmental changes (Huang et al., 2013) and discover critical ovary development nutrients (Leng et al., 2019; Zhu et al., 2020).

We retrieved three studies in salmonids with a focus on this subject. Dietrich et al. (2019), compared *O. mykiss* and common carp (*Cyprinus carpio*) seminal plasma quality. Adipic acid, picolinic acid, homocitrulline and niacin were identified as *O. mykiss* specific seminal plasma metabolites. Most metabolites were identified to participate in oxidative stress regulation, energy metabolism and production, sperm cell apoptosis and survival, osmoregulation, and inflammation via free radical scavenging (Dietrich et al., 2019). Metabolomics may thus allow specific biomarker-based quality assessment of semen prior to cryopreservation for subsequent aquaculture use in seed multiplication or stock enhancement programmes (Dietrich et al., 2019). Second, CE-MS metabolomics revealed elevated muscle glycolysis in transgenic *O. kisutch* as a good biomarker for increased carbohydrate utilisation to supply energy in rapidly growing engineered salmon (Nakano et al., 2019). In the third study, ^{1}H-NMR profiling demonstrated potential to aid the identification of fillet and whole fish nutritional value biomarkers important in selective breeding programmes (Gribbestad et al., 2005). In this review, these two studies presented the only evidence of potential metabolomics application in salmon breeding, an approach that could benefit industry by boosting the discovery of biomarkers for favourable traits.

This is an emerging area of metabolomics research in salmonid aquaculture and the number of studies is expected to increase with time. Metabolomics is a useful tool that may be adapted to aid the design of selective breeding programmes for enhanced salmon performance and production.

2.6.6.2 Effects of handling stress

Although metabolomics studies on farmed fish welfare are scarce, research highlighting the effects of handling practices is emerging. The following papers reveal how metabolomics has enabled the study of physiological mechanisms of handling stress. Among salmonids, the first study used ^{1}H-NMR spectroscopy to assess the physiological effects of long-term daily netting for 15 s to *S. salar* (Karakach et al., 2009). Plasma metabolomics established that long-term handling stress, upregulated and downregulated levels for LDL, VLDL, and lipids at week one and two, which suggested changes to lipid and phospholipid membrane metabolism. Elevated plasma levels for TMAO depicted lipid metabolism, while increased valine, alanine, carbohydrates, and lactate at week two suggested stress. Higher lactate and AAs were used as glucose precursors, to replenish the energy needed to cope with handling stress (Karakach et al., 2009).

In a pioneering study, Young et al. (2019) used GC-MS to assess the effects of acute handling, euthanasia, and sedation on *O. tshawytscha* plasma metabolome. Results revealed that acute stress disrupted plasma osmoregulation, induced anaerobic metabolism, altered protein metabolism, and changed ammonia recycling. Sedation with clove oil and AQUI-S anaesthetics induced spikes in plasma glycolytic, respiratory, citric acid cycle carbon flux, and FA profile, all

indicative of mild stress activation. The study signified the need to standardise fish handling practices when taking samples for physiological assessments and during routine husbandry practices (Young et al., 2019).

NMR was used to investigate the physiological effects of clove oil in *O. mykiss* (Rahimi et al., 2020). Plasma metabolite PLS-DA modelling established that clove oil elevated levels for valine, isoleucine, creatine, N, N-dimethylglycine, and glucose, while N-acetylglucosamine and succinate were downregulated. Results suggested that clove oil induced changes in fish energy, AA, and phospholipid metabolism (Rahimi et al., 2020).

In an exemplary industry study, Lazado et al. (2020) used LC-MS to investigate the effects of low and high doses of therapeutic exposure to peracetic acid for 5 min, and re-exposure for 30 min two weeks later on *S. salar* metabolome. External welfare assessments including damage to the skin, pectoral fin and dorsal fin were not compromised by the peracetic acid doses used at both exposure times. Plasma metabolite analysis yielded no major effects of peracetic acid. However, individual metabolite examination revealed that oxidative stress regulatory tryptophan, methionine, citrulline, histidine, and trans-4-hydroxyproline, were perturbed irrespective of dose. This demonstrated a response mechanism by fish to increased release of reactive oxygen species in the water following exposure to the oxidant. Thus, peracetic acid did not induce strong systemic changes, re-enforcing its chemotherapeutic use in *S. salar* production as an oxidising therapeutic agent to improve culture conditions, prevent infection and promote welfare (Lazado et al., 2020).

Taken together, these studies suggested that novel metabolomics approaches may aid our understanding of metabolic effects induced by daily fish handling practices at the farm level, with an eventual contribution to future welfare improvement and production optimisation.

2.6.6.3 Effects of starvation

Starvation is a practice used to manage fish stocks prior to grading, harvesting and slaughter or marketing (Bugeon et al., 2004). Starvation also limits water quality deterioration during transport (Caruso et al., 2012). In temperate regions, fish are starved in winter by lowering their metabolic activity to conserve energy (Echevarría et al., 1997). Starvation induces the use of glycogen, lipid, protein, and liver gluconeogenesis in teleosts. In the first 90 days of starvation, fatty fish show increased lipid metabolism and white muscle protein catabolism, while lipid metabolism dominates in lean fish. From 91 to 150 days, fatty fish resort to carbohydrate metabolism, while lean fish utilize protein due to lipid and carbohydrate exhaustion (Echevarría et al., 1997). The starvation process involves a short transition period, a long protein conservation phase where lipids are used for energy, and a protein-energy catabolic state. The shift from lipid to protein catabolism is determined by the critical fat levels, which range between 0.7-5.0% (Bar, 2014). Metabolomics is used in farmed teleosts to investigate effects of short-term (Baumgarner & Cooper, 2012; Jiao et al., 2020; Kokushi et al., 2011; Kullgren et al., 2010; Mekuchi et al., 2017) and long-term (Cipriano et al., 2015; Gillis & Ballantyne, 1996; Segner et al., 1997)

starvation, albeit with a paltry number of studies involving salmonids. Once again, this is an emerging area of interest in salmonids, and papers are anticipated to increase over time.

Investigating short-term starvation effects, Kullgren et al. (2010), deprived *O. mykiss* juveniles of food for 28 days. Plasma, muscle, and liver metabolites were analysed by ^{1}H-NMR metabolomics. Plasma results showed increased and decreased levels for VLDL and HDL, respectively. Results confirmed lipid involvement in energy metabolism during fasting. Starvation also elevated muscle levels for alanine, which suggests protein catabolism to support liver glycogen levels. In the liver, glycogen increased in fasted fish signified a metabolic strategy to cope with fasting (Kullgren et al., 2010). The study showed the importance of metabolomics in understanding physiological state during periods of limited food availability such as during winter and under routine husbandry practices of fasting prior to grading and harvesting.

In addition, tandem GC×GC-MS was used to test the effect of starvation on *O. mykiss* serum, liver, and muscle metabolism. Starvation downregulated serum LC-FA levels for octadecanoic acid, tetradecanoic acid, PUFAs of arachidonic acid (ARA), DHA and several short-chain fatty acids (SC-FA). Serum levels of alanine, glutamine, glycine, and serine also decreased in starved fish. Observed FA and AA results indicated use in energy metabolism. In the liver, starvation upregulated levels for oleic acid and tetradecanoic acid, while depleted octadecanoic acid and pentanoic acid suggested selective hepatic lipid energy catabolism during starvation. Starvation also elevated liver levels for methionine, proline, and lysine by-product 2-piperidine carboxylic acid suggestive of increased hepatic protein catabolism. Starvation induced muscle differential lipid metabolism as demonstrated by depleted levels for hexadecanoic acid and elevation of octadecanoic acid and tetradecanoic acid (Baumgarner & Cooper, 2012). Findings divulge the effectiveness of metabolomics in whole salmon physiological assessments.

Long-term starvation (>30 days) effects have also been examined. Cipriano et al. (2015), used GCxGC-MS to compare serum metabolomes of a cohort of inappetent sea-run and captive *S. salar*. Serum profiling found no starvation stress (ketosis) in inappetent fish. However, sugar alcohols and omega-9 monounsaturated FAs were elevated, while long chain polyunsaturated fatty acid (LC-PUFA) of EPA, DHA, and ALA were downregulated in inappetent fish. Findings suggested differential FA metabolism for energy during fasting; while depleted serum BCAA proposed general use in proteolysis to support energy demands in inappetent *S. salar*. Diminished alanine and other AA alterations reflected changes to metabolism and biosynthesis (Cipriano et al., 2015). The paper demonstrated the usefulness, suitability, and sensitivity of metabolomics in unravelling physiological mechanisms in salmonids under less known conditions.

In salmonids, lipid reserves in the mesentery, muscle and liver serve in sequence as depots for energy metabolism and production (Sheridan et al., 1985). Overall, the literature suggested that metabolomics is a suitable tool for understanding the effects of starvation practices on FA, protein, and glycogen metabolism in salmonids.

2.6.7 Areas for improvement in salmonid metabolomics

In less than two decades since initiation in salmonid aquaculture research, metabolomics has shown to have a huge potential to enhance our understanding of salmonid physiology and metabolism. We envision that metabolomics will help to hasten the process involved in identifying sustainable solutions to some of the industry's growing list of challenges. We identified some research gaps and future research directions in this regard.

NMR and MS-based platforms coupled to chromatography were the most popular, with multi-platform use being rare. Application of multiple platforms and other instruments (e.g., CE-MS, and DFI-MS) may broaden the repertoire of detectable metabolites. While platform selection is often driven by funding limitations and/or instrument availability, efficient experimental designs may overcome some of these difficulties. For instance, a clear study goal with a budget in mind will direct sample choice (e.g., plasma or serum), size and the appropriate analytical platform (Young & Alfaro, 2018). We highly recommend that future studies involve the use of two or more analytical platforms for discovery-based metabolomics projects. Among omics, metabolomics generally receives the least funding, and more equitable distribution would enhance the immense value this approach brings onboard (Pinu et al., 2019). To fast-track industry uptake, institutional collaborations need to be enhanced, while manufacturers should focus in part on the development of handheld or bench top instruments for field application (Giraudeau, 2020).

Salmon muscle, liver and plasma were the most common sample types in metabolomics research. However, studies in other teleosts have established the use of non-invasively collected skin mucus and stool samples (Ekman et al., 2015; Hano et al., 2021). While the use depends on the specific research question, such samples may provide informative data, for repeated measures and temporal studies. Meanwhile, lack of specificity for liver, kidney, muscle, and brain sampling locations may also make data verification difficult since organ metabolomes are heterogeneous by nature. There is also the need for streamlining sampling and laboratory sample processing protocols. This is due to the wide variation in how researchers handle fish prior to or during sampling. For instance, recent studies have shown that the use of sedatives and handling stresses prior to sampling alter the fish metabolome which may affect interpretation of results (Rahimi et al., 2020; Young et al., 2019). Furthermore, the lack of standardisation can make cross-study comparisons difficult. Thus, standardisation of pre-sampling protocols and best-practice reporting in fish metabolomics is still an area for improvement. The Metabolomics Standards Initiative provides general guidelines for this purpose (Fiehn et al., 2007), and the use of the ARRIVE guidelines (Kilkenny et al., 2010) for fish-based research should be implemented during the design and reporting stages.

Salmonid ecotoxicology literature is underrepresented regarding studies investigating the effects of current use pesticides and microplastics (Chen et al., 2020; Vázquez et al., 2021). Although their toxicities may be established from other biological endpoints, metabolomics may provide insights at a species and/or environment-specific level. In addition, the biodegradation

mechanisms of sex reversal hormones used in commercial salmon production is another area falling within this research theme where metabolomics could deliver value (Judycka et al., 2021).

A changing physical environment due to climate change is of concern in many regions where water temperatures already are close to or fluctuate at the boundary of thermal limits for different species. As salmon aquaculture grows, extending our reach into new regions and climes necessitate detailed understanding of fish resilience to changes in key physico-chemical parameters (Melis et al., 2017; Wen et al., 2019). We may feed metabolomics resilience data into selective breeding programmes. In addition, studies of synergistic effects of starvation/nutrition and thermal stress (Chauton et al., 2015; Jiao et al., 2020; Silva et al., 2014) at metabolic levels are important aspects to investigate. These approaches may provide scope to better design winter diets aimed at enhancing growth and pathogen resistance.

Nutritional studies showed that single cell proteins (Molnár & Pal, 2020), functional feeds (Encarnação, 2016), supplementary choline (Hansen et al., 2020), tryptophan (Cerqueira et al., 2020), taurine (G. Shen et al., 2018; Shen et al., 2019), and methionine (Espe et al., 2008) benefits fish health and wellbeing. However, the precise mechanisms are not resolved and is an area where salmon metabolomics could provide insights. Metabolomics also offers novelty to identify sustainable FO/FM replacement ingredients (Jin et al., 2015; Schock et al., 2012; Watson et al., 2020). In addition, use of proton or carbon (^{1}H or ^{14}C) labelled supplements in metabolomics could allow precise tracking of these additives. Thus, metabolic flux remain unexploited in aquaculture nutrition (Lee & Go, 2005).

Biochemical characterisation of farmed salmon will help to rectify the long held belief that wild fish is of superior quality to farmed counterparts (López-Mas et al., 2020; Ma et al., 2020). Another interesting field of research regards the metabolic profiling of vegetable oil FAs effects on salmon metabolome and fillet texture. Studies with targeted approaches have linked high aqua-feed inclusion levels of vegetable origin ALA with fillet softness and gapping in farmed fish (Torgersen et al., 2014; Totland et al., 2017). Metabolomics may also aid characterisation of bioactive compounds of nutritional or medical value from farmed fish by-products or waste.

Identification of disease-specific biomarkers and mechanisms could enable design of amelioration strategies to enhance fish resistance against pathogens. Functional roles of metabolites may be exploited to identify prospective drugs or critical metabolites for rectifying a pathology through metabolome reprogramming (Peng et al., 2015a). Metabolome reprograming involves exogenous administration of the key metabolites to normalise a pathological state, with subsequent improvement of the biological phenotype (Peng et al., 2015b). The strategy remains under researched in salmonids and offers a more sustainable and greener strategy for managing aquaculture pathogens when compared to antibiotic prophylaxis and therapy.

Husbandry metabolomics offers huge potential in salmonid breeding and production improvement, similar to application in terrestrial animals (Goldansaz et al., 2017). For instance, metabolomics can be used to identify biomarkers of importance to gamete quality and

development, broodstock fecundity, critical larval nutrition, and enhanced survival. Metabolomics may also aid discovery of biomarkers for fast growth, feed efficiency, and fillet yield, helping to shape breeding programmes. Despite its limited use in salmon breeding programmes (Gribbestad et al., 2005; Nakano et al., 2019), integration with genomics data can enhance desirable trait prediction accuracies (Goldansaz et al., 2017). Further integration of metabolomics data with methylome (epigenetics) data (Gavery & Roberts, 2017) may unearth novel environmental mechanisms of aquaculture value. In addition, studies on handling stress may focus on identifying biomarkers of different culture systems, environments, and daily husbandry practices to improve welfare and optimise performance.

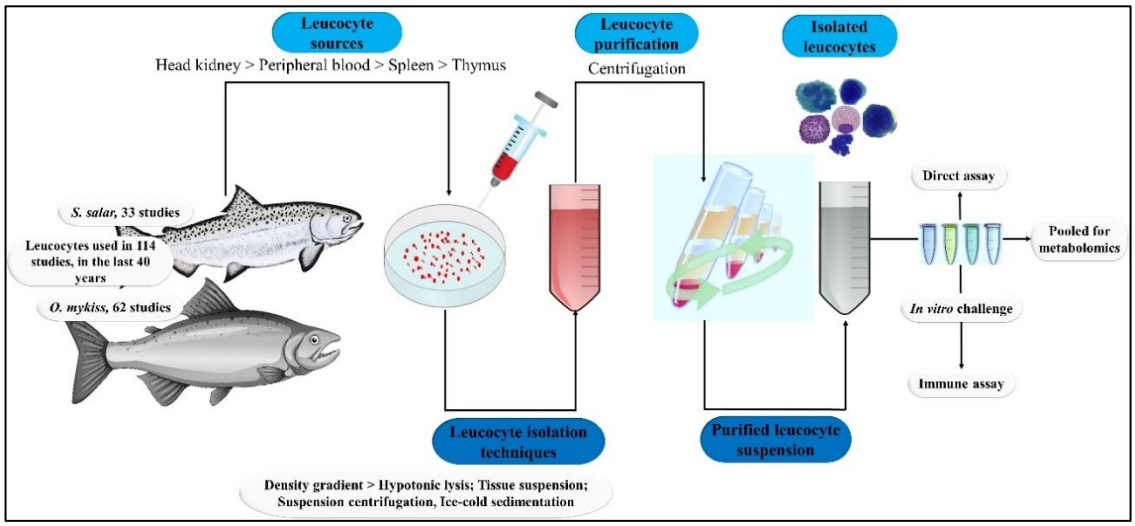
Although metabolite structures are the same across taxa, metabolic pathways can differ, even if subtly between vertebrates at species level (Palma et al., 2019). Some studies used databases constructed for human metabolism to aid analysis and interpretation of findings. Using such databases may lead to spurious findings when assessing metabolomics data in fish models at the biochemical pathway level. However, increasing availability of fish metabolite data should facilitate development of a fish metabolome database akin to the existing proteomic and genomic databases such as MetaFishNet (Li et al., 2010) and The Livestock Metabolome Database (Goldansaz et al., 2017). Coordination across major laboratories will enable identification of the growing list of unknown metabolic features. This approach will enable aquatic scientists to improve their accuracy and precision regarding synthesis and interpretation of fish metabolomics data.

2.6.8 Conclusions

Metabolomics can provide insights into physiological processes via identification of metabolites responsible for differences in individual fish exposed to a specific stimulus. Metabolomics has evolved over the last couple of decades with improvements in analytical platforms and computation. To enhance our knowledge of the salmon metabolome and increase industry benefit, future studies need to increase the use of MS-based platforms, adopt NMR innovations, and ideally employ multiplatform use in discovery-based studies. To enhance industry uptake, institutional collaborations need to be enhanced, while manufacturers should focus in part on the development of handheld or benchtop NMR instruments for field application (Giraudeau, 2020; van Beek, 2021). Adoption of non-invasive sampling will be very useful in longitudinal and repeated measures studies. Researchers will also have to streamline pre-sampling and sample processing protocols and embrace standard reporting guidelines. We propose studies on current use pesticides, sex reversal hormones and toxin amelioration strategies. Others should focus on mechanisms of climate change adaptation, starvation, functional feeds, dietary supplements, bioactives, alternatives to FM/FO, and incorporate metabolic flux analysis. Metabolomics can also be used to characterise farmed products, including effects of vegetable oils on fillet quality. Other areas include studying host-pathogen interactions and metabolome re-programming. In husbandry, researchers should place emphasis on identification of biomarkers

for welfare and predicting major production traits via integration with epigenetics and genomics tools. Laboratory coordination should facilitate establishment of a fish metabolome database and enable identification of unknown features. These will help aquatic researchers improve on accuracy and interpretation of metabolomics findings.

3 Chapter 3: Literature Review 2. Advances in Salmonid Fish Immunology: a review of lymphoid tissue and peripheral blood leucocytes methods and techniques.



Graphical representation of the techniques used to isolate salmonid leucocytes for assaying.

This chapter has been published:

Lulijwa, R., Alfaro, A. C., Merien, F., Meyer, J., & Young, T. (2019). Advances in salmonid fish immunology: A review of methods and techniques for lymphoid tissue and peripheral blood leucocyte isolation and application. *Fish & Shellfish Immunology*, *95*, 44-80. https://www.sciencedirect.com/science/article/pii/S1050464819309659

3.1 Prelude to literature review 2

Rapid aquaculture development has come with the emergence of previously less known aquatic pathogens, driving the need for developing immunological techniques in aquaculture. Fish health assessments are routinely accomplished for instance using lymphoid and peripheral blood mononuclear cells (PBMCs) functional assessments. But despite several researchers analysing and interrogating published literature on fish immunology, there has been less focus towards reviewing literature on the use of salmonid isolated lymphoid leucocytes and PBMCs in immunological studies. Indeed, previous reviews focussed on characterisation of the fish mucosal immunity and immune system development. Others focussed on the fish innate humoral immunity, profiling granulocytes, understanding of macrophage biology and defence mechanisms, immune genes, and cytokines. Despite the limited reviews on salmonid isolated leucocytes use in immunological studies, these cells have extensively been applied to understand immunomodulatory effects caused by for example dietary manipulations, environmental stressors, pathogen challenges and vaccine development. This was therefore the first extensive salmon review that focussed on existing scientific evidence regarding the use of isolated leucocytes in immunology. It informs researchers and aquaculture practitioners in the industry of less costly, time saving and ethically flexible techniques that may be embraced to isolate fish leucocytes for immunological assessments. The review also provided information on the most used immunological assays. Using data from salmonids, this chapter provided a compilation of immunological techniques that could be consulted or modified for use by scientists working with under researched species. The review also suggested the integration of metabolomics, flow cytometry with traditional assays to open innovative approaches in fish immunology studies.

[1]Abbreviations

[1] PBL: peripheral blood leucocytes, TKL: total kidney leucocyte, HKL: head kidney leucocytes, MKL: middle kidney leucocyte, PKL: posterior kidney leucocyte, ATK: anterior part of trunk kidney, SPL: spleen leucocytes and THL: thymus leucocytes, F: frequency. HK: head kidney, PB: peripheral blood, SP: spleen, TH: thymus. DDGC: discontinuous density gradient centrifugation, CDGC: continuous density gradient centrifugation. HL: hypotonic lysis, ICSC: ice-cold sedimentation and centrifugation, SC: suspension centrifugation, and TS: tissue suspension. CL: calorimetry, FC: flow cytometry, PCM: phase contrast microscopy, LM: light microscopy, EFM; epifluorescence microscopy, FM: fluorescence microscopy, SEM: scanning electron microscopy, SP: spectrophotometer, MMR: multimode microplate reader, LU: Luminometer, CC: colony count, MPF: Microplate fluorometer; ER: ELISA reader.

3.2 Introduction

For almost four decades, global aquaculture has been responsible for the increased amount of fish consumed, surpassing population growth to become the world's fastest growing food production primary industry (FAO, 2014, 2018c). In the past five years, over 50% of food fish has come from aquaculture (FAO, 2014), and is tipped to contribute 60% of all food fish by 2030 (FAO, 2018c). The sector is thus poised to be the main protein source for the world's estimated 8.6 billion people by 2030 (Worldometers, 2019). To meet the growing global demand for seafood, aquaculture farms aim to produce high quantity of healthy and fast-growing fish, under optimal husbandry and management practices in limited water volumes (Martinez et al., 2016). Husbandry practices include, but are not limited to maintaining good water quality, proper feeding, and biosecurity protocols with the aim to safeguard fish health.

Several intrinsic and extrinsic factors, including physical and chemical stressors have the ability to alter the fish innate immunity (Uribe et al., 2011). One of the sustainable practices recommended to enhance fish immunity and promote growth involves the administration of dietary supplements and immunostimulants (Magnadottir, 2010; Magnadóttir, 2006). Application of immunostimulants is preferred over the use of antibiotics, which have been associated with food safety concerns (Chen et al., 2018) and occupational health hazards (Phu et al., 2016). Antibiotic use in aquaculture has also been associated with antimicrobial resistance (X. Liu et al., 2017), residue accumulation (Wang et al., 2015), aquatic toxicity, antibiotic resistant microbial community selection (Buschmann et al., 2012; Samuelsen et al., 2014), and the emergence of multi-antibacterial resistant strains (Chuah et al., 2016). Farmed fish often co-exist with a multitude of bacteria, viruses, fungi, and parasites, which exploit lapses in optimal culture conditions to proliferate and compromise the innate immune system. Indeed, disease outbreaks routinely lead to heavy industry losses [reviewed in Fazio (2019)]. These scenarios make fish immunology understanding an indispensable asset to allow routine detection of health problems [reviewed in Uribe et al. (2011)].

Salmonid production is dominated by Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), with a contribution of 6% of the world's finfish aquaculture production in 2016 (FAO, 2018c). *S. salar* is the ninth most farmed finfish species globally (FAO, 2018a). Major farmed salmonid producers include Norway, Chile, Scotland, and Canada (Asche et al., 2013). In the salmon farming industry, major losses have been caused by the caligid copepod sea lice (*Lepeophtheirus salmonis* and *Caligus elongatus)* (Pike, 1989). *In Chile, b*acterial kidney disease (BKD), *Piscirickettsia salmonis*, and infectious salmon anaemia virus (ISAV) have devastated those industries (Lozano et al., 2018) and *P. salmonis* remains the biggest industry health challenge (Miranda et al., 2018). In Norway, *L. salmonis* remains a major salmon health challenge (Overton et al., 2019), and pancreases disease (PD) caused by *Salmonid alphavirus* (SAV) and ISAV (Norwegian Veterinary Institute, 2018). In Scotland, *L. salmonis*, is the number one challenge (Kenyon & Davies, 2018; Marine Scotland, 2012); while furunculosis caused by

Aeromonas salmonicida, and Piscine Reovirus (PRV) poses the biggest problem in British Colombia, Canada (Pens, 2017).

The role of immunity in fish is to act as a barrier that ensures protection against pathogens and maintains homeostasis (Lieschke & Trede, 2009; Zhu et al., 2013). The immune system consists of the innate components, which form the first line of defence [reviewed in Ángeles Esteban (2012); Martin and Król (2017)]. The adaptive immune components complement the fish immune machinery and rely on humoral and cellular responses, characterized by specific antigen recognitions, which invoke a quick secondary pathogen-specific response (Martin & Król, 2017). Thus, fish rely on both immune components for defence (Sahoo et al., 2021; Whyte, 2007). For literature on the cellular specifics and functions of the innate and adaptive immune components of fish, see literature in (Lulijwa et al., 2019a) and Figure 3.1. For details on specific molecules produced by the physical and humoral components, see (Gomez et al., 2013; Salinas & Miller, 2015). The lymphoid organs of fish include the head kidney (HK), spleen (SP) and thymus (TH) a paired organ, located in the dorsolateral area of the opercular cavity in teleosts (Trede & Zon, 1998). Teleost's T cell development takes place in the thymus and B cells in the head kidney (Lieschke & Trede, 2009; Trede & Zon, 1998). Thus, to conduct immunological studies, fish leucocytes must be purified from lymphoid organs and peripheral blood by hypotonic lysis and density gradient centrifugation (Crippen et al., 2001; Hu et al., 2018; Pierrard et al., 2012). Possession of the adaptive and innate immune capacity makes fish an important immunological model to investigate vaccine design, efficacy and vaccination strategies, dietary manipulations, including effects induced by pathogens and stressors.

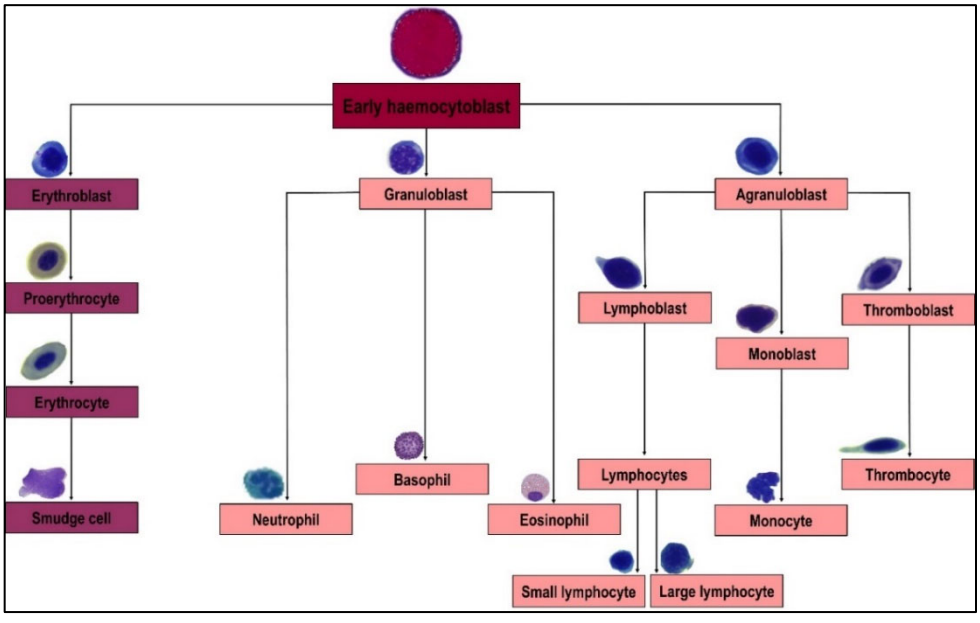


Figure 3.1 Schematic representation of salmonid red blood and white blood cells haematopoiesis. Most of the cells come from slide smears of Chinook salmon (*Oncorhynchus tshawytscha*) peripheral blood. Only basophil, eosinophils are adapted from Claver and Quaglia (2009) and haemocytoblast from (Mumford et al., 2007). NB: as monocytes age, they differentiate into macrophages (Merien, 2016). Other cells not shown include dendritic cells (DC) and mast cells (MC).

In studying salmonid fish immunology, several approaches are commonly used, including characterisation of physical barriers (Martin & Król, 2017), peripheral blood (PB) haematology (Lulijwa et al., 2019a; Markkula et al., 2009; Petropoulos et al., 2009; Vikeså et al., 2017), HK, PB and SP isolated cellular functional characterisation (Arkoosh et al., 2018; Johansson et al., 2016; Rønneseth et al., 2013; Ulvestad et al., 2018), and humoral parameters (Alcorn et al., 2003; Desvignes et al., 2002; Sharifuzzaman & Austin, 2010). Consequently, integrated haematology, and the study of humoral and cellular parameters have been employed in salmonids to investigate immunomodulatory effects of: dietary manipulations (Peña et al., 2016; Soto et al., 2016; Ulvestad et al., 2018); pathogen challenges (Julin et al., 2015; Rønneseth et al., 2013); physical and chemical stressors (Arkoosh et al., 2015; Arkoosh et al., 2018; Jokinen et al., 2011; Taylor et al., 2007).

In fish immunology, previous reviews have focussed on: developmental aspects of the fish immune system (Bruce & Brown, 2017; Lieschke & Trede, 2009; Zapata et al., 2006), innate immunity (Magnadóttir, 2006; Rebl & Goldammer, 2018; Uribe et al., 2011), humoral immunity (Magnadóttir, 2006), cellular granulocytes (Ainsworth, 1992; Hine, 1992), macrophage biology (Hodgkinson et al., 2015) and macrophage antimicrobial mechanisms (Grayfer et al., 2018), immune related genes (Zhu et al., 2013), cytokines (Secombes, 2016), plus mucosal immunology (Gomez et al., 2013; Rombout et al., 2014; Salinas & Parra, 2015; Salinas et al., 2011). However, no review has previously focussed on the use of isolated salmonid lymphoid tissue or PBL to illustrate immunological effects induced by dietary manipulations, vaccines and vaccination strategies, pathogen challenges, and effects induced by stressors. Literature on the use of isolated lymphoid tissue and PBL in immunological investigations has been accumulating across salmonid species since the 1980s, forming the basis for the current review. To achieve our objective, we searched using the terms “fish leucocytes*immunology/immunological assays” in the Web of Science and “fish AND leucocytes AND salmonid AND immunology” in Scopus. Only papers on salmonids were considered by title and quick abstract scan for the key words isolated leucocytes, immune or immunological response, and/or characterisation. Subsequently, we highlight the major findings of this review with respect to salmon immune understanding. We also mention developed and characterised salmonid immunological cell lines, identify research gaps, and make recommendations for affordable, nonlethal, and ethically appropriate techniques.

3.3 Commonly studied salmonids and target tissues/organs

The current review found 114 studies that discussed the isolation and use of organs and PBL (Table 3.1). These studies spead accross seven salmonid species, including Coho salmon (*Oncorhynchus kisutch*), *O. mykiss*, *O. tshawytscha*, sockeye salmon (*Oncorhynchus nerka*), brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*), and *S. salar*. Most of the immunological studies were carried out using farmed *O. mykiss* and *S. salar* (Table 3.1). Although lymphoid organs, including SP, HK, and the TH have been used to illustrate immunological functions of isolated leucocytes among different salmonids, the majority of immunological

investigations involved isolating cells from the HK, PB and SP in order of popularity are listed in Table 3.1.

Table 3.1. Commonly studied salmonid species and popular lymphoid tissues or organs for isolation of fish leucocytes in fish immunology. PBL: Peripheral blood leucocytes, HKL: Head kidney leucocytes, SPL: Spleen leucocytes, and THL: Thymus leucocytes. F: Frequency. Data comes from 114 reviewed salmonid studies.

Studied salmonid species	Studies reviewed	PBL	HKL	SPL	THL
	F	F	F	F	F
O. kisutch	4	1	3	2	1
O. mykiss	62	27	41	9	1
O. tshawytscha	11	3	10	6	-
O. nerka	1	-	1	-	-
S. trutta	2	-	2	1	-
S. fontinalis	1	-	1	-	-
S. salar	33	17	27	4	1
Total	**114**	**48**	**85**	**22**	**3**

Indeed, teleost HK is the most important organ as it is comparable to adult mammalian bone marrow (Crippen et al., 2001). In fish, the HK is the primary hematopoietic tissue where all blood cells form during larval and adult stages; blood forms in the liver in the mammalian foetus, and the bone marrow in adults (Bennett et al., 2001; Thisse & Zon, 2002).

3.3.1 Techniques used to isolate salmonid leucocytes

The use of fish lymphoid and PBL requires isolation from a mass of red blood cells present in PB, the HK, TH and SP, through density gradient centrifugation and hypotonic lysis (Hu et al., 2018; Pierrard et al., 2012). The present review found that most studies used discontinuous density gradient centrifugation (DDGC) (44%) and continuous density gradient centrifugation (CDGC) (41%) to isolate fish leucocytes form lymphoid organs and PB (Figure 3.2).

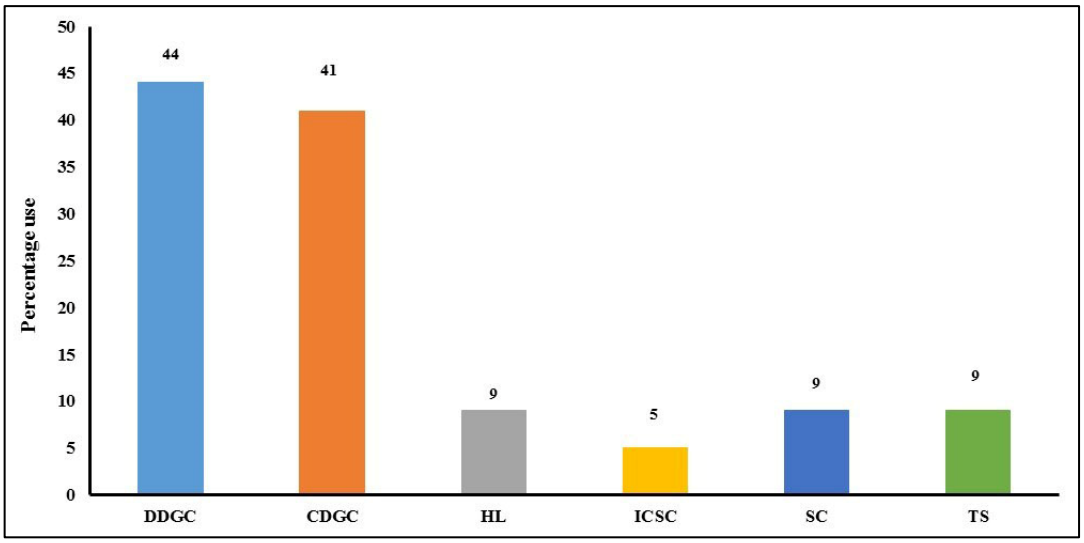


Figure 3.2. Techniques commonly used to isolate salmonid fish leucocytes for immunological assays. DDGC: Discontinuous density gradient centrifugation, CDGC: Continuous density gradient centrifugation, HL: Hypotonic lysis, ICSC: Ice-cold sedimentation and centrifugation, SC: Suspension centrifugation, and TS: Tissue suspension. Data comes from 114 reviewed salmonid studies.

Generally, DDGC involves layering density gradients of at least two different densities in a centrifugation vessel, usually a 15 mL centrifuge tube and topping up with buffer diluted PB or tissue single cell suspension to be separated into red blood cells and leucocytes. For instance, we found that Percoll density gradient was commonly layered in the range 1.060g mL^{-1} over 1.075 g mL^{-1} and 34% over 51% for S. *salar* and *O. mykiss*, respectively.

The centrifugation speed, duration and temperature settings varied greatly among studies and species, although all protocols yielded fish leucocytes (Table 3.2). Overall, the density gradient centrifugation process mostly lasted between 15 – 40 min at 400 g, and at 4 – 15°C (Table 3.2). CDGC involved layering buffer diluted blood or PB or tissue suspension over a pure layer of density gradient medium (1.077 g mL^{-1}) or a dilute density gradient layer (e.g., 50-54%) followed by centrifugation as detailed below (Table 3.2).

The most commonly used density gradient medium was Percoll (GE Healthcare) by 63 of the studies, although other media such as Histopaque 1077 (Sigma-Aldrich Co. LLC) was used by 14 of the studies, Ficoll or Ficoll-Paque PLUS (GE Healthcare), and Lymphoprep (STEMCELL Technologies) were also applied (Table 3.2). All isolation gradient products have a standard density of 1.077 g mL^{-1} and are designed to separate cells based on weight by centrifugation. Percoll density gradient is described by GE Healthcare as a low-viscosity, nontoxic medium suitable for density gradient centrifugation of cells, viruses and subcellular particles. Similarly, Histopaque 1077 is a sterile, endotoxin-tested solution of polysucrose and sodium diatrizoate. In addition, Ficoll-Paque PLUS is a sterile, ready-to-use aqueous medium composed of a mixture of Ficoll PM400 and sodium diatrizoate, for high yield isolation of lymphocytes from peripheral blood. However, Ficoll use in higher vertebrate leucocyte isolation alters cellular surface markers, functional responses and morphology (Ogle et al., 1985). Density gradient isolation of leucocytes also limits granulocyte recovery, with consequences for adaptive immune responses when compared to hypotonic lysis, and reported to increase respiratory burst in PBL (Hu et al., 2018; Samaï et al., 2018).

Despite the dominance of density gradient centrifugation as a technique for leucocyte isolation, these commercial reagents are expensive. Development of modified micro-scale techniques that require a minimal density gradient medium and < 300 µL of PB is a cost-effective solution, avoids euthanisation, in some cases, and can be applied on small fish (Lulijwa et al., 2019a). Similarly, the hypotonic lysis technique originally developed by Crippen et al. (2001) has been recently improved by Hu et al. (2018) to facilitate fast and affordable isolation of organ and PBL for fish immunological studies. The technique has recently gained use (Attaya et al., 2019; Hu et al., 2019; Hu et al., 2018; Wang et al., 2018) and reported to give superior cellular composition, with limited erythrocyte contamination (Hu et al., 2018) allowing a holistic assessment of fish immune parameters at innate and adaptive levels in immune and vaccine studies (Hu et al., 2018; Samaï et al., 2018).

Table 3.2. Techniques commonly used in salmonid leucocyte isolation and intended assays. PBL: Peripheral blood leucocyte, TKL: Total kidney leucocyte, HKL: Head kidney leucocyte, MKL: Middle kidney leucocyte, PKL: Posterior kidney leucocyte, SPL: Spleen leucocyte, and THL: Thymus leucocytes. Unless otherwise stated, the antibiotic solution used consisted of a combination of penicillin/streptomycin (P/S) at ratios e.g., 1% P/S, P 100 UmL^{-1} / S 100 µg L^{-1}, etc., while singly used antibiotics included oxytetracycline, gentamycin sulphate and streptomycin.

Species	Cells	Technique	Intended assays	Reference
O. kisutch	PBL	Heparinised PB 1 mL of 4, 5 or 6% Ficoll-Sodium Metrizoate Solution (SMS Sigma) were purified by Ficoll-SMS DCDGC for 20 min at 100 g and 15°C.	Microscopic characterisation	(Kawahara et al., 1990)
	HKL	Unfiltered HK suspension in holding medium (HM) on ice was sediment to remove debris, the supernatant, was removed and washed once in HM for 10 min at 500 g and 4°C. The resulting cell pellet was resuspended in TCM and cells counted on a haemocytometer.	Plaque forming cells Antibody production Total IgM	(Kaattari & Yui, 1987)
	HKL, SPL	Unfiltered HK and SP suspensions in tissue culture medium, and leucocyte suspensions were kept on ice to allow debris sedimentation. Leucocytes were purified according to Yui and Kaattari (1987). The resulting cell pellet was resuspended in TCM and adjusted to 10^7 mL^{-1} by haemocytometer.	Lymphocyte mitogen stimulation Plaque forming cell assay	(Yui & Kaattari, 1987)
	HKL, SPL, THL	Unfiltered single cell suspensions were obtained by repeated aspiration of tissue fragments with a 1 mL syringe on ice in L-15 complete medium with 10% FCS, L-glutamine at pH 7.4. The suspension was washed twice at 1000 g at 4°C for 10 min and the pellet resuspended in L-15 complete medium for assaying.	Plaque forming cell assay	(Kaattari & Irwin, 1985)
O. mykiss	PBL	Heparinised PB diluted 1:2 in heparinised MEM with Eagle's salt with 0.2% sodium heparin, buffered with 7.5% sodium hydrogen carbonate (pH 7.6) and leucocyte suspension purified by Percoll (54%) CDGC at 400 *g* for 25 min. The leucocytes were washed with PBS (pH 7.6) at 500 g for 5 min. Leucocytes were counted by haemocytometer fixed with RNA later® and stored at −20°C.	Cell proliferation and apoptosis Immune gene expression	(Yada et al., 2019)
	PBL	PBL were purified according to Hu et al. (2018).	Immune gene expression Cell proliferation Phagocytosis	(Wang et al., 2018)
	PBL	PBL were purified according to Hu et al. (2018).	Immune gene expression Flow cytometry	(Attaya et al., 2019; Hu et al., 2019)
	PBL	Heparinised PB diluted 1: 8 in 1x phosphate buffered saline (PBS) and leucocytes purified by Histopaque (1.077 g mL^{-1}) CDGC at 500 g for 40 min. PBL were collected from the interface, washed twice and counted. Also, PBL were prepared by modified hypotonic lysis via erythrocytes lysis in ice-cold water mixing for 20 sec, then immediate return to isotonic condition with 10x PBS. The PBL preparation was left on ice for 5-10 min to allow debris and nuclear materials to settle. The PBL were separated by passing through an EASYstrainer (70 µm) pelleted at 200 g, for 5 min, washed once and resuspended in complete culture medium.	Marker gene expression Phagocytosis Cell proliferation Immune gene expression	(Hu et al., 2018)
	HKL	Filtered (70 µm) HK suspension in L-15 medium with 2% FBS, were centrifuged into single cells for 10 min at 600 g and 4°C. Cell pellet was resuspended in L-15 medium and leucocytes purified by Percoll (60%) CDGC for 60 min at 700 g and 20°C. Leucocyte were posteriorly centrifuged for 10 min at 400 *g* and 4°C; collected and resuspended in a supplemented L-15 medium. This procedure was repeated twice, and cells counted on a haemocytometer.	Immune gene expression	(Peña et al., 2016)
	HKL, SPL	Nylon mesh filtered suspensions were purified according to Chung and Secombes (1988). Leucocytes were adjusted to 10^6 mL^{-1} in PBS with 2% FCS, and 0.1% sodium azide.	Flow cytometry characterisation of trout polyclonal antibody to rainbow C-type lectin (CLEC4T1+).	(Johansson et al., 2016)
	HKL	Nylon mesh (100 µm) filtered HK in incomplete heparinised L-15 medium with antibiotic solution, 0.5% FBS, was purified by centrifugation at 200 g for 5 min, the pellet washed once with complete medium with 10% FBS and adjusted to 10^6 mL^{-1}.	Immune gene expression	(Monte et al., 2015)
	PBL	Heparinised PB was purified to obtain PBL by Percoll (1.075 g mL^{-1}) CDGC at unspecified settings.	Monoclonal antibody (MAb) detection	(Korytář et al., 2013)
	PBL, SPL, HKL & PKL	Cell strainer (70 µm) filtered cell suspension in L-15 medium with 10% bovine growth serum (BGS), gentamicin (50 µg mL^{-1}), and fungizone (0.25 µg mL^{-1}) on ice and adjusted to the right density used for direct assay. PB diluted 1:5 in heparinised PBS was allowed to settle for at least 20 min at room temperature. Leucocyte rich plasma was removed from settled erythrocyte and purified by Nycoprep (1.077 g mL^{-1}) CDGC. Cells were adjusted to 10^6 mL^{-1} by Trypan Blue in supplemented media.	Phagocytosis Antibody detection Transmission electron microscopy	(Bassity & Clark, 2012)
	HKL	HKL isolated aseptically according to Secombes (1990). Nylon mesh (100 µm) filtered tissue cell suspension in unheparinised medium and leucocytes purified by Percoll (51%) CDGC for 30 min at 400 g and 4°C. Cells were washed twice with L-15 medium by centrifugation at 800 g for 10 min, and adjusted to 10^6 mL^{-1} by haemocytometer in L-15 medium with 0.1% FBS.	Flow cytometry of PB Respiratory burst Bactericidal activity	(Sharifuzzaman & Austin, 2010)
	HKL	Nylon mesh (100 µm) filtered HK suspension in heparinised RPMI 1640 with antibiotic solution and 0.1% FCS were purified by Percoll (34/51%) DCDGC for 25 min at 400 g and 4°C according to Sakai et al. (1995). Cells were washed twice with RPMI 1640, adjusted to 10^5 mL^{-1} by haemocytometer.	Phagocytosis Phagocytic index	(Nya & Austin, 2010)
	HKL	Nylon mesh (100 µm) filtered HK cell suspension in heparinised RPMI 1640 with antibiotic solution and 0·1% FCS were purified by Percoll (34/51%) DCDGC for 25 min at 2000 g and 4°C, according to Sakai et al. (1995) washed twice in RPMI 1640 and adjusted to 10^5 mL^{-1} with a haemocytometer.	Phagocytosis Phagocytic index	(Nya & Austin, 2009)
	PBL, HKL	PB and HK leucocytes were isolated according to Markkula et al. (2006). Briefly, nylon net (80 µm) filtered HK cell suspension in heparinised HBSS were purified by Percoll (1.040/1.090 g mL^{-1}) DDGC. For PBL, the blood pellet was resuspended in heparinised HBSS medium with Ultroser G serum substitute, sodium	Respiratory burst Lymphocyte proliferation	(Markkula et al., 2009)

	pyruvate and Hepes, pH 7.4 and the suspension purified by Percoll (1.075 g mL^{-1}) CDGC at 400 g for 30 min. Leucocytes were washed twice and resuspended in RPMI-1640 with Ultroser G Serum substitute, sodium pyruvate, L-glutamine, antibiotic solution and Hepes, and adjusted accordingly with a haemocytometer.		
HKL	Nylon cell strainer (37 µm) filtered HKL cell suspension in MEM were purified by Percoll (34/55%) DCDGC at 400 *g* for 50 min. The leucocyte were harvested, washed with PBS (pH 7.6), and suspended in MEM containing 0.5% trout serum. Isolated leucocytes were adjusted to 10^7 mL^{-1} MEM with 0.5% trout serum.	Superoxide anion production Phagocytosis Immune gene expression	(Yada, 2009)
HKL	Filtered HK cell suspension in heparinised L-15 medium were purified by Percoll (1.070/1.080 g mL^{-1}) DCDGC for 40 min at 400 g as described by Braun-Nesje et al. (1981).	Respiratory burst	(Morgan et al., 2008)
HKL	Nylon mesh (100 µm) filtered HK suspension diluted 1: 10 in heparinised sterile filtered L-15 medium with antibiotic solution and 2% FCS were purified by Percoll (34/51%) DCDGC for 25 min at 400 g and 4°C according to Secombes (1990). Leucocytes at the interface were collected and washed thrice in L-15 medium with 0.1% FCS and antibiotic solution, and adjusted to 10^7 mL^{-1}.	Phagocytosis Respiratory burst	(Brunt et al., 2007)
HKL	Nylon mesh (100 µm) filtered HK suspension in heparinised RPMI 1640 medium with 2% FCS, and antibiotic solution were purified by Percoll (34/51%) DCDGC for 25 min at 400 g and 4°C according to Secombes (1990). Leucocytes at the interface were collected and washed twice in HBSS, adjusted to 10^6 mL^{-1} with a haemocytometer in RPMI 1640 with 0.1% FCS and antibiotic solution.	Phagocytosis Respiratory burst	(Kim & Austin, 2006)
HKL	Nylon mesh (100 µm) filtered HK suspension in heparinised L-15 medium were directly used for assay	Respiratory burst	(Taylor et al., 2007)
HKL	Nylon net (80 µm) filtered HK cell suspension in heparinised RPMI 1640 with Ultroser G serum substitute, sodium pyruvate, mercaptoethanol , sodium chloride, Hepes, pH 7.4 were purified by Percoll (1.040/1.080 g mL^{-1}) DCDGC, at unspecified centrifuge parameters. Cells were washed twice and resuspended in culture medium: incubation medium supplemented with L-glutamine, antibiotic solution and sodium bicarbonate and adjusted to desired density by a haemocytometer.	Respiratory burst Natural cytotoxicity	(Markkula et al., 2007)
HKL	Nylon net (80 µm) filtered HK cell suspension in heparinised RPMI 1640 with Ultroser G serum substitute, sodium pyruvate and Hepes were purified by Percoll (1.040/1.090 g mL^{-1}) DCDGC at 400 g for 30 min. Cells were washed twice and resuspended in supplemented RPMI-1640 culture media, and adjusted accordingly with a haemocytometer.	Respiratory burst Natural cytotoxicity	(Markkula et al., 2006)
HKL	Sterile nylon mesh (200 µm) bags filtered HK suspension in Dulbecco's Modified Eagle Medium with high glucose, 10% heat inactivated FCS and antibiotic solution were adjusted to 10^7 mL^{-1} on a haemocytometer, for direct assay use.	Immune gene expression Microarray analyses	(MacKenzie et al., 2006)
PBL	PBL were isolated by Histopaque (1.077 g mL^{-1}) CDGC at unspecified settings and stored at −80°C until needed.	Gene expression Indirect epifluorescent detection of surface antigens	(Kales et al., 2006)
PBL	Leucocyte buffy coat was isolated from heparinised PB by centrifuging at 1000 rpm for 10 min at room temperature, collected and resuspended in heparinised RPMI 1640 with antibiotic solution. Leucocytes were purified by Histopaque 1077 CDGC at 1500 rpm for 30 min. Leucocytes were resuspended in Trizol for RNA extraction and in PBS for protein extraction.	Northern blot analysis Gene expression	(Nath et al., 2006)
HKL	Nylon mesh (70 µm) filtered HK cell suspension in heparinised L-15 medium with 2% FCS, L-glutamine, and antibiotic solution were purified by Percoll (34/51%) DCDGC at 400 *g* for 25min at 4°C. Cells were collected and washed twice in heparinised L-15 medium with 0·1% FCS, L-glutamine, antibiotic solution and adjusted by haemocytometer to 10^7 mL^{-1} according to Secombes (1990).	Phagocytosis Pinocytosis Antibody synthesis	(Cecchini et al., 2005)
HKL	Nylon mesh (100 µm) filtered HK cell suspension in heparinised RPMI 1640 with 0.1% FCS and oxytetracycline were purified by Percoll CDGC at 2500rpm for 25 min at 4°C, washed thrice in RPMI 1640 and adjusted to 10^5 mL^{-1} according to Sakai et al. (1995).	Phagocytosis Respiratory burst	(Brunt & Austin, 2005)
HKL	Nylon mesh filtered HK cell suspension in heparinised L-15 medium with 0.1% FCS and antibiotic solution were purified by Percoll (34%/51%) DCDGC for 25 min at 400 g and 4°C according to Chung and Secombes (1988). Cells were adjusted to 10^6 and 10^7 mL^{-1} in L-15 medium with antibiotic solution for further use.	Phagocytosis Superoxide anion production	(Panigrahi et al., 2004)
HKL	HKL in heparinised L-15 medium with sodium salt and antibiotic solution were purified by Percoll (34%/51%) DCDGC for 20 min at 400 g and 4°C according to Secombes (1990). Cells were adjusted to 10^6 mL^{-1} in L-15 medium, with antibiotic solution.	Phagocytosis Oxidative burst Lymphocyte proliferation	(Hoeger et al., 2004)
PBL	PBL were isolated according to Espelid et al. (1996). Briefly, PB diluted 1:2 in heparinised MEM with Eagle's salt, and leucocyte suspension purified by Percoll (54%) CDGC at 400 *g* for 25 min. The leucocytes were washed with PBS (pH 7.6) and suspended in MEM with 0.5% trout serum, and adjusted to 10^7 mL^{-1}.	Cell proliferation PBL lysozyme activity Immune gene expression	(Yada et al., 2004)
HKL	Sterile nylon mesh (200 µm) bags filtered HKL suspension in Dulbecco's Modified Eagle Medium with high glucose, 10% heat inactivated FCS and antibiotic solution was adjusted to 10^7 mL^{-1} on a haemocytometer, for direct assay use.	Immune gene expression	(Goetz et al., 2004)
HKL	Sterile filtered HK suspension in ice-cold incomplete L-15 medium with L-glutamine, and Hepes, pH 7.1, were partially purified for 10 min at 500 *g* and 4°C, and washed thrice. Cells were resuspended in complete L-15 medium with ciprofloxacin, antibiotic solution, fungizone, M β-mercaptoethanol and 10% FCS. Cells were adjusted to 10^6 mL^{-1} using a haemocytometer. Due to the toxicity by Ficoll-Paque, no density gradient was used further in the study.	Lymphocyte mitogen stimulation	(Leonardi & Klempau, 2003)
PBL	Heparinised PB dilute 1: 2 with isolation medium Histopaque (1.077 g mL^{-1}) with bacto haemagglutination buffer. The suspension was purified by Histopaque (1.077 g mL^{-1}) with bacto haemagglutination buffer DCDGC for 15 min at 500 *g* and 4°C. Leucocytes were gently collected and dispensed into a siliconized tube, washed twice in phenol red-free HBSS and adjusted to 10^6 mL^{-1}.	Respiratory burst Phagocytosis	(Dügenci et al., 2003)
HKL, PBL	Nylon mesh filtered HK cell suspensions in heparinised RPMI 1640 with antibiotic solution were centrifuged at 500 g for 5 min and washed thrice with medium. Purified macrophages were obtained by Percoll (34/51%) DCDGC for 30 min at 300 g and 4°C, cells were collected, washed twice at 300 g for 10 min with HBSS and adjusted to 10^6 mL^{-1}. For PBL, heparinised PB diluted 1:3 in HBSS medium was washed thrice for 5 min at 14°C and 500 g, the cloudy layers removed following each centrifugation, and the free erythrocyte pellet (95%) was gently resuspended in RPMI 1640 to 10^6 mL^{-1}.	Phagocytosis assay Cellular killing capacity Binding capacity	(Passantino, Altamura, et al., 2002)

HKL	HKL were isolated according to Sakai et al. (1995). Briefly, unfiltered HK suspension diluted 1:10 in filter sterilized heparinised RPMI 1640 with oxytetracycline, and 0.1% FCS were centrifuged for 5 min at 500 g and 4°C, washed thrice in RPMI and adjusted to 10^7 mL^{-1} in heparinised RPMI 1640 with oxytetracycline and 0.1% FCS.	Phagocytosis	(Irianto & Austin, 2002)
PBL	PBL were isolated according to Espelid et al. (1996). Briefly, PB was diluted 1:2 in heparinised MEM with Eagle's salt, sodium biphosphate (pH 7.6). The leucocyte suspension was purified by Percoll (54%) CDGC at 400 g for 20 min. Leucocytes were harvested, washed with PBS (pH 7.6), and suspended in MEM with 10% *O. mykiss* serum.	Respiratory burst	(Yada et al., 2001)
HKL	Nylon mesh (100 µm) filtered HK cell suspension in heparinised RPMI-1640 with antibiotic solution were purified by Percoll (34%/51%) DCDGC for 20 min at 400 g and 4°C according to Secombes (1990). Leucocytes were washed and resuspended in HBSS medium to desired concentration by Trypan Blue exclusion.	Phagocytosis Pinocytosis Chemiluminescence	(Clerton et al., 2001)
HKL	Sterile mesh screen filtered HK cell suspension in heparinised medium with antibiotic solution, and gentamycin were purified by Percoll (51%) CDGC at 400 *g* for 25 min. Cells were washed twice in serum-free medium and centrifuged at 200 *g* for 10 min. Leucocytes were adjusted to appropriate concentration with haemocytometer.	Nitric oxide production Respiratory burst HKL flow cytometry characterisation	(Stafford et al., 2001)
HKL, PBL	PB diluted 1:2 in cold isolation medium, erythrocytes lysed with cold sterile distilled water for 20 s and immediately returned isotonic with 10x PBS and the suspension centrifuged for 10 min at 750 g and 4°C. The cell pellet was resuspended in MEM and kept on ice to sediment out debris. Leucocyte supernatant was collected and further centrifuged as above , the pellet resuspended in MEM with sodium bicarbonate, 5% FCS antibiotic solution, streptomycin and L-glutamine. After a 1:12 dilution of PB in isolation medium, PBL were purified by Histopaque (1.077 g mL^{-1}) CDGC for 40 min at 400 g and 4°C. Unfiltered HK leucocyte suspension were prepared by hypotonic lysis in MEM and were purified by Percoll (34%/51%) DCDGC for 40 min at 2000 g and 4°C.	Viability Leucocyte differential counts Flow cytometry characterisation Sudan Black B Neutral Red Staining	(Crippen et al., 2001)
HKL, PBL	Heparinised PB was diluted 1:2.5 in heparinised HBSS with 2% FCS and purified by Percoll (51%) CDGC. Unfiltered HK cell suspension in HBSS with 2% FCS were purified by Percoll (34%/51%) DDGC. PBL and HKL suspensions were centrifuged for 20 min at 800 g and 4°C. PB and HK leucocytes were harvested, diluted 10x in HBSS, washed for 10 min at 800 g and 4°C. PB and HK leucocyte pellets were resuspended in medium with Hepes, sodium chloride, pH 7·4 and homogenised on ice for 1 min. PBL were homogenised with a glass piston tissue grinder and the homogenate diluted with an equal volume of Hepes, sodium, pH 7·4 and centrifuged for 10 min at 800 g and 4°C to remove cellular debris. Similarly, HKL homogenate was diluted with an equal volume as used for PBL and centrifuged for 10 min at 13,000 g and 4°C.	Antibacterial activity HKL lysozyme content	(Smith et al., 2000)
HKL, PBL	Stainless steel mesh (100 *µm*) filtered HK cell suspension in heparinised HBSS and leucocyte buffy coat preparation diluted in heparinised HBSS were purified by Histopaque (1.077 g mL^{-1}) CDGC for 30 min at 400 *g* and 9°C. Cells were collected at the interface, washed thrice with HBSS, and resuspended in RPMI 1640 medium with Hepes, L-glutamine, 10% FCS and antibiotic solution.	HKL respiratory burst HKL phagocytosis HKL lymphocyte proliferation PBL Surface IgM marking	(Karrow et al., 1999)
PBL & HKL	Heparinised PB diluted 1:20 in MEM and stainless steel filtered HK cell suspensions were prepared in cold serum-free MEM. Leucocytes were purified by Ficoll (1·077 g mL^{-1}) CDGC for 20 min at 400 g, cells were collected, washed in PBS and resuspended in MEM 10.	Flow cytometry characterisation Phagocytosis Oxidative burst Lymphoproliferation Flow cytometry cell population analysis	(Chilmonczyk & Monge, 1999)
PBL & HKL	HK and PB leucocyte suspensions were purified by Lymphoprep CDGC at unspecified settings. Cells were collected at the interface, washed twice and diluted in heparinised L-15medium with antibiotic solution, glutamine and 5% FCS.	Leucocyte proliferation	(Boesen et al., 1997)
PBL & HKL	Heparinised PB diluted 1:5 in L-15 medium with 5% FCS were purified by Percoll (51%) CDGC for 20 min at 400 g and 4°C. Leucocytes were washed twice in RPMI-1640 with sodium hydrogen carbonate (pH 7·2), antibiotic solution and 2-Mercaptoethanol, and adjusted to 10^6 mL^{-1}. Nylon mesh (100 µm) filtered HK cell suspensions in L-15 medium, were purified by Percoll (51%) CDGC for 35 min at 400 g and 4°C. Leucocytes were collected, washed and adjusted to 10^6 mL^{-1} in L-15 medium with antibiotic solution and 2-Mercaptoethanol.	Respiratory burst	(Marsden & Secombes, 1997)
HKL	Nylon mesh (100 µm) filtered HK cell suspensions in heparinised L-15 medium, with antibiotic solution and 2% FCS were purified by Percoll (34%/51%) DCDGC for 30 min at 400 g and 4°C following Chung and Secombes (1988). Leucocytes were collected, washed twice for 5 min at 400 g and 4°C in L-15 with 0.1% FCS.	Respiratory burst assays	(Novoa et al., 1996)
HKL	Nylon mesh filtered HK suspension in heparinised RPMI 1640 medium with antibiotic solution were purified by centrifugation at 500 g for 5 min and washed thrice RPMI 1640, cells counted by Trypan Blue exclusion.	Phagocytosis Chemiluminescence assay Superoxide anion production	(Sakai et al., 1995)
PBL	PBL were purified according to Marsden et al. (1995) below.	Leucocyte proliferation Antibody production	(Marsden et al., 1996)
PBL	Heparinised PB was diluted 1:5 with L-15 medium with 5% FCS, and leucocytes purified by Percoll (51%) CDGC for 20 min at 400 g and 4°C. Leucocytes were washed for 5 min at 400 g and 4°C in L-15 medium with 5% FCS. Leucocytes were counted by Trypan Blue and adjusted to 10^6 mL^{-1} in L-15 with 5% FCS.	Leucocyte proliferation Lymphocyte separation	(Marsden et al., 1995)
HKL	Nylon mesh (100 µm) filtered HK cell suspension in in L-15 medium were purified by Percoll (51%) CDGC for 35 min at 400 g and 4°C. Leucocytes were collected at the interface, washed and adjusted to 10^6 mL^{-1} in L-15 medium with antibiotic solution and 2-mercaptoethanol.	Respiratory burst Leucocyte proliferation	(Marsden et al., 1994)

	SPL	Metallic nets pressed SP suspensions in RPMI 1640 with Hepes, glutamine, gentamycin, and 5% FCS were adjusted to 10^6 mL^{-1} with a haemocytometer for direct assay use.	Respiratory burst	(Thuvander et al., 1993)
	HKL	Unfiltered HKL cell suspension in heparinised RPMI 1640 with antibiotic solution, sodium carbonate were purified by Percol (51%) CDGC at 400 g for 25 min at 4°C. The cell pellet was adjusted to 10^6 mL^{-1} in RPMI for further analysis.	Lymphocyte proliferation Respiratory burst	(Hardie et al., 1993)
	PBL	Heparinised PBL were separated on a Percoll (54%) CDGC according to Thuvander et al. (1987).	Lymphocyte membrane total IgM	(Thuvander & Caristein, 1991)
	PBL, SPL	Unfiltered SP suspensions were obtained according to Kaattari and Irwin (1985). PB diluted 1 in RPMI-1640 were purified by centrifugation at 500 g for 10 min at 17°C, and the resulting pellet suspended in RPMI-1640. The suspension was purified by Histopaque 1077 CDGC for 20 min at 800 g and 17°C. Cells at the interface were washed once in TCM and adjusted to 10^7 mL^{-1} in TCM.	Plaque forming cells assay	(Arkoosh & Kaattari, 1991)
	HKL, SPL, THL, PBL	PB diluted 1:5 in RPMI 1640, and metal nets pressed HK, SP and TH suspensions in medium were purified according to Thuvander et al. (1987). Cells were purified by Percoll (54%) CDGC at 400 g. Cells at the interface were washed, counted and resuspended in RPMI 1640 with Hepes, glutamine, antibiotic solution, gentamicin and 5% FCS until used.	Cell proliferation Antibody production	(Reitan & Thuvander, 1991)
	HKL	Fine wire mesh filtered HK suspensions in cold L-15 medium were drawn into sterile Strumia capillary tubes and centrifuged at 13600 g for 3 min. Tubes were cut at the cell fluid interface, tubes with cells fixed to the bottom of tissue culture wells using Vaseline. The medium was added to each well through a Millipore Filter Unit and incubated at 12°C for 12-I6h.	Cell migration Migration inhibition	(Thomas & Woo, 1990)
	SPL	Metallic nets pressed SP suspensions in RPMI 1640 with Hepes, glutamine, mercaptoethanol, gentamicin, sodium pyruvate and 5% heat inactivated *S. trutta* serum were adjusted to 10^6 mL^{-1} with a haemocytometer for direct assay use.	Phagocytosis Lymphocyte stimulation	(Thuvander, 1989)
	PBL	PB diluted 1:3 in cold complete medium following the procedure by Boyum (1968). Briefly, leucocytes were purified by Ficoll-Paque CDGC for 30 min at 500 g and 10°C. Lymphocytes at the interface were collected, washed twice in cold, complete medium by centrifugation for 10 min at 100 g and 10°C.	Lymphocyte proliferation Flow cytometry characterisation	(Tillitt et al., 1988)
	HKL	Nylon mesh filtered HK cell suspension in heparinised L-15 medium with 0.1% FCS and antibiotic solution were purified by Percoll (34%/51%) DCDGC for 25 min at 400 g and 4°C. Leucocytes were adjusted to 10^7 mL^{-1} in L-15 medium with 0.1% FCS and antibiotic solution.	Respiratory burst Hydrogen peroxide production Superoxide dismutase inhibition	(Chung & Secombes, 1988)
	HKL, SPL	Unfiltered HK and SP suspensions in tissue culture medium, and leucocyte suspensions were kept on ice to allow debris sedimentation. Single cell suspension was collected and washed once in TCM by centrifugation for 10 min at 500 g and 4°C. Leucocyte pellets were resuspended in TCM to 10^7 mL^{-1}, using Trypan Blue exclusion assay.	Same assays as for O. kisutch above	(Yui & Kaattari, 1987)
	PBL	PB diluted 1:3 in RPMI 1640, was purified by Percoll (54%) CDGC at 400 g for 11 min at room temperature, a leucocyte fraction collected at the interface, washed once in RPMI 1640 at 280 g for 8 min. The resulting pellet was resuspended in RPMI 1640 with Hepes, sodium bicarbonate, glutamine, antibiotic solution and either 5% FCS, 5% *S. trutta* serum (BTS) or 1% Ultroser G serum supplement at 10^6 mL^{-1}.	Phagocytosis	(Thuvander et al., 1987)
	SPL	Unfiltered SP suspensions in TCM with FCS and gentamicin, were centrifuged for I0 min at 500 g and 4°C. The pellet was collected, washed and resuspended in the modified TCM, cells were counted on a haemocytometer and adjusted to 10^6 mL^{-1} in TCM.	Plaque forming cells Lymphocyte stimulation	(Kaattari et al., 1986)
	HKL, SPL, PBL, THL	PB diluted 1:1 with sodium citrate cold anticoagulant buffer, and organ filtered cell suspension, were purified by Percoll (30%) CDGC at 500 g for 8 min at 5°C to isolate leucocytes for cell cultures.	Lymphocyte stimulation	(Warr & Simon, 1983)
	HKL	Stainless steel mesh (0.3 mm) filtered HK cell suspension in L-15 medium with 10% FCS, glucose and antibiotic solution were purified by Percoll (1.070/1.080 g mL^{-1}) DCDGC for 40 min at 400 g at 5°C or on ice.	Phagocytosis Microscopic characterisation	(Braun-Nesje et al., 1981)
O. nerka	HKL	Stainless steel screen filtered HK suspension in heparinised L-15 medium with 10%FCS, glucose, and antibiotic were passed through a glass wool mat to remove any remaining large aggregates. Leucocytes were purified by passing through Percoll (34%/51%) DCDGC according to Braun-Nesje et al. (1981). Cells were washed once in heparinised L-15 medium at 1000 *g* for 10 min, and resuspended in L-15 with heparin.	Respiratory burst Phagocytosis HKL myeloperoxidase content	(Alcorn et al., 2002)
O. tshawytscha	PBL	PBL were isolated according to Lulijwa et al. (2019a)	Phagocytosis Respiratory burst Immune gene expression	(Lulijwa et al., 2019b)
	PBL	PBL were isolated by a modified method from Pierrard et al. (2012). Briefly, heparinised PB diluted 1:1 in sterile filtered (40 µm) PBS, pH 7.4 were purified from micro blood volume (284 µL) by Lymphoprep (1.077 g mL^{-1}) CDGC at 971 g for 20 min in a 1.5 mL Eppendorf tube at room temperature. Leucocytes at the interface were aspirated with a pipette and washed twice in PBS at 674 g for 7 min. The cell pellet was adjusted to 10^5 - 10^6 mL^{-1} in PBS with 2% FCS and kept at 4°C.	PBL viability PBL microscopic characterisation PBL flow cytometry characterisation	(Lulijwa et al., 2019a)
	HKL	Unfiltered HK suspension in L-15 medium with 0.1% FBS and antibiotic solution on ice, leucocytes were purified by Histopaque (1.077 g mL^{-1}) CDGC at unspecified settings and macrophages separated by plastic adherence.	Respiratory burst Phagocytosis	(Arkoosh et al., 2018)
	HKL	Unfiltered HK suspensions in L-15 medium with 0.1% FBS and antibiotic solution were sediment on ice to remove debris for 3–5 min. The leucocyte supernatants were purified by Histopaque (1.077 g mL^{-1}) CDGC for 15 min at 500 g and 16°C. The interface layer with WBCs was washed in L-15 medium with 0.1% FBS and resuspended in L-15 medium with 0.1% FBS at 10^6 mL^{-1} by haemocytometer.	Phagocytosis Respiratory burst	(Arkoosh et al., 2015)

	HKL & SPL	HKL and SPL were isolated according to Crippen et al. (2001). Nylon cell strainer (40 µm) filtered suspensions in isolation medium (HBSS) and Alsever's solution (AS) and leucocytes partially purified for 7 min at 500 *g* and 4°C. The cell pellet was resuspended in ice-cold isolation medium, and clumps removed. For hypotonic lysis of erythrocytes, 2 mL of cell suspension were diluted with 9 mL sterile deionized water for 20 sec; 1 mL of sterile 10x PBS was immediately added to stop lysis. Cells were washed twice for 7 min at 500 *g* and 4°C, cells were resuspended in ice-cold TCM, and adjusted to 10^6 mL^{-1} by Trypan Blue assay.	Cell viability Cell apoptosis IgM response	(Misumi et al., 2009)
	HKL & SPL	HKL and SPL were isolated according to Crippen et al. (2001). Nylon cell strainer (40 µm) filtered suspensions in isolation medium (HBSS) and Alsever's solution (AS) and leucocytes partially purified for 7 min at 500 *g* and 4°C. The cell pellet was resuspended in ice-cold isolation medium, and clumps removed. For hypotonic lysis of erythrocytes, 2 mL of cell suspension were diluted with 9 mL sterile deionized water for 20 sec; 1 mL of sterile 10x PBS was immediately added to stop lysis. Cells were washed twice for 7 min at 500 *g* and 4°C, cells were resuspended in ice-cold TCM, and adjusted to 10^6 mL^{-1} by Trypan Blue assay.	Cell viability Cell apoptosis Cell proliferation IgM response	(Misumi et al., 2005)
	HKL, SPL, & PBL	PBL were isolated by hypotonic lysis (Crippen et al., 2001). Briefly, PB erythrocytes were lysed with distilled water for 20 sec, immediately returned to isotonic with 10x PBS, erythrocyte debris removed and the PBL washed with isolation media, and finally re-suspended in RPMI for assay use. Unfiltered HK and SP suspensions in ice-cold TCM (MEM) with 5% FCS, antibiotics, sodium bicarbonate, essential and none-essential amino acids and sodium pyruvate. Leucocyte suspensions were allowed to settle, and cell suspensions aspirated off. Cells were adjusted by Trypan Blue to 10^7 mL^{-1} in sterile TCM on ice for direct assay use.	MAb Surface IgM Flow cytometry characterisation of isolated cells	(Milston et al., 2003)
	HKL	Stainless steel filtered HKL cell suspension in heparinised L-15 medium with 10% FCS, glucose, and antibiotic solution were purified by Percoll (34–51%) DCDGC for 40 min at 400 g and 5°C. Cells were washed once, resuspended in L-15 medium with 20 IU mL^{-1} heparin, and adjusted to the right concentration using a haemocytometer.	Respiratory burst Phagocytosis	(Alcorn et al., 2003)
	HKL, SPL	Unfiltered suspension in holding medium (HM) on ice was sediment to remove debris, the supernatant, was removed and washed once in HM for 10 min at 500 g and 4°C according to Yui and Kaattari (1987) above. The resulting cell pellet was resuspended in TCM and cells counted on a haemocytometer.	Same assays as for *O. kisutch* above	(Yui & Kaattari, 1987)
	HKL, SPL	HK and SP leucocytes were isolated according to Arkoosh and Kaattari (1991).	Plaque forming cell assay	(Arkoosh et al., 1994)
	HKL	Unfiltered HKL cell suspension on ice was sedimented to remove debris in RPMI-1640 with 10% FCS, L-glutamine, gentamycin, 2-meracaptoethanol, uracil, cytosine, adenosine and guanine. The suspension was obtained by repeated aspiration of fragments using a 1 mL syringe. Leucocytes were adjusted to 10^7 mL^{-1} with a haemocytometer in supplemented RPMI-1640 for assaying.	Plaque forming cell assay	(Slater & Schreck, 1993)
	HKL, SPL	Unfiltered suspension obtained by repeatedly forcing tissue through a 1 cc syringe in TCM was sedimented on ice, supernatant was centrifuged at 1400 g for 10 min, pellet collected and suspended in TCM. Cells were counted on a haemocytometer and adjusted to 10^7 mL^{-1} in TCM until needed for assay.	Plaque forming cell assay	(Arkoosh et al., 1991)
S. salar	PBL, HKL, SPL	PB and organ leucocytes were isolated according to Jørgensen et al. (2001). Heparinised PB suspension leucocytes were purified by Percoll (54%) CDGC for 40 min at 400 g and 4°C. Cell strainer (100 µm) filtered HK and SP suspensions were purified by Percoll (25/54%) DCDGC and centrifuged as above. Leucocytes at the interface were collected and washed twice in L-15 medium with P/S prior to use.	Flow cytometry sorting Surface protein characterisation Immune gene expression	(Peñaranda et al., 2019)
	HKL	Cell strainer (100 µm) filtered HK cell suspension in heparinised L-15 medium with antibiotic solution, and 2% inactivated FBS were purified by Percoll (25%/54%) DCDGC for 40 min at 400 *g* and 4°C according to Braun-Nesje et al. (1981). Leucocytes at the interface were collected and washed twice in L-15 medium at 450 *g* for 15 and 10 min respectively, and adjusted to 10^6 mL^{-1} in L-15 with 1% FBS.	Respiratory burst activity Nitric oxide production Immune gene expression	(Ulvestad et al., 2018)
	HKL	Cell strainer (100 µm) filtered HK suspensions in heparinised L-15 medium with 2% FCS and antibiotic solution were purified by Percoll (25/54%) DCDGC for 45 min at 400 g and 4°C according to Jørgensen et al. (2001). Leucocytes at the interface were collected and washed twice in L-15 medium and seeded at 10^6 mL^{-1}.	Luciferase reporter gene assay	(Julin et al., 2015)
	HKL, PBL	Briefly, heparinised PBL was diluted 1: 2 in heparinised L-15+ medium with sodium carbonate, D-glucose, gentamycin sulphate, and L-gluamin. Homogenised and unfiltered HK suspensions were prepared in heparinised cold L-15+ medium. Leucocytes from PB and HK suspensions were purified by Percoll (1·075/1·060 g mL^{-1}) DCDGC for 30 min at 400 g according to Pettersen et al. (2000). Isolated leucocytes were diluted in L-15 + medium and analysed in a CASY-TT, adjusted to 10^6 mL^{-1}.	Surface MAb detection Leucocyte flow cytometry characterisation HK tissue gene expression	(Rønneseth et al., 2013)
	HKL, SP	Nylon mesh (80 µm) filtered HK and SP cell suspensions in heparinised RPMI-1640 with 2% healthy *S. salar* serum and antibiotic solution were purified by Percoll (34/51 %) DCDGC at 400 *g* for 45 min. Cells were collected at the interface and centrifuged again at 400 *g* for 10 min. Leucocytes were washed thrice in medium and suspended in the same medium to 10^7 mL^{-1}.	Phagocytosis Respiratory burst HKL lysozyme activity	(Paredes et al., 2013)
	PBL	Heparinised PB was diluted 1:2 in L-15+ medium with gentamicin sulphate, L-glutamine and HEPES. Leucocyte were then purified by Percoll (1·075/1·060 g mL^{-1}) DCDGC for 40 min at 400 g as previously described by Pettersen et al. (1995)	Phagocytosis Flow cytometry characterisation Respiratory burst PBL myeloperoxidase Acid phosphatase Mitogen stimulation Cell surface marker gene expression	(Haugland et al., 2012)

HKL	Nylon mesh (37 µm) filtered HKL cell suspension in heparinised L-15 medium with bovine serum were purified by Percoll (34/51%) DCDGC at 3000 *g* for 30 min. Leucocytes were collected, washed with PBS (pH 7.6) and suspended in L-15 with 10% bovine serum.	Respiratory busrt Phagocytosis Immune gene expression	(Romero et al., 2012)
PBL, SPL & HKL	PB diluted 1:3 in L-15 medium and cell strainer filtered (50-100 µm) homogenised organ cell suspension were purified by Percoll (1.080 g mL^{-1}) DDGC for 40 min at 800 *g* and 4 °C. Leucocyte were collected, washed once in PBS for 10 min at 400 *g* and 4 °C. Cells were resuspended in L-15 medium, counted using a Kova slide chamber and adjusted to 10^7 mL^{-1}.	Antibody detection Flow cytometry characterisation	(Hedfors et al., 2012)
PBL & HKL	PBL and HKL were isolated as described in Øverland et al. (2010). Heparinised PB diluted in L-15+ medium and cell strainer (100 µm) filtered HK cell suspension in heparinised L-15+ medium with gentamicin sulphate, L-glutamine, and HEPES were purified by Percoll (1·060/1·075 g mL^{-1}) DCDGC for 35 min at 400 g and 4°C.The cell pellet was resuspended in L-15 medium and counted by CASY Cell Counter.	Respiratory burst Microscopic characterisation	(Kalgraff et al., 2011)
PBL & HKL	Cell strainer (100 µm) filtered HK cell suspension in heparinised L-15 medium with gentamicin sulphate, L-glutamine, and HEPES. Peripheral blood and HK leucocytes were isolated on Percoll (1·060/1·075 g mL^{-1}) DCDGC for 35 min at 400 g and 4°C according to Pettersen et al. (2000).	Phagocytosis Flow cytometry Immunofluorescence	(Øverland et al., 2010)
PBL & HKL	Heparinised PB diluted 1:4 in L-15 medium were purified by Histopaque 1077 CDGC at 400 g for 45 min. Leucocytes were collected using a Pasteur pipette. If erythrocyte contamination of PBL was considered to be excessive (>2%), then the PBL fraction was centrifuged again on 4 mL of fresh Histopaque 1077. Cells were washed twice in 0.1% L-15 medium, at 600 g for 10 min and stored at −20°C. Nylon mesh (100 µm) filtered HKL suspension in heparinised L-15 medium were isolated and cells suspended in bijoux kept on ice until used.	Respiratory burst Total lipid analysis	(Petropoulos et al., 2009)
PBL	Heparinised PBL were purified by Percoll (1.060/1.075 g mL^{-1}) DCDGC for 35 min at 400 g according to Pettersen et al. (2000). Heparinised PB was diluted 1:4 in PBS, and leucocytes purified by Percoll (1·060/1·075 g mL^{-1}) DCDGC at 400 g for 30 min, and leucocyte fraction collected from the interface were washed in PBS by centrifugation at 200 g for 10 min. The cell pellet was resuspended in PBS-380 and kept on ice.	Nitrite oxide production Respiratory burst Phagocytosis Immune gene expression Bactericidal assay Flow cytometry Immunofluorescence Sudan Black B PBL myeloperoxidase	(Pettersen et al., 2008)
PBL	PBL were isolated by Histopaque (1.077 g mL^{-1}) CDGC at unspecified settings and stored at −80 °C.	Same assays as for *O. mykiss* above	(Kales et al., 2006)
HKL & PBL	Heparinised PB was diluted to 2 mL in PBS and cell strainer (100 µm) filtered HK cell suspension in RPMI 1640 with L-glutamine and gentamicin. Leucocytes from PB and HK were purified by Percoll (1.060/1.075 g mL^{-1}) DCDGC for 35 min at 400 g according to Pettersen et al. (2000).	MAbs labelling Flow cytometry characterisation	(Rønneseth et al., 2006)
HKL & PBL	Unfiltered HK cell suspension in RPMI 1640 with gentamicin sulphate, L-glutamin and heparinised PB diluted 1:3 in PBS (pH 7.3) on ice were isolated by discontinuous Percoll (1.060/1.075 g mL^{-1}) centrifugation for 35 min at 400 g according to Pettersen et al. (2000). Leucocyte at the interface were collected and washed with PBS at 200 g for 10 min, and cells adjusted to 10^6 mL^{-1} in PBS with 1% BSA, 0.1% sodium azide and EDTA.	MAbs labelling Flow cytometry characterisation	(Pettersen et al., 2005)
HKL, PBL	Mesh filtered HK in heparinised L-15 medium with 2% FCS and antibiotic solution on ice were purified by Percoll (51%) CDGC according to Secombes (1990); adjusted to 10^7 mL^{-1}. For PBL, PB pellet was resuspended 1:2 in PBS and centrifuged at 100 g for 10 min. The buffy coat was aspirated, diluted in PBS, and leucocytes purified by Histopaque (1.077/0.001 g mL^{-1}) DCDGC for 30 min at 400 g and 15°C. Leucocytes were collected, washed twice in PBS for 10 min at 400 g and 4°C and once in modified phenol red-free L-15 medium with antibiotic solution.	Phagocytosis Respiratory burst PBL proliferation	(Gross et al., 2004)
PBL & HKL	Cell strainer (100 µm) filtered HK cell suspension in cold wash medium and PB diluted to 2 mL in PBS. HKL and PBL were isolated by Percoll (1.060/1.075 g mL^{-1}) DCDGC for 35 min at 400 g according to Pettersen et al. (2000). The leucocyte suspension was washed at 200 g for 10 min in PBS and resuspended in EDTA PBS with 1% BSA, 0.1% sodium azide at 10^6 mL^{-1}.	MAb assay Flow cytometry characterisation	(Pettersen et al., 2003)
HKL	Sterile steel mesh (100 µm) filtered HK cell suspension in RPMI 1640 were purified by Percoll (34/51%) DCDGC for 30 min at 400 g and 4°C. Leucocytes were aspirated at the interface and washed twice for 5 min at 1200 g and 4°C. The pellet was resuspended in Cortland saline at 1200 g for 5 min at 4°C, and the cell pellets diluted at 10^7 mL^{-1}, with the help of a Thomas' haemocytometer.	Phagocytosis	(Desvignes et al., 2002)
PBL & HKL	Heparinised PB was diluted 1:4 in PBS, were purified by Percoll (1.060/1.075 g mL^{-1}) DDGC at 400 *g* for 30 min. PBL were collected from the interface, washed with PBS at 200 *g* for 10 min, cells were resuspended in PBS with 1% BSA, sodium azide and Titriplex III and kept on ice until used. Unfiltered HKL suspension in RPMI 1640 with gentamycin, L-glutamine and 10% FBS were purified by Percoll CDGC for 30 min at 400 *g* and 4 °C. Cells were washed once in RPMI 1640, at 200 g for 10 min at 4°C, resuspended in TCM with RPMI 1640, gentamicin, L-glutamine, mercaptoethanol and 10% FCS.	PBL MAb characterisation PBL flow cytometry characterisation HKL MAb characterisation	(Melingen et al., 2002)
HKL-TO cells	Unfiltered HK suspension in RPMI 1640 with gentamicin sulphate, L-glutamine, and 10% FCS were purified by Ficoll CDGC for 30 min at 1000 g and 4°C. HKL were suspended in RPMI 1640, centrifuged for 10 min at 900 g and 4°C. The collected cells were diluted in Eagle's MEM, containing Earle's BSS, gentamicin sulphate, fungizone, L-glutamine, mercaptoethanol, 1 % MEM Eagle non-essential amino acid and 10 % FCS.	Virus production	(Wergeland & Jakobsen, 2001)
HKL	Cell strainer (100 µm) filtered HK cell suspension in heparinised L-15 medium with antibiotic solution and 2% FCS were purified by Percoll (25/54%) DCDGC at 400 g for 40 min at 4°C with minor modification to the protocol by Graham and Secombes (1988). Leucocytes at the interface were collected and washed twice in L-15 medium, and seeded at 10^6 mL^{-1} in L-15 medium with 5% FCS, and antibiotic solution.	IFN cytokine like activity	(Jørgensen et al., 2001)

	HKL	Nylon mesh (70 µm) filtered HK cell suspension in heparinised L-15 medium with antibiotic solution and 2% heat-inactivated FCS were purified by Percoll (37%/51%) DCDGC for 35 min at 400 *g* according to Braun-Nesje et al. (1981). Cells at the interface were collected and washed in heparinised L-15 medium with 0·1% FCS at 200 g, for 10 min, and finally resuspended in medium, at 10^6 μL^{-1}.	HKL lysozyme activity Northern blotting	(Paulsen et al., 2001)
	PBL	Heparinised PB was diluted 1:4 in PBS, and leucocytes purified by Percoll (1·060/1·075 g mL^{-1}) DCDGC at 400 g for 30 min, according to Pettersen et al. (1995). The leucocyte fraction collected from the interface were washed in PBS by centrifugation at 200 g for 10 min. The cell pellet was resuspended in PBS-380 and kept on ice.	MAb assay Immunofluorescence double staining Flow cytometry	(Pettersen et al., 2000)
	HKL (SHK-1) cell line	Unfiltered HK suspension in HEPES-buffered saline with collagenase were purified by Percoll (25-42 %, 42-50 %, & 50-58 %) DCDGC at 400 g for 60 min as previously described (Dannevig et al., 1995; Dannevig et al., 1994). Leucocytes were washed by centrifugation and resuspended in L-15 medium with 5% FCS, gentamycin and mercaptoethanol until needed for assays.	Phagocytosis Bacterial killing Enzyme cytochemistry Immunohistochemistry	(Dannevig et al., 1997)
	HKL	Cell strainer (70 µm) filtered HK suspension in L-15 medium with antibiotic solution and 2% FCS were washed twice in L-15 medium counted and resuspended to a concentration of 10^6 mL^{-1} in L-15 +ab and 5% FCS.	Leucocyte proliferation	(Midtlyng et al., 1996)
	PBL	Heparinised PB was diluted 1:2 in L-15 medium with glutamate and sodium bicarbonate. Medium osmolality was adjusted to 380 mOsm. The suspension leucocytes were purified by 54% Percoll CDGC at 400 *g* for 25 min. The leucocytes were washed with medium and adjusted to 10^6 mL^{-1}.	Lymphocyte proliferation Leucocyte IgM detection	(Espelid et al., 1996)
	PBL	Heparinised PB diluted 1:1 in PBS (pH 7·2), were purified by Percoll (1·060 /1·075 g mL^{-1}) DCDGC at 400 g, for 40 min, according to Braun-Nesje et al. (1981). Cellular fractions were collected for assaying following suspension in PBS with 0.1% BSA.	MAb assay Antibody inhibition Immunofluorescence Western blotting Deglycosylation of Igm	(Pettersen et al., 1995)
	HKL	Stainless steel mesh (0.3 mm diameter) filtered HK cell suspension in L-15 medium with FCS and antibiotic solution were purified by Percoll (37%/ 51%) DCDGC for 30 min at 400 g. Cells were adjusted to the right density in L-15 medium with 0.1% FCS, and antibiotic solution.	Respiratory burst Bactericidal assay Superoxide inhibitors	(Jørgensen & Robertsen, 1995)
	HKL	Stainless steel mesh (0.3 mm diameter) filtered HK cell suspension were purified by Percoll (37%/51%) DCDGC for 30 min at 400 g, according to Braun-Nesje et al. (1981). Cells at the interface were collected adjusted to 10^7 mL in L-15 medium with 0.1% FCS, antibiotic solution.	Respiratory burst Phagocytosis Bacterial killing Hydrogen peroxide production NADPH-oxidase inhibition	(Solem et al., 1995)
	HKL	Stainless steel mesh (0.3 mm diameter) filtered HK cell suspension in L-15 medium with glucose, gentamycin and 2% FCS were purified by Percoll DCDGC for 20 min at 400 *g* and 4°C, following the protocol by Braun-Nesje et al. (1981). Cells at the interface were collected, washed in pure medium, and adjusted to 10^6 mL^{-1}.	Phagocytosis Pinocytosis Superoxide anion production Cellular lysosomal enzyme activity Cellular acid phosphatase	(Dalmo & Seljelid, 1995)
	HKL	Metal nets pressed HKL in medium were purified by 54% Percoll CDGC at 400 g according to Reitan and Thuvander (1991). Cells at the interface were washed, counted and resuspended in RPMI 1640 with HEPES, glutamine, antibiotic solution, gentamicin and 5% FCS and used for later assays.	ELISA Leucocyte proliferation	(Erdal & Reitan, 1992)
	HKL, SPL, THL, PBL	PB diluted 1:5 in RPMI 1640, and metal nets pressed HK, SP and TH suspensions in medium were purified by 54% Percoll CDGC at 400 g as described by Thuvander et al. (1987). Cells at the interface were washed, counted and resuspended in RPMI 1640 with HEPES, glutamine, antibiotic solution, gentamicin and 5% FCS and used for later assays.	Same assays as for *O. mykiss* above	(Reitan & Thuvander, 1991)
	HKL	Stainless steel mesh (0.3 mm diameter) filtered HK suspension in heparinised L-15 medium with 10% FCS, glucose and antibiotic solution were sedimented to remove debris and cell viability was done via Trypan Blue exclusion (0.5%). Leucocytes were purified by Percoll (1.060/1.068 g mL^{-1}) DCDGC for 40 min at 400 g at 5°C in the laboratory or on ice. Cell suspensions were washed in L-15 medium with FCS, counted and adjusted to the appropriate density.	Same assays as for *O. mykiss* above	(Braun-Nesje et al., 1981)
S. fontinalis	HKL	Unfiltered HK cell suspensions in DMEM with 10% FCS, glutamine and antibiotic solution were sedimented and the cellular supernatant was recovered, washed in PBS and leucocytes were purified by Ficoll-Histopaque CDGC at unspecified parameters. Collected cells were resuspended in DMEM with 10% heat-inactivated FCS at a density of 10^6 mL^{-1} for cultures.	Northern analysis Phagocytosis Flow cytometry characterisation	(MacKenzie et al., 2003)
S. trutta	HKL	Unfiltered HK cell suspensions were purified according to MacKenzie et al. (2003) above.	Same assays as for *S. fontinalis* above	(MacKenzie et al., 2003)
	HKL, SPL	Metallic filtered (100 µm) issue cell suspension were purified by Ficoll-paque (Histopaque 1.077 g mL^{-1}) CDGC at 600 g for 30 min at 20°C. Lymphocytes were collected at the RPMI/Ficoll interface, washed twice, and adjusted to 10^6 viable lymphocytes mL^{-1} in RPMI1640 with 2 mM L-glutamine and 10% FCS.	Phagocytosis Lymphocyte cytotoxicity	(Marc et al., 1995)

3.4 Popular immunological assays in salmonid leucocyte immunology

Within the 114 reviewed articles, over 200 immunological tests were conducted to study isolated organ and PBL in salmonids (Table 3.3). Findings showed that phagocytosis was the most applied assay to investigate cellular immunological functional capacity, with this technique being followed by respiratory burst activity (Table 3.3). We briefly discuss the most popular assays and give appropriate examples, where possible, below.

Table 3.3. Commonly studied immunological tests in salmonid fish immunology. LM: Light microscopy, TEM: Transmission electron microscopy, SEM: Scanning electron microscopy. Data comes from 114 reviewed studies. Same assays reported by the same author in one paper on different species are reported once.

Immunological assays	Assay counts	% of assays
Flow cytometry characterisation	21	9.5
Microscopy (LM, TEM, SEM)	5	2.3
Leucocyte differential counts	1	0.5
Bacterial killing/Cytotoxicity	6	2.7
Phagocytosis, phagocytic index	41	18.6
Pinocytosis	3	1.4
Respiratory burst, superoxide anion and hydrogen peroxide production	29	13.2
Nitric or Nitrite oxide production	3	1.4
Lysozyme activity	4	1.8
Acid phosphatase	2	0.9
NADPH-Oxidase	1	0.5
Superoxide dismutase	1	0.5
Cell proliferation	20	9.1
Cell migration	1	0.5
Monoclonal antibodies	10	4.5
Antibody production or detection	6	2.7
Immunoglobulins	7	3.2
Plaque forming cells	8	3.6
Peroxidase and myeloperoxidase	3	1.4
Immunohistochemistry, immunofluorescence, epifluorescence, chemiluminescence	8	3.6
Enzyme cytochemistry	1	0.5
ELISA	1	0.5
Immune gene expression, cytokine activity	23	10.5
Northern or western blot	3	1.4
Sudan Black B	2	0.9
Mitogen stimulation	3	1.4
Virus production	1	0.5
Total lipid analysis	1	0.5
Cell viability, apoptosis	4	1.8
Binding capacity	1	0.5
Total assays reviewed	**220**	**100.0**

3.4.1 Phagocytosis assay

Phagocytosis is one of the most important fish immunological responses to pathogens, making its characterisation a good indicator of cellular defence capability. Previous studies show that phagocytosis is the most important innate immune defence mechanisms in poikilothermic fish, as it is the least affected parameter by temperature [reviewed in (Uribe et al., 2011)]. The phagocytic process involves pathogen uptake via engulfment into phagosomes (Øverland et al., 2010). Phagocytes can take up harmful foreign particles as well as dead cells, macromolecules and cellular debris (Grayfer et al., 2014; Sirimanapong et al., 2014; Uribe et al., 2011). Among leucocytes, phagocytic cell-types include B lymphocytes, monocytes, macrophages, neutrophils, MC, DC and non-specific cytotoxic cells (Uribe et al., 2011; Whang et al., 2011; Wu et al., 2019).

Macrophages and neutrophils are professional phagocytes (Mathias et al., 2009; Neumann et al., 2001), with a high phagocytic ability and capacity (Neumann et al., 2001). Professional phagocytes eliminate bacterial pathogens through reactive oxygen species (ROS) production during respiratory burst (Uribe et al., 2011) and reactive nitrogen intermediates (RNI) via pattern recognition receptors (PRR) and cytokines (Grayfer et al., 2014). Inside macrophages, lysosomes are well-equipped with bactericidal molecules, including lysozyme, nucleases, proteases, lipases, and hydrogen peroxide, among others (Neumann et al., 2001). Neutrophils contain granular myeloperoxidase which kills bacteria via halogenation of cell walls in the presence of hydrogen peroxide and halides, and lysozymes with hydrolytic enzymes (Fischer et al., 2006).

The phagocytic process is initiated by receptor-mediated endocytosis or through non-specific hydrophobic interactions of the cell membrane with the target particle (Neumann et al., 2001; Rabinovitch, 1995) through pathogen associated molecular patterns (PAMPs). The steps involved in neutrophil and macrophage destruction of pathogens sequentially involve: pathogen recognition via chemotaxis, pathogen adhesion, pathogen ingestion and formation of a phagosome, followed by a phagolysosome, digestion and exocytosis of residues (Neumann et al., 2001).

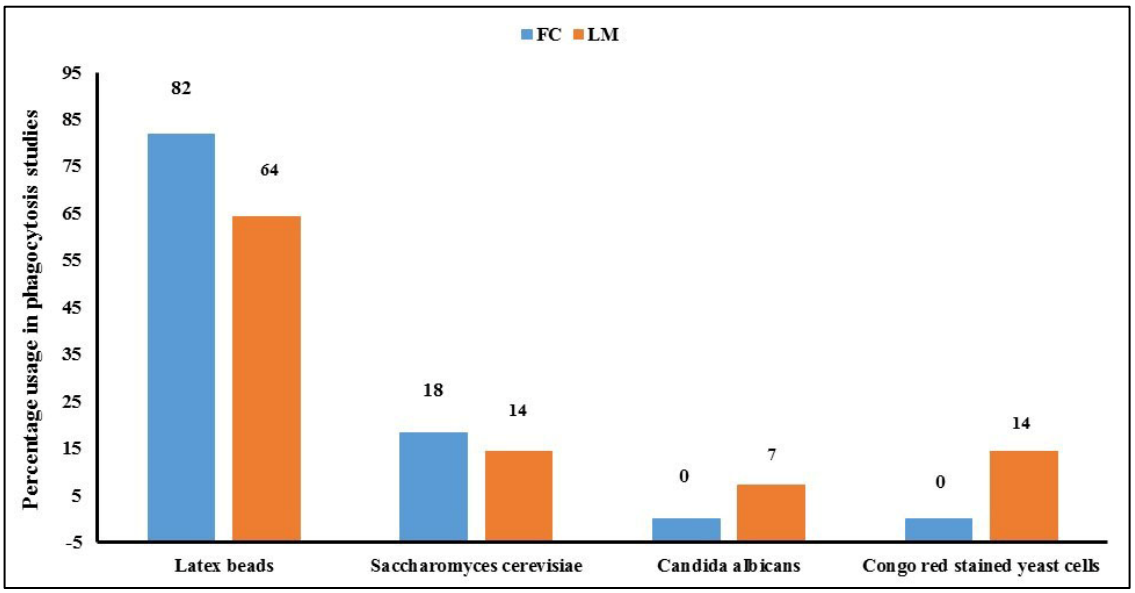


Figure 3.3 Techniques commonly used to study phagocytosis involving isolated salmonid fish leucocytes. FC: Flow cytometry, LM: Light microscopy. Data comes from 114 reviewed studies.

In this paper, leucocyte phagocytosis was the most applied immunological assay to illustrate immunomodulation in farmed salmonids, reported in 40 studies. The most commonly used technique to study phagocytosis was flow cytometry, followed by light microscopy, using fluorescent latex beads (Figure 3.3). In addition, studied leucocytes were mostly isolated from the HK and PB (Table 3.1); while the most studied species were *O. mykiss* and *S. salar* (Table 3.1). Recent examples that employed isolated leucocyte phagocytosis to investigate salmonid immunological responses included engulfment of fluorescence-emitting beads by *O. mykiss* PBL,

following stimulation with interleukin-1β (*il-1β*) and interleukin-6 (*il-6*) (Hu et al., 2018). In addition, sheep red blood cells (SRBC) were phagocytosed by *O. tshawytscha* HKL (Arkoosh et al., 2018), and yeast cells by *S. salar* HK (Paredes et al., 2013). The complete list of particles employed in assessing isolated leucocyte phagocytosis among salmonids is included in Table 3.4.

Table 3.4. Techniques used to study phagocytosis using isolated salmonid organ and PBL. CL: calorimetry, FC: flow cytometry, PCM: phase contrast microscopy, LM: light microscopy, EFM; epifluorescence microscopy, FM: fluorescence microscopy, SEM: scanning electron microscopy, SP: spectrophotometer, MMR: multimode microplate reader, LU: Luminometer, CC: colony count, MPF: Microplate fluorometer; ER: ELISA reader. For blood tissues, HK: head kidney, PB: peripheral blood, MK: Middle kidney, and SP: Spleen.

Species	Tissue	Targeted cells	Inducers	Technique	Reference
O. mykiss	PB	Leucocytes	Fluorescent latex beads 1.0 µm	FC	(Hu et al., 2018; Wang et al., 2018)
	HK,SP,MK	Leucocytes	Green fluorescent 1.0 µm latex beads	FC	(Bassity & Clark, 2012)
	HK	Macrophages	Latex beads	LM	(Nya & Austin, 2010)
	HK	Macrophages	Latex beads	LM	(Nya & Austin, 2009)
	HK	Macrophages	Zymosan A	SP	(Yada, 2009)
	HK	Leucocytes	Pathogenic bacteria (*Streptococcus iniae*, *Lactococcus garvieae, A. salmonicida, Yersinia ruckeri, Vibrio anguillarum* and *Vibrio ordalii*)	CC	(Brunt et al., 2007)
	HK	Leucocytes	Latex beads 0.85 µm	LM	(Kim & Austin, 2006)
	HK	Leucocytes	Latex beads 0.8 µm	LM	(Brunt & Austin, 2005)
	HK	Leucocytes	*S. cerevisiae*	LM	(Cecchini et al., 2005)
	HK	Macrophages	Fluorescent latex beads 2.0 µm	LM	(Panigrahi et al., 2004)
	HK	Macrophages	Fluorescein-labelled *Escherichia coli*	MPF	(Hoeger et al., 2004)
	PB	Macrophages	*S. cerevisiae*	SP	(Dügenci et al., 2003)
	HK, PB	Leucocytes Free erythrocytes	*C. albicans*	LM	(Passantino, Altamura, et al., 2002)
	HK	Leucocytes	Latex beads 0.8 µm	LM	(Irianto & Austin, 2002)
	HK	Leucocytes Macrophages	*S. cerevisiae*	LM	(Clerton et al., 2001)
	HK	Leucocytes	Fluorescent latex beads 1.03 µm	FC	(Karrow et al., 1999)
	PB, HK	Leucocytes	Fluoresceinated (FITC) latex beads	FC	(Chilmonczyk & Monge, 1999)
	HK	Leucocytes	Latex particles (0.85 µm)	LM	(Sakai et al., 1995)
	SP	Leucocytes	Fluoresceinated (FITC) latex beads, 1.72 µm	FCM,	(Thuvander, 1989)
	PB	Leucocytes	Fluoresceinated (FITC) latex beads, 1.72 µm	FC, FCM, SEM	(Thuvander et al., 1987)
	HK	Macrophages	SRBC, latex beads, heat-killed *Candida*, live yeast cells & *V. anguillarum*	PCM	(Braun-Nesje et al., 1981)
O. *nerka*	HK	Leucocytes	FITC-labelled *Staphylococcus aureus*	FM	(Alcorn et al., 2002)
O.tshawytscha	PB	Leucocytes	Fluorescent latex beads 1.0–1.3 µm	LM	(Lulijwa et al., 2019b)
	HK	Macrophages	SRBC	SP	(Arkoosh et al., 2018)
	HK	Macrophages	SRBC	SP	(Arkoosh et al., 2015)
	HK	Leucocytes	FITC-labelled *S. aureus*	FM	(Alcorn et al., 2003)
S. salar	HK	Macrophages	Yeast cells	LM	(Paredes et al., 2013)
	HK	Macrophages	Fluorescein-labelled *E. coli*	ER	(Romero et al., 2012)
	PB	Leucocytes	Fluorescent latex beads 1.00 µm	FC	(Haugland et al., 2012)
	PB, HK	Leucocytes	Fluorescent latex beads 1.00 µm	FM, FC	(Øverland et al., 2010)
	HK	Macrophages	Fluorescent latex beads 2 or 3 µm	LM, FC	(Pettersen et al., 2008)
	HK	Phagocytes	Yeast cells	LM	(Gross et al., 2004)
	HK	Macrophages	Opsonised zymosan	LU	(Desvignes et al., 2002)
	HK	Macrophages	*A. salmonicida*	EFM	(Dannevig et al., 1997)
	HK	Macrophages	Glutaraldehyde-fixed sheep red blood cells	CL	(Solem et al., 1995)
	HK	Macrophages	Fluorescein labelled Latex microbeads	PCM	(Dalmo & Seljelid, 1995).
	HK	Leucocytes and macrophages	Fixed sheep red blood cells, latex beads, heat-killed *Candida*, live yeast cells & *V. anguillarum*	PCM	(Braun-Nesje et al., 1981)
S. fontinalis	HK	Macrophages	*S. cerevisiae* FITC	FC	(MacKenzie et al., 2003)
S. trutta	HK	Macrophages	*S. cerevisiae* FITC	FC	(MacKenzie et al., 2003)
	HK	Leucocyte	Opsonised zymosan	LU	(Marc et al., 1995)

3.4.2 Respiratory burst

Respiratory burst or oxidative burst involves rapid production of ROS (superoxide molecules and hydrogen peroxide) by different leucocytes during innate immune responses to pathogens. The technique is one of the most important salmonid immunological assays, frequently assessed together with phagocytosis. This technique may require stimulation with PAMPs and macrophage-activating factors (MAF), such as phorbol myristate acetate (PMA) or concanavalin A (con A) either in synergy or singly. In this review, respiratory burst was the second most studied cellular innate immunological assay employed in innate immune responses of salmonids (Table 3.3). During fish immunological responses to pathogens, respiratory burst plays an important biochemical role that facilitates successful phagocytosis and eventual pathogen destruction. The technique is a monopoly of phagocytic neutrophils, monocytes and macrophages, and aims at ROS production, catalysed by NADPH oxidase complex that lines the inner phagocyte cell membranes and cytoplasm following stimulation (Briggs et al., 1975). However, as monocytes differentiate into macrophages, they lose capacity to produce ROS (Cassatella et al., 1985).

The process of respiratory burst has been reviewed thoroughly by Grayfer et al. (2014) and the process for ROS production was illustrated earlier in a review in teleosts by Neumann et al. (2001). Briefly, the reaction starts with formation of superoxide anion (O_2^-), which eventually gets catalysed into hydroxyl radical (OH.), hydrogen peroxide (H_2H_2), hypochlorus acid (OCl^-) and peroxynitrite ($ONOO^-$) [reviewed in Grayfer et al. (2014)]. In salmonids, genes for the enzyme NADPH oxidase complex have been linked with ROS production in *O. mykiss*, *S. salar*; and were cloned and sequenced in *O. mykiss* [reviewed in Grayfer et al. (2014)]. In addition, Grayfer et al. (2014) provided numerous evidences of the induction of phagocyte ROS production by cytokines, such as tumor necrosis factor alpha (*tnf-α*) and interferon gamma (*ifnγ*) and *il-1β* in *O. mykiss*.

Consequently, leucocyte respiratory burst has been widely used in several *in vitro* and *in vivo* experiments to illustrate immunomodulatory effects induced by dietary manipulations in *O. mykiss* (Nya & Austin, 2010; Sharifuzzaman & Austin, 2010), *S. salar* (Ulvestad et al., 2018); immunostimulants in *O. mykiss* (Brunt & Austin, 2005); *O. tshawytscha* (Lulijwa et al., 2019b) and in *S. salar* (Paredes et al., 2013). Respiratory burst has also been used to assess: immunomodulatory effects induced by chemical and physical stressors in *O. tshawytscha* (Arkoosh et al., 2018), in *O. mykiss* (Markkula et al., 2009; Taylor et al., 2007); vaccines efficacy in *O. mykiss* (Marsden et al., 1994; Marsden & Secombes, 1997); and effects of pathogens in *O. mykiss* (Rønneseth et al., 2013) and *S. salar* (Desvignes et al., 2002).

3.4.3 Flow cytometry assays

Flow cytometry is a popular laser-based technology that allows multiple characterisation of cells in biofluids and has been used in fish to rapidly, accurately, and consistently analyse fish leucocyte subpopulations and cellular immune functions (Chilmonczyk & Monge, 1999). For example, flow cytometry has been used to characterise fish leucocytes in *O. mykiss* (Crippen et

al., 2001; Hu et al., 2019; Korytář et al., 2013; Pettersen et al., 2000; Scharsack et al., 2001; Tillitt et al., 1988), *O. tshawytscha* (Lulijwa et al., 2019a), *S. salar* (MacKenzie et al., 2003; Melingen et al., 2002; Peñaranda et al., 2019; Pettersen et al., 2003). Flow cytometry has also been used to assess cellular functional characteristics (Chilmonczyk & Monge, 1999) of respiratory burst (Kalgraff et al., 2011), antibody production (Hedfors et al., 2012; Johansson et al., 2016; Milston et al., 2003; Pettersen et al., 2005; Pettersen et al., 2008; Pettersen et al., 2003) and phagocytosis (Haugland et al., 2012; Øverland et al., 2010; Thuvander et al., 1987) among others. Leucocyte flow cytometry has also been used to assess salmonid fish immune response to pathogens (Chilmonczyk & Monge, 1999; Rønneseth et al., 2013; Rønneseth et al., 2006); and chemical stressors (Misumi et al., 2005; Misumi et al., 2009). Recently, a portable flow cytometer, the Muse® Cell Analyser, has been used in aquaculture as an accurate and fast quantitative assessment tool of single cells compared to traditional manual methods (Lulijwa et al., 2019a).

3.4.4 Immune gene and cytokine expression

Detection of immune genes is yet another important technique used to assess immunomodulation in aquaculture, via leucocyte cytokine transcript expression. Zhang and An (2007), described cytokines as proteins released by cells and have a specific effect on the interactions and communications between cells. Depending on the cells that produce them, cytokines are called lymphokine, monokine, or chemokine if they have chemotactic capacity and interleukin (IL) if produced by a specific leucocyte type and act on another leucocyte type. Stimulated macrophages produce pro-inflammatory cytokines, which accelerate inflammatory reactions during pathological or stressful conditions. Pro-inflammatory cytokines of *il-1β*, *il-6*, and *tnf-α*, are involved in inflammation; while anti-inflammatory cytokines such as *il-1* receptor antagonist, *il-4*, *il-10*, *il-11*, and *il-13* are produced to control actions of the pro-inflammatory cytokines (Zhang & An, 2007).

Fish have major pro-inflammatory cytokines such as *tnf-α*, *il-1β*, *il-6*, downstream effectors *il-8* and *il-12*; and the classical anti-inflammatory *il-10* and transforming growth factor (TGF-β1), but lack most of the mammalian TNF-β and *il-1* members, such as *il-1f1*, *il-1f3* and *il-1f5-11* [reviewed in Secombes (2016)]. In fish, *il-1β* stimulates T-lymphocytes [reviewed in Magnadottir (2010)], while IL-1 receptors have been suggested to regulate *il-1β* in stress responses, as they have been consistently regulated in *O. mykiss* and *S. salar* HK and SP following LPS and *tnf-α* stimulation (Sangrador-Vegas et al., 2000; Subramaniam et al., 2002). Recently, PBL were used to demonstrate IL-2 modulated gene expression and cytokine induced cellular proliferation (Wang et al., 2018). Thus, gene expression has been employed in salmonids to characterise *S. salar* TO cell line, small mononuclear cells for dendritic like properties (Haugland et al., 2012; Pettersen et al., 2008), *O. mykiss* HK and PB leucocytes for immune response (Hu et al., 2018; Monte et al., 2015) and viral pathogen transcript detection (Julin et al., 2015; Rønneseth et al., 2013). Immune gene expression has also been used in salmonids to assess immunomodulatory effects induced by steroid hormones (MacKenzie et al., 2006; Peña et al., 2016; Romero et al.,

2012; Yada, 2009; Yada et al., 2004), LPS and β-glucan administration (Goetz et al., 2004; MacKenzie et al., 2006; Ulvestad et al., 2018), and temperature perturbations (Kales et al., 2006; Nath et al., 2006) in farmed salmonids. However, the potential effects of nucleic acids and lipoproteins as crude LPS contaminants may be co-inducers of pro-inflammatory activity (Hong et al., 2013).

3.4.5 Cell proliferation assay

The cell proliferation assay is a measure of an increase in cell population, considered an indicator of cell health, and is commonly used to monitor the response of cells following immunostimulation (Adan et al., 2016). In salmonids, isolated leucocyte proliferation is one of the important immunological assays widely applied. The technique was recently used to characterise *O. mykiss* PBL following isolation by hypotonic lysis and density gradient centrifugation (Hu et al., 2018). Frequently, leucocyte proliferation was used to assess effects induced by steroid hormones (Espelid et al., 1996; Yada et al., 2019; Yada et al., 2004), pathogens (Chilmonczyk & Monge, 1999; Gross et al., 2004; Marsden et al., 1994; Marsden et al., 1995), mitogens and or pathogens (Reitan & Thuvander, 1991; Tillitt et al., 1988), vaccines (Boesen et al., 1997; Erdal & Reitan, 1992; Marsden et al., 1996; Midtlyng et al., 1996), vitamin C (Hardie et al., 1993), hydrocarbon creosote (Karrow et al., 1999), sewage effluents (Hoeger et al., 2004), and long term UVB irradiation (Markkula et al., 2009) in *O. mykiss* and *S. salar*.

3.4.6 Antibody production assay

Antibodies (Ab) or immunoglobulins (Ig) are surface cellular markers that provide a suitable surface for attachment of specific pathogens to B-lymphocytes. They form part of the adaptive immune system that is very pathogen specific, thanks to their high affinity [reviewed in Hawlisch and Köhl (2006)]. Antibodies help to facilitate a proper response to pathogens (Dunkelberger & Song, 2010) in association with the complement system. In teleosts, the complement system is composed of different serum proteins that play a vital role in fish defence and is divided into three different pathways. These include the classical complement activity, which is triggered by antibody binding to cell surfaces (Holland & Lambris, 2002). The alternative pathway is directly activated by foreign pathogens (Boshra et al., 2006), and the lectin pathway is activated by binding of a protein complex made of mannose or mannan binding lectin in bacterial cells (Sakai, 1992). In fish, the alternative complement pathway is the most important innate immune defence mechanism against pathogens (Boshra et al., 2006), through pathogen killing via opsonisation and phagocyte activation, usually through Gram-negative bacterial cell wall LPS (Rauta et al., 2012). In their review, Uribe et al. (2011) noted that the complement proteins enhanced salmonid antibody neutralisation of encapsulated viruses; while LPS present in Gram-negative pathogenic bacteria stimulated complement proteins to facilitate phagocytosis.

A number of studies characterised salmonid isolated leucocytes for the presence of Ig (Bassity & Clark, 2012; Hedfors et al., 2012; Kaattari & Yui, 1987; Korytář et al., 2013; Milston et al., 2003; Pettersen et al., 2000; Pettersen et al., 1995). Isolated leucocyte Ig or Ab production

was used to study immunomodulatory effects induced by recombinant interleukin-4/13A in *O. mykiss* HKL cultures (Johansson et al., 2016) and rearing temperature (Pettersen et al., 2005). Other leucocyte Ig or Ab production studies looked at immune effects of seawater and freshwater shifts on MAb+ and IgM+ cells in PB and HK leucocytes in *S. salar* (Pettersen et al., 2003), photoperiod and immune response in *S. salar* PB and HK leucocytes (Melingen et al., 2002). Isolated leucocyte MAbs production were also used to detect infectious pancreatic necrosis virus (IPNV) in vaccinated *S. salar* HK and PB leucocytes (Rønneseth et al., 2013; Rønneseth et al., 2006). Other studies used leucocyte Ig and MAbs production to assess immunomodulatory effects of bovine lactoferrin (Cecchini et al., 2005), vaccines (Marsden et al., 1996), mitogens and pathogens (Reitan & Thuvander, 1991), hydrocarbon creosote (Karrow et al., 1999), polychlorinated biphenol (Thuvander & Caristein, 1991), tributyltin (Misumi et al., 2009), and hormones (Espelid et al., 1996).

3.5 Leucocyte application to assess effects of dietary manipulations

Intensive aquaculture produces fish under high density, and may subject animals to a multitude of stressors and conditions that support pathogenic infections. Good nutrition, and welfare must be ensured to support growth and enhance disease resistance (Ashley, 2007). Good nutrition prior to disease outbreaks increases fish resistance and reduces mortality (Waagbø, 1994). Thus, immune system enhancement is a central aspect in aquaculture operations. In this regard, several *in vivo* and *in vitro* dietary manipulations using for instance probiotics, immunostimulants, including but not limited to nucleotides, β-glucans, LPS and polysaccharides have been commonly applied in aquaculture to enhance fish innate immunity and prevent infection [reviewed in Magnadottir (2010); Uribe et al. (2011)].

3.5.1 Illustrating immune benefits of probiotics

Probiotics are live microbial adjuvants which confer beneficial effects to the host when administered in sufficient quantities (FAO/WHO., 2001). In the last three decades, several studies have used isolated leucocytes functional properties to assess effectiveness of singular and synergistic probiotics administration against pathogens. At a cellular immune functional level, singular oral administration of Kocuria SM1 in *O. mykiss* resulted in effective defence against *Vibriosis* via enhanced respiratory burst and HKL bacterial killing activities (Sharifuzzaman & Austin, 2010). Offered synergistically, probiotics *Carnobacterium maltaromaticum* B26 and *Carnobacterium divergens* B33, significantly enhanced HKL phagocytosis and respiratory burst , respectively, against *A. salmonicida* and *Y. ruckeri* (Kim & Austin, 2006). In addition, enhanced HKL phagocytic and respiratory burst activities, resistance against *L. garvieae* and *S. iniae* were reported following probiotics *Aeromonas sobria* GC2 administration in *O. mykiss* (Brunt & Austin, 2005). Also, oral administration of probiotics JB-1 and GC2, equated to *Bacillus* sp. and *A. sobria*, respectively, stimulated HKL phagocytosis and respiratory burst activities against infectious *A. salmonicida, L. garvieae*, *S. iniae, V. anguillarum, V. ordalii* and *Y. ruckeri* in *O.*

mykiss (Brunt et al., 2007). Besides, a potential probiotic *Lactobacillus rhamnosus* (JCM 1136) induced increased HKL phagocytosis in *O. mykiss* (Panigrahi et al., 2004).

3.5.2 Studying immune benefits of immunostimulant and MAFs effects

Evolutionally important, PAMPs are conserved signal molecules found in bacteria and viruses (Castro & Tafalla, 2015). They include viral double stranded ribonucleic acid (dsRNA) and bacterial deoxyribonucleic acid (DNA), fungal β1,3-glucans (β-glucan), bacterial cell wall peptidoglycans, polysaccharides and Gram-negative bacterial endotoxin or lipopolysaccharides (LPS), not expressed in multicellular life forms (Elward & Gasque, 2003). PAMPs are recognised by the host immune system through the pattern recognition receptors (PRRs) on cell surfaces, following breach of physical barriers. The binding of the host cellular PRRs and the PAMPs initiates cellular responses specifically designed to kill and eliminate the microbial pathogen (Rieger & Barreda, 2011). MAFs are receptor based signals that initiates pathogen clearance (Mosser & Edwards, 2008). Indeed, salmonid isolated leucocytes have been used to assess immunostimulatory effects of PAMPs, such as viral double stranded (dsRNA), LPS, β-glucan, cytokines, MAFs such as PMA, con A and phytohaemagglutinin (PHA) following singular or synergistic administration *in vivo* and *in vitro*.

Functionally, singular administration of LPS *in vivo* enhanced oxidative burst, phagocytosis, and bactericidal activities against pathogenic *Aeromonas hydrophila* in *O. mykiss* (Nya & Austin, 2010). However, conflicting results were obtained as singular LPS did not alter phagocytic capacity in *O. mykiss* HK and PB macrophages *in vitro* (Passantino, Tafaro, et al., 2002). Furthermore, *in vitro* exposure to different bacterial LPS products enhanced respiratory burst activity, phagocytic activity and ability to kill an avirulent A-layer lacking strain of *A. salmonicida in S. salar* HKL macrophages and enhanced pathogen resistance (Solem et al., 1995). Using β-glucan *in vitro*, singular administration induced higher ROS production compared to singular LPS stimulation in *S. salar* HKL macrophages (Ulvestad et al., 2018), and *in vitro*, administration of β-glucan enhanced respiratory burst in *S. salar* HK macrophages, but did not induce increased bactericidal activity against avirulent and virulent strains of *A. salmonicida* (Jørgensen & Robertsen, 1995). Offered synergistically, LPS and β-glucan enhanced phagocytosis, pinocytosis and superoxide anion production in *S. salar* HKL macrophages (Dalmo & Seljelid, 1995). Similarly, combined yeast β-glucans and bacterial LPS enhanced respiratory burst activity in *S. salar* HKL macrophages (Paulsen et al., 2001; Ulvestad et al., 2018).

While applying MAFs, *O. mykiss* HKL macrophages were stimulated into respiratory burst with PMA to produce oxygen and hydrogen peroxide, which was catalysed by exogenous superoxide dismutase (SOD) and its inhibitor (diethyldithiocarbamate; DDC) reduced hydrogen peroxide production (Chung & Secombes, 1988). Similarly, *O. mykiss* HKL macrophages, stimulation with PMA induced higher respiratory burst activity than stimulation with LPS, *tnf-α* or β-glucans, while a synergistic response was even higher (Novoa et al., 1996). Likewise, MAF con A induced stronger *O. mykiss* PBL proliferation than did LPS, PHA or pokeweed mitogen

(PKWM) *in vitro* (Tillitt et al., 1988) and earlier work had revealed that *O. mykiss* leucocytes isolated from HK, TH, PB and SP, responded very well to LPS and con A *in vitro* (Warr & Simon, 1983).

Also, in *O. kisutch*, IgM production was induced following PHA treatment, which suggested that this PAMP may not be limited to T cell activation in salmonids, but may alternatively induce the production of lymphokines capable of polyclonal activation of B cells, while fetal calf serum (FCS) induced production of large amounts of IgM without antigenic stimulation (Kaattari & Yui, 1987). In addition, *O. mykiss* SPLs were stimulated *in vitro* for antibody production following antigen B-lymphocyte stimulation with Trinitrophenylated (TNP) forms of *E. coli* LPS and keyhole limpet haemocyanin (KLH) (Kaattari et al., 1986).

At the gene level, isolated leucocytes have been used to demonstrate that synergistic β-glucan and bacterial LPS administration enhanced accumulation of lysozyme gene transcripts in *S. salar* HK macrophages (Paulsen et al., 2001). In addition, *S. salar in vitro* combined stimulation with bacterial LPS and β-glucan highly induced gene expression for arginase-1 and pro-inflammatory IL-1β in the stimulated cells (Ulvestad et al., 2018). In established *S. salar* TO cell line, LPS induced significant upregulation of cluster of differentiation 83 (CD83)gene transcript, indicative of DC origin, while expression of TCR-α or the macrophage marker M-CSFR were not detected *in vitro* (Pettersen et al., 2008). Using bacterial LPS *O. mykiss* macrophages stimulated *in vitro* by *E. coli* LPS resulted into upregulation of genes for vascular cell adhesion molecule, the CCAAT/enhancer binding protein β, nuclear factor kappa B (NF-kB α) inhibitor, DCs cell restricted marker (CD209e), major histocompatibility complex class II (MHC II), cyclin L1, acute phase serum amyloid A, and prostaglandin endoperoxide synthase 2 (Goetz et al., 2004). Finally, plasmid DNA and synthetic oliogodeoxynucleotides (ODNs) with unmethylated CpG induced antiviral IFN cytokine activity in *S. salar* HKL; particularly among adherent macrophages than suspension leucocytes (Jørgensen et al., 2001).

Fish leucocytes have been used to study and classify different cytokines in *O. mykiss* HKL incubated with LPS and Poly I:C or the pro-inflammatory cytokine IFN-γ, which induced class 2 cytokine receptors named R1 type receptors (IL-10R1/CRFB7, IL-20R1a/CRFB8a and IL-20R1b/CRFB8b) and one R2 type receptor (IL-10R2/CRFB4), as antiviral molecules against VHSV (Monte et al., 2015). In addition, isolated leucocytes were applied to assess directional immune response as *O.mykiss*, RTS 11 cells, recombinant *il-1β* and IFN-γ reportedly induced upregulation of genes involved in inflammation and major histocompatibility complex class I (MHC I) antigen presentation pathway respectively (Martin et al., 2007).

3.5.3 Illustration of immunomodulatory benefits of plant and animal extracts

During the 1990s and early 2000s, salmonid isolated leucocytes were used to demonstrate immune effects of plant- and animal-based extracts at cellular immune functional levels. For example, PBL showed that dietary plant extracts from mistletoe (*Viscum album*), nettle (*Urtica dioica*), and ginger (*Zingiber officinale*) in *O. mykiss* significantly enhanced phagocytosis and

extracellular respiratory burst in fish fed a diet containing 1% powdered ginger root extracts (Dügenci et al., 2003). In addition, enhanced HKL phagocytosis, respiratory burst and bactericidal activities demonstrated enhanced immunity following oral administration of Garlic (*Allium sativum*) in *O. mykiss* (Nya & Austin, 2009). Enhanced HKL phagocytes chemiluminescence and phagocytosis in *O. mykiss*, administrated *in vivo* and *in vitro* with bovine lactoferrin was demonstrated (Sakai et al., 1995), including increased HKL endocytosis in *O. mykiss* administered with bovine lactoferrin (Cecchini et al., 2005). Conversely, the administration of bovine lactoferrin did not affect HKL antibody production against human-γ-globulins (HGG) *in vivo* in *O. mykiss* (Cecchini et al., 2005).

In vitamin C deficient *O. mykiss*, isolated HKL showed that *in vitro* supplementation enhanced leucocyte proliferation and response to PAMPs, while intraperitoneal (i.p.) administration enhanced the same parameters and *in vitro* supplementation enhanced leucocyte proliferation in fish fed a commercial diet (Hardie et al., 1993). In addition, *in vivo* supplementation or *in vitro* HKL incubated with or without pantothenic acid (vitamin B_5) induced strong cell mediated migration against infection with *Cryptobia salmositic* in *O. mykiss* (Thomas & Woo, 1990).

Similarly, in *O. tshawytscha* fed with experimental feeds with either fish meal as a control, fish meal plus cooked fish by-products, or fish meal plus hydrolyzed fish protein alone, or with hydrolyzed fish protein and processed fish bones, there was enhanced phagocytosis, respiratory burst and myeloperoxidase activity in all fed groups (Murray et al., 2003). Moreover, feeding level affected *O. tshawytscha* cellular function, as phagocytic activity appeared to be inversely proportional to feeding level (Alcorn et al., 2003).

3.5.4 Understanding the effects caused by hormonal supplements

Hormonal supplements are applied in animal production to enhance growth (Yu et al., 1979) and enhance immune function (Paredes et al., 2013). Indeed, cultured and isolated salmonid leucocytes have been used to demonstrate immune enhancement both *in vitro* and *in vivo*. For example, *S. salar* SHK-1 cell line and HK leucocytes exhibited increased ROS production and phagocytosis following synthetic prolactin (PRL)-releasing peptide (PrRP) administration *(*Romero et al., 2012*)*. Administered *in vitro* and *in vivo* HK macrophages treated with PRL resulted into enhanced ROS production and cellular phagocytosis (Paredes et al., 2013). In addition, *in vitro* administration of insulin-like growth factor-I (IGF-I) stimulated increased superoxide production in zymosan-stimulated *O. mykiss* HKL (Yada, 2009). Synergistic *in vitro* PRL and growth hormone (GH) administration did not affect cellular proliferation (Yada et al., 2004); while *in vivo* singular i.p. administered of GH induced production of superoxide anions in *O. mykiss* PBL in both freshwater and seawater (Yada et al., 2001).

At the gene expression level, isolated salmonid leucocytes have been used to validate immunostimulatory and immunosuppression effects of hormones through assessment of immune related cytokine expression. For instance, PRL supplementation immediately upregulated

expression of toll-like receptors (TLRs), myeloid differentiation factor 88 (MyD88) gene transcripts and prolonged the expression of *il-1β* to regulate long term immune response in *P. salmonis* infected *O.mykiss* (Peña et al., 2016). Similarly, PRL upregulated the expression of genes for *il-1β*, inhibitor of kappa B (IκBα), TLR1, and TLR5M (membrane-bound form) in *S. salar* SHK-1 cells infected with *P. salmonis* (Soto et al., 2016), and supplementary PRL-releasing peptide (PrRP) induced expression of pro-inflammatory cytokines *il-1β*, *il-6*, *il-8*, *il-12* and PRL in SHK-1 cells and *S. salar* HKL (Romero et al., 2012). Conversely, cortisol administration significantly inhibited the LPS-dependent upregulation for expression of *tnf-α2*, a pro-inflammatory cytokine in *O. mykiss* macrophages (MacKenzie et al., 2006). Furthermore, salmon insulin like growth factor-1 (sIGF-I), induced expression of insulin like growth receptor (IGFR) type Iβ in *O. mykiss* HKL *in vitro* but did not enhanced expression of another IGFR type Iα (Yada, 2009). Administered synergistically, PRL and GH did not affect PBL lysozyme gene transcript in *O. mykiss* (Yada et al., 2004).

3.5.5 Exemplary case studies

To demonstrate the application of fish leucocytes in illustrating immunomodulatory effects of dietary manipulation, an *in vitro* study by Ulvestad et al. (2018) and an *in vivo* one by Nya and Austin (2010) are used. The *in vitro* study investigated the effects of singular and synergistic *in vitro* LPS and β-glucan administration on *S. salar* HK macrophages respiratory burst and immune gene expression. HK were aseptically obtained from healthy and unvaccinated *S. salar* and leucocytes were isolated by discontinuous Percoll (GE Healthcare, Sweden) according to the protocol by Braun-Nesje et al. (1981). Isolated cells were adjusted to 10^5 mL^{-1} using a NucleoCounter® NC-200™ (Chemometec, Denmark). Cells were plated and kept in medium for 24 h, to separate adherent from suspension cells. Isolated by washing, adherent cells were cultivated in cell medium supplemented with antibiotic solution and 2% FBS. 24 h post isolation, HK macrophages were stimulated with singular LPS /β-glucan or synergistically for 24 h (single stimulation) or 48 h (multiple stimulation), and control cells received only the cell medium. Functionally, cellular HK macrophages treated *in vitro* by LPS and β-glucan exhibited enhanced ROS production compared to the control, while stimulation and costimulation by β-glucan prompted higher ROS production than with LPS (Ulvestad et al., 2018). At the gene level, the arginase-1 and IL-1β gene transcripts were highly expressed in treated *S. salar* HK macrophages than in the control fish leucocytes (Ulvestad et al., 2018).

Secondly, the *in vivo* study applied isolated leucocytes to demonstrate immunomodulatory effects of immunostimulant LPS supplementation (Nya & Austin, 2010). The authors investigated the efficacy of supplementary LPS against infectious *A. hydrophila* in O. *mykiss* fingerlings. Fish fingerlings were fed a commercial diet supplemented with zero (control), 1·875, 3·75, 7·5 and 15.0 mg LPS 100 g^{-1} of feed. Five groups of 20 fish each were fed twice daily to satiety for two weeks. 24h post the dietary feeding schedule, fish were challenged by i.p. injection with 0.1 mL suspensions of *A. hydrophila.* To assess the efficacy of LPS as an immunostimulant,

HKL were isolated by discontinuous Percoll gradient (Sigma-Aldrich) (Sakai et al., 1995) to assess innate cellular immune functions of phagocytosis, respiratory burst, lysozyme activity and bactericidal activity from groups of 10 fish. Isolated HKL demonstrated that LPS administration *in vivo*, effectively enhanced the fish innate immune capacity against *A. hydrophila* via enhanced phagocytosis and phagocytic index in supplemented *O.* mykiss (Nya & Austin, 2010).

3.6 Leucocytes used to assess effects of physical and chemical stressors

Farmed fish and shellfish may face a multitude of intrinsic and extrinsic stressors that influence the innate immune capacity [reviewed in Uribe et al. (2011)]. Major extrinsic factors that modulate fish innate immune capacity include changes in temperature (Kayansamruaj et al., 2014); variation in oxygen levels, suspended solids, pH, and salinity (Wang et al., 2018b), photoperiod (Tian et al., 2015), handling stress and stocking density (Yarahmadi et al., 2016). However, temperature is even more important due to fish's poikilothermic lifestyle (Guijarro et al., 2015). Relatedly, ultraviolet radiations have also been reported to modulate salmonid immunity (Jokinen et al., 2008; Markkula et al., 2007; Markkula et al., 2009; Salo et al., 2000). Furthermore, immune effects of environmental contaminants have been reviewed by Rehberger et al. (2017) and include pesticides, organic compounds such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (HAHs), polybrominated biphenyl ethers (PBDEs), heavy metals, pharmaceutical compounds and natural hormones (Peña et al., 2016).

3.6.1 Studying the effects induced by physical stressors

Among salmonids, isolated leucocytes have been applied to illustrate immunomodulatory effects of physical stressors, including but not limited to temperature, osmotic shock, ultraviolent (UV) irradiation at cellular functional and gene levels. For example, isolated HK macrophages showed that cold stress in *O. nerka* reared at 8°C resulted into increased HK macrophage phagocytosis than in fish at kept at 12°C (Alcorn et al., 2002) and fish reared at 6°C exhibited higher percentages of Ig+ cells in PBL compared to fish reared at 18°C in *S. salar* post-smolts (Pettersen et al., 2005). At the molecular level, cold stress at 2°C downregulated the expression of genes for MHC-II β and α genes in *O. mykiss* (Nath et al., 2006), confinement in shallow water downregulated *O. mykiss* PBL corticosteroid receptors (Yada et al., 2019), while fish maintenance at 2°C did not affect expression of *beta-2 microglobulin* (β_2m*)* in *S. salar* and *O. mykiss* (Kales et al., 2006).

In addition, osmotic stress during transfer from freshwater to seawater significantly enhanced HK macrophage respiratory burst activity in non-smolting *O. mykiss* (Taylor et al., 2007) and in *S. trutta* (Marc et al., 1995). Also, immunomodulatory effects of ultraviolet B (UVB) irradiation perturbed immune functions in salmonids and increased fish susceptibility to pathogens (Markkula et al., 2007; Markkula et al., 2009). Conversely, long term UVB exposure did not affect PHA induced lymphocyte proliferation in *O. mykis* (Markkula et al., 2009), high

ultraviolet B (UVB) irradiation (1000 mJ cm^{-2}) exposure supressed respiratory burst following exposure in *O. mykiss* HKL (Markkula et al., 2006).

3.6.2 Studying the effects caused by chemical stressors

Other than physical stressors, isolated salmonid leucocytes are also employed in revealing immunosuppressive effects of environmental contaminants, such as heavy metals, sewage effluents, polyaromatic hydrocarbons (PAHs). For example, isolated leucocytes showed that heavy metals cadmium, mercury and zinc altered cellular phagocytosis, respiratory burst, and lymphocyte differentiation [reviewed in (Rehberger et al., 2017)]. In *O. mykiss* exposed to lower cadmium (2ppb), leucocytes exhibited altered macrophage phagocytosis and ROS production, in a time-dependent manner (Zelikoff et al., 1995); while *O. mykiss* PBL characterised as phagocytic by flow cytometry and electron microscopy, showed enhanced phagocytosis in a cellular function validation test with an arsenic (Thuvander et al., 1987). In *O. mykiss* exposed to treated sewage effluents, increased *in vitro* lymphocyte proliferation was noted; while HKL oxidative burst, phagocytosis were unaffected (Hoeger et al., 2004).

Regarding immunological effects of PAHs, different concentrations of dichlorodiphenyltrichloroethane (DDT) metabolite 1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (*p*,*p*′-DDE) significantly reduced cell proliferation in *O. tshawytscha* (Misumi et al., 2005) and tributyltin induced apoptosis in SP and HK leucocytes (Misumi et al., 2009). For PBDEs, dietary manipulations containing binary combinations of the contaminant congers BDE-47 and BDE-99 induced increased HK macrophage phagocytosis, superoxide anions and ROS production in *O. tshawytscha* fed the binary diet compared to the control diet, while susceptibility to V. anguillarum infection was higher in the test group *(*Arkoosh et al., 2018*)*. Similarly, oral exposure of *O. tshawytscha to* PBDE congeners (BDE-47 and BDE-99) resulted in reduced HK macrophages phagocytosis in fish exposed to BDE-99, while both congers induced increased production of superoxide anion, and salmon exposed to the pollutant exhibited increased susceptibility to *Listonella anguillarum* (Arkoosh et al., 2015). In *O. mykiss*, i.p. administration with polychlorinated biphenol (PCB) mixture Clophen A50, SP leucocyte response to both T and B cells to LPS and PHA increased significantly in the PCB exposed fish after immunisation with keyhole limpet haemocyanin (KLH) (Thuvander et al., 1993).

In *O. mykiss* exposed to PCB mixture Clophen A50, antibody levels to *V. anguillarum* were significantly lower in the groups that had been fed the Clophen A50-containing diet, while IgM levels remained unaffected (Thuvander & Caristein, 1991). In addition SP and HK leucocytes isolated from *O. tshawytscha* exposed to 59 ppm p,p′-DDE *in vitro* showed significantly lower percentage of Ig+ blasting cells than controls, although the response was biphasic (Misumi et al., 2005) and comparable findings were noted following tributyltin (TBT) exposure *in vitro* (Misumi et al., 2009). Similarly, creosote PAH exposure in *O. mykiss* , resulted into PBL dose dependent reduction in the number of sIg+ cells following treatment with different mixtures (Karrow et al.,

1999); and i.p. administration of stress hormone, cortisol downregulated the number of Ig+ leucocytes in *S. salar* (Espelid et al., 1996).

3.6.3 Exemplary case studies

To exemplify the application of salmonid isolated leucocytes in studying immunomodulatory effects of environmental stressors, two studies by Arkoosh et al. (2015); Arkoosh et al. (2018) are used. In the first study, authors investigated the independent effects of two of the most predominant PBDE congeners, BDE-47 and BDE-99, on the health and survival of juvenile *O. tshawytscha*. Health fish were held in 300 L tanks with flow-through seawater and fed daily rations of BDE-47 or BDE-99 at 2% body weight for 40 days. Experimental diets contained environmentally acceptable levels at low (32), medium (100), and high (280) ng BDE-47 g^{-1} of food; and low (40), medium (120), and high (300) ng BDE-99 g^{-1} of food. The control group received food with 0.4 ng BDE-47 g^{-1} food and 0.1 ng of BDE-99 g^{-1} food as well as 0.1 ng of BDE-49 g^{-1} and 0.1 ng of BDE-100 g^{-1} food. 24 h post feeding, subsets of treated fish were sampled, HKs aseptically removed, and HKL isolated by Histopaque 1077 CDGC according to Solem et al. (1995). Isolated HK macrophages were separated from suspension cells by plastic adherence according to Secombes (1990), incubated for 18 h prior to assay use. Using HK macrophages, immunosuppression was demonstrated in fish exposed to BDE-99, but not BDE-47 via reduced phagocytic capacity against SRBCs, although both congers enhanced HKL macrophage *in vitro* superoxide anion production, survival in fish was compromised following challenge with pathogenic *V. anguillarum* (Arkoosh et al., 2015).

In the second study, investigators studied the effects of a synergistic formulation of the most predominant PBDEs congers (BDE-47/99 mixture) on the innate immune system and disease susceptibility of juvenile *O. tshawytscha.* Five BDE-47/99 mixture diets and a control were produced and labelled as control zero, and one, two, three, four to five for the treatments. Fish were fed daily on the BDE-47/99 mixture diets, thrice a day for 39 days in triplicates of 285 animals. At the end of the experiment, fish HK were sampled aseptically and HKL and adherent macrophages isolated according to Arkoosh et al. (2015) and assayed for phagocytosis and respiratory burst. Isolated HK macrophages demonstrated enhanced cellular functional properties of phagocytosis, respiratory burst in fish that received the BDE-47/99 mixture diets than control diet fed fish. However, the mechanism for this enhanced innate immune function in HK macrophages following PBDE exposure remains a mystery although it has been suggested to result from the ability of PBDEs to act as an endocrine receptor agonist and/or antagonist. Conversely, the risk of mortality in BDE-47/99 mixture diets fed fish was higher than in the control fish following challenged with pathogenic *V. anguillarum* (Arkoosh et al., 2018).

3.7 Study of pathogen and parasite effects, vaccine design, and assessment of efficacy and vaccination strategies

From a pathological and immunostimulation perspective, isolated leucocytes have also been used to assess vaccine efficacy, immune response to parasites, bacterial and viral pathogens,

design vaccines and vaccination strategies in farmed salmonids as illustrated in the subsequent sections.

3.7.1 Studying the effects of parasites and bacterial pathogens

Among salmonids, the cellular functional immunosuppression has been reported in fish following exposure to parasites. For instance, parasitic Myxosporean (PKX), the causative agent for proliferative kidney disease (PKD) led to impaired phagocytosis but not respiratory burst in *O. mykiss*, HK and PB leucocytes (Chilmonczyk & Monge, 1999), while *Neoparamoeba* sp reinfection in *S. salar* previously infected with gill disease, depressed phagocytic activity of HK and PB leucocytes (Gross et al., 2004). Other than parasitic infestations, isolated leucocytes have also been used to study immunological effects induced by bacterial challenges and infections. For instance, *V. anguillarum*, *in vitro* challenge in *O. kisutch*, *O. mykiss* and *O. tshawytscha*, induced mitogens and polyclonal activities in SP and HK lymphocytes (Yui & Kaattari, 1987), *A. salmonicida* or *Y. ruckeri* induced IL-22 production in PBL (Hu et al., 2019); while unchallenged *O. mykiss,* HK and PB leucocytes *in vitro* resulted into noticeable antibacterial activity against Gram-positive bacteria (Smith et al., 2000).

3.7.2 Aiding vaccine design, assess efficacy and vaccination strategies

Isolated leucocytes have also aided design of bacterial vaccines, as *O. mykiss* injected with formalin-killed *V. anguillarum*, HK and PB leucocyte proliferated following challenge with formalin-killed bacteria outer membrane proteins (OMP) preparation (Boesen et al., 1997). Using *A. salmonicida* strain (MT004) preparations, the vaccine prepared in PBS (MT004/PBS) exhibited higher antigen specific proliferation and MAF production than A10H adjuvant (MT004/A10H) preparation in *O. mykiss* PBL following i.p. injection (Marsden & Secombes, 1997). Recently, Attaya et al. (2019) used *O. mykiss* PBL to screen for *A. salmonicida* based vaccines.

Vaccine efficacy has also been assessed using isolated leucocytes as an earlier study with *A. salmonicida* vaccine induced continued production of MAF in *O. mykiss* HKL post-immunisation, and the trend correlated very well with lymphocyte proliferation (Marsden et al., 1994). Relatedly, *O. mykiss* challenged with *A. salmonicida* antigens *in vitro*, all HKL primed demonstrated enhanced responses to *A. salmonicida* antigens, indicative of both T and B memory cell formation in vaccinated individuals. Furthermore, T and B cells from primed fish were able to respond to an A-layer positive strain (MT423) of the antigen, hence a shared common immunogenic T and B cell epitopes by both antigens (Marsden et al., 1995). In addition, vaccination with a mutant *A. salmonicida* vaccine against furunculosis in *O. mykiss*, live and not heat killed vaccine, significantly enhanced T-cell proliferation compared to B-cells (Marsden et al., 1996). In addition, vaccination against furunculosis in *S. salar* and *O. mykiss* PB, SP and HK leucocytes enhanced antibody production following stimulation with *E. coli* LPS and PHA (Reitan & Thuvander, 1991), and a test for a mutant *A. salmonicida* vaccine against furunculosis

in *O. mykiss*, showed that live bacteria significantly primed lymphocyte antibody production (Marsden et al., 1996).

Regarding vaccination strategy choices, *S. salar* vaccinated by single and repeated administration by i.p. injection, immersion or oral route, and revaccination by combinations of these methods, against furunculosis: the i.p. injection strategy resulted into higher *in vitro* proliferation of HKL (Midtlyng et al., 1996). In addition, i.p. administration of adjuvant vaccine, preferably in a polyvalent formulation, was optimal to stimulate immunity and thus recommended for immunoprophylaxis against furunculosis (Midtlyng et al., 1996). Similarly, *S. salar* i.p. administered with an adjuvant furunculosis vaccine, HKL from vaccinated fish showed significantly stronger response to whole *A. salmonicida* than did unvaccinated controls, with minimal differences to response among the different mitogens (Erdal & Reitan, 1992).

3.7.3 Studying the effects of viral pathogens

Isolated leucocyte experimental studies involving viral pathogens have demonstrated impaired and enhanced cellular functional immune parameters. Viral haemorrhagic septicaemia virus (VHSV) impaired phagocytosis, but not respiratory burst in *O. mykiss*, HK and PB leucocytes (Chilmonczyk & Monge, 1999), while i.p. injection of *S. salar* parr with salmon pancreas disease virus (SPDV) (P42p isolate of SPDV) invoked stimulation of innate immunity via increased HK leucocyte phagocytosis (Desvignes et al., 2002). In addition, *in vitro S. salar* TO cells exhibited capacity to provide a high yield of infectious salmon anaemia virus (ISAV) and showed cytopathic effects (CPE) within 9 days; *S. salar* injected with diluted virus supernatant exhibited mortalities, hematocrit values and clinical signs in accordance with ISAV infection (Wergeland & Jakobsen, 2001). Besides, leucocytes flow cytometry analysis aided detection of IPNV in HK and PB leucocytes from IPNV vaccinated, non-vaccinated and i.p. infected *S. salar* (virus shedders) and carrier fish (Rønneseth et al., 2013). Furthermore, both flow cytometry and quantitative real time polymerase chain reaction (qRT-PCR) indicated that i.p. infected fish were carriers as B-cells and neutrophils and other leucocytes harboured IPVN following cohabitation challenge, hence enhanced protection against future infection (Rønneseth et al., 2013).

Isolated salmonid leucocytes have also been employed to assess gene expression following viral pathogen challenges. To illustrate, *S. salar*, challenged with a high virulence (HV) and low virulence (LV) IPNV, exhibited higher levels of viral transcripts in HK of fish infected with LV IPNV compared to HV during the freshwater phase (Julin et al., 2015). *In vitro*, *S. salar* HKL derieved cell lines, SHK-1 and TO, challenged with Salmon AlphaVirus (SAV) an aetiological agent of SPD, SAV nsP1 gene transcripts for strain P42P increased rapidly in TO cells with subsequent development of CPE, but not in SHK-1 cells (Gahlawat et al., 2009). However, SAV P42P strongly upregulated expression of type I interferon (IFN) and IFN-induced antiviral Mx protein transcripts in SHK-1 cells. However, higher IFN expression appeared in TO cells than in SHK-1 cells, and lower Mx gene transcripts in TO cells (Gahlawat et al., 2009).

3.7.4 Exemplary case study

To demonstrate the application of fish isolated leucocytes in studying immunological effects of pathogens, a study by Desvignes et al. (2002) is adapted. The study investigated the host immunological response following experimental infection with SPDV cultured in *O. tshawytscha* embryo (CHSE-214) cell line. 75 healthy *S. salar* yearling parr, were i.p. injected with 100 µL of an infectious dose of 10^5 tissue culture infectious dose 50% ($TCID_{50}$). The control group consisted of 75 healthy fish i.p. injected with 100 µL of virus free CHSE-214 cell culture supernatant. Treated and control fish were separated into triplicate groups, kept in covered 50 L tanks, at a density of 25 fish, supplied with oxygenated filtered water at 14°C. Fish were sampled at 2, 4, 9, 16 and 30 days post infection (dpi) using five fish per tank.

HK were sampled and leucocytes isolated by Percoll (Sigma, U.S.A.) DCDGC, washed twice, and viable cells resuspended in Cortland saline at 10^7 mL^{-1} with the help of a Thomas' haemocytometer until needed for immunological assay, while blood plasma was used for humoral assessments. Overall, *in vivo* infection of *S. salar* with cultured SPDV induced an immune response at specific and non-specific levels. Isolated HKL exhibited higher phagocytosis in infected fish compared to control fish, while plasma lysozyme and complement increased in the treatment group. In addition, neutralising antibodies against SPDV appeared two weeks post infection and increased with time in content and prevalence among treated fish, although SPDV infection did not induce IFN activity in treated fish (Desvignes et al., 2002).

3.8 Salmonid fish immunological cell development

As earlier illustrated, fish immune cells can be isolated from lymphoid organs (Trede & Zon, 1998), by density gradient centrifugation and hypotonic lysis (Crippen et al., 2001; Hu et al., 2018; Pierrard et al., 2012). Indeed, several investigators have successfully isolated, cultured, maintained and characterised leucocytes from PB, HK, SP and TH (Table 3.5), to aid farmed fish immunology understanding. Thus far, a number of cell lines have previously been developed from salmonid HKL, resulting into DC like TO cells in *S. salar* (Wergeland & Jakobsen, 2001), trout primary kidney monocyte-like cultures (T-PKM) in *O. mykiss* (Stafford et al., 2001) and *S. salar* SHK-1 (Dannevig et al., 1997). Other HK leucocyte cell lines were isolated from *S. salar* and *O. mykiss* HK macrophages (Braun-Nesje et al., 1981); DC cells from *O. mykiss* (Bassity & Clark, 2012), and HK macrophages in *S. fontinalis* and *S. trutta* (MacKenzie et al., 2003).

In addition, salmonid leucocyte cell lines originating from the SP were developed for *O. mykiss* cell line (RTS11) in *O. mykiss* (Ganassin & Bols, 1996, 1998); *O. Mykiss* PB, kidney and SP for DC cell lines (Bassity & Clark, 2012), and small monnuclear cells with DC like characteristics from *S. salar* PBL (Haugland et al., 2012). Some investigators established long-term cell lines, and others short-term immune cell lines to enable fish immunological understanding using adherent and suspension cultures. These cell lines were characterised for cellular morphology, and functional properties of phagocytosis, respiratory burst, ROI and RNI

production, immune enzyme activity, antibody production and immune gene expression (Table 3.5).

Table 3.5. Vertebrate fish organ and peripheral blood developed immunological cell lines: ATK: anterior part of trunk kidney, SP: Spleen, TH: thymus, HK: Head kidney, PB: peripheral blood.

Fish species	Origin	Cell line and immunological characterisation	Reference
1. Salmonid immunological cell lines (n = 10)			
O. mykiss	SP, HK, ATK	Short term *O. mykiss* trout DC cells: exhibited irregular membrane processes and expressed surface MHCII, had tree-like morphology, expressed DC markers, phagocytosed small particles, were activated by TLR-ligands, and migrated *in vivo*.	(Bassity & Clark, 2012)
	HK	Long-term T-PKM cells: were positive for ROI and RNI negative with PMA, Con A and LPS.	(Stafford et al., 2001)
	SP	Long-term *O. mykiss* cell line (RTS11): Majority of cells were small, non-adherent and a few larger, granular cells termed macrophages-like cells. Larger cells were phagocytic, and engulfed DiI-acetylated low-density lipoprotein and acridine orange and stain for non-specific esterase. Supernatants exhibited lysozyme activity. Small cells were induced to proliferate by RTS11 supernatant and LPS.	(Ganassin & Bols, 1996, 1998)
S. salar	HK	Long-term Salmonid cell line (TO): Cells showed quick adherence to the plastic surfaces, very phagocytic and bactericidal activity; no respiratory burst, and NO production; reacted positive to a leucocyte specific MAb but did not bind to neutrophil specific MAb or stain for myeloperoxidase. Cells also expressed CD83 gene but not the T-cell receptor alpha (TCR-α) or the macrophage marker (M-CSFR).	(Pettersen et al., 2008)
	PB	Short-term small, mononuclear blood cells from *S. salar*: highly expressed CD83 gene transcript and MHC-II; highly phagocytic, positive for acid phosphatase; lacked respiratory burst and myeloperoxidase activity; and have a high morphological plasticity.	(Haugland et al., 2012)
	HK	Long-term Salmonid cell line (TO): Cells were cultured and passed more than 150 times and exhibited no changes in morphology, growth or virus production	(Wergeland & Jakobsen, 2001)
	HK	Long-term Salmon HK, SHK-1 cell line: cells proliferated; negative for alkaline phosphatase; positive for acid phosphatase, non-specific esterase, and $Mg2^{+}$ dependent adenosine triphosphatase and 5' nucleotidase. Cells reacted with MAb directed against *S.salar* PBL, and did not phagocytose *A. salmonicida*.	(Dannevig et al., 1997)
O. mykiss, S. salar	HK	Long-term HK macrophages: Cells were macrophage like and engulfed a range of particles	(Braun-Nesje et al., 1981)
S. fontinalis, *S. trutta*	HK	Short-term primary trout monocytes and in vitro differentiated macrophages: showed phenotypic changes, strong phagocytosis of Zymosan; and LPS induced trout *tnf-α* (*tnf-α*) mRNA expression.	(MacKenzie et al., 2003)
2. Non-Salmonid immunological cell lines (n = 13)			
G. morhua	HK	Short-term HK macrophages: were highly phagocytic; acid phosphatase and non-specific esterase positive; d alkaline phosphatase and peroxidase negative; and enhanced respiratory burst by LPS.	(Sørensen et al., 1997)
Goldfish *(Carassius auratus)*	HK	Long-term *in vitro*-derived goldfish kidney macrophages (IVDKM): were positive for ROI and RNI with PMA, Con A and LPS.	(Stafford et al., 2001)
C. auratus	HK	Long-term HK macrophages: stained positive for non-specific esterase; phagocytic on SRBC, amastigotes and promastigotes of Leishmania major; PMA & or LPS stimulated ROI & RNI.	(Wang et al., 1995)
	HK	Short-term HK macrophages: produced NO when stimulated by PMA, Con A or LPS, and co-stimulation with both factors induced synergistic NO production.	(Neumann et al., 1995)
Catla catla	TH	Long-term Catla thymus macrophage (CTM) cell line: phagocytic on yeast cells and fluorescent latex beads; PMA & or LPS stimulated ROI & RNI; positive for alpha-naphthyl acetate esterase enzyme; and supernatant showed lysozyme activity.	(Chaudhary et al., 2014)
Common carp (*Cyprinus carpio*) and Ginbuna (*Carassius auratus langsdorfii*)	TH	Long-term Carp thymus (KoT), and ginbuna thymus (GTS6 and GTS9): Cells proliferated and RT-PCR detected marker genes for myeloid/erythroid progenitors (*gata1*), haematopoietic stem cells (*gata2*), neutrophils (*mpx*/*mpo*), B-cells (*IgH*) and T-cells (*lck*, *TCRβ* and *gata3*) in primary cells, majority of which were lost after the third passage, except T-cell markers.	(Katakura et al., 2009)
C. carpio	PB	Long-term Carp leucocyte cell line (CLC): reacted with MAbs against carp HK macrophages; binding to an antibody against complement component C3; had acid-phosphatase positive granule; phagocytic on SRBC; and LPS, PMA & co-culture with carp PBL enhanced respiratory burst; and secreted *il-1*.	(Weyts et al., 1997)
Rohu (*Labeo rohita*)	TH	Long-term *L. rohita* thymic macrophages (LRTM): tested positive for alpha naphthyl esterase acetate positive; exhibited nonspecific esterase and surface expression of Fc receptors for IgG; MHC-I, MHC-II antigens; engulfed yeast cells and latex beads; PMA and LPS induced ROI & RNI production;	(Rebello et al., 2014)
L. rohita	HK	HK macrophages (HKM): Showed strong plastic adherence; phagocytosed yeast cells; LPS enhanced ROI & RNI, and lysozyme activity.	(Awasthi et al., 2014)
C. carpio	PB	Long-term Carp Leucocyte Culture (CLC): tested positive for peroxidase & periodic Acid Schiff (PAS); phagocytosed iron particles; cell growth enhanced with *tnf-α*, IL-2 and FBS enhanced multiplication and *inf-γ* depressed growth. Meanwhile, cell proliferation was highly enhanced by LPS than Con A; while PHA and SAC did not stimulate cells.	(Faisal & Ahne, 1990)
Channel catfish (*Chanos chanos*)	PB	Long-term monocyte like cell line from channel catfish: resembles mammalian monocytes or macrophages; stained positively for nonspecific esterase and peroxidase; phagocytosed latex beads; LPS induced *il-1* production; and showed effective antigen-presentation to autologous PBL for antigen specific *in vitro* proliferative and antibody responses.	(Vallejo et al., 1991)
Half smooth tongue sole (*Cynoglossus semilaevis*)	PB	Short-term monocyte-derived macrophages: Showed proliferative characteristics; highly enhanced respiratory burst against PMA, and *V. anguillarum tests; highly* phagocytic against latex beads and yeast cells; reacted positive to esterase activity; and enhanced expression of M-CSFR, MHC-II, *il-6*, *il-10*, TNF and arginase genes.	(Sha et al., 2017)

Nile tilapia (*Oreochromis niloticus*)	HK	Long-term Tilapia HK (THK): THK proliferated and THK cells ingested latex bead; HK cells exhibited monocytic leucocyte markers transcripts for CD33, CD53, CD82, MCSFR, and CD205; THK cells exhibited transcripts of haematopoietic stem cells (*gata2*) (GATA2), GATA4 , and GATA6 but not myeloid/erythroid progenitors (GATA1).	(Wen, 2016)

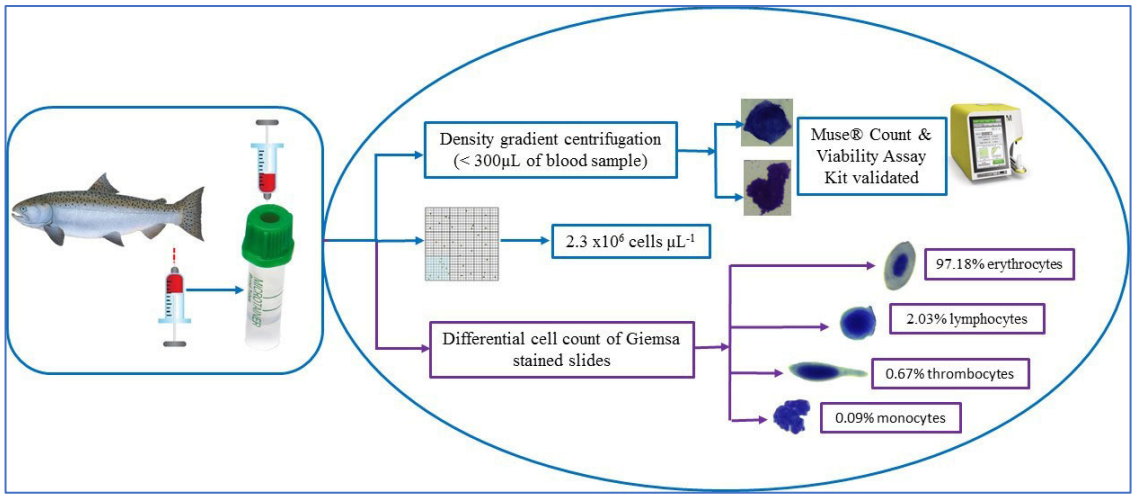
3.9 Future research and recommendations

In this review, we illustrated the techniques used to isolate salmonid immune leucocytes and their use to demonstrate immunomodulation following dietary manipulations, exposure to environmental stressors, effects of pathogens and parasites, vaccine design, efficacy and administration assessment. We also present findings on development of fish immune cell lines and their potential uses in aquaculture immunology. We mined 114 papers, among which, the most common technique used to isolate fish leucocytes was DDGC with Percoll density gradient.

Leucocytes were mostly isolated from fish HK and PB, with *O. mykiss* and *S. salar* as the most studied species. Phagocytosis, followed by respiratory burst, were the most popular immunological assays employed in salmonid immunology. Regarding dietary manipulations, leucocyte commonly demonstrated enhanced cellular functions and immune gene expression following fish administration with probiotics, PAMPs, plant and animal extracts, and hormonal supplements. In addition, leucocytes demonstrated immunosuppressive effects following fish exposure to physical and chemical stressors, while parasites, viral and bacterial pathogens depressed fish immunity and upregulated pro-inflammatory cytokine transcripts. Overall, we found 23 fish organ developed and characterised cell lines, of which 10 originate from *S. salar* and *O. mykiss* HKL.

We observed in this review that most developed cell lines involved sacrificing fish. Also, large numbers of fish are sacrificed during vaccine trials, which can be avoided by using nonlethal PBL isolation techniques such as hypotonic lysis and micro-volume blood by density centrifugation. Hypotonic lysis gives a granulocyte enriched cell suspension compared to density gradient centrifugation, due to cell size fractionation. Thus, granulocyte quality and quantity in gradient isolated cells may affect assessment of adaptive immune parameters. We suggest that these observations be checked at species level for each method, as isolated leucocytes may be affected by fish gender, age, physiological state and isolation steps, among others. However, we suggest that techniques for leucocyte isolation need to move towards more affordable, time saving, nonlethal and ethically flexible methods such as hypotonic lysis in tandem with micro blood volumes use in the face of expensive gradient medium reagents. Finally, as this review retrieved no study of metabolomics using isolated leucocytes, we suggest that this approach be integrated with traditional techniques, and the newer novel Muse® Cell Analyser assays to open up new research avenues in fish physiology and immunology *in vitro*.

4 Chapter 4. Case study 1: Characterisation of Chinook salmon (*Oncorhynchus tshawytscha*) Blood and Validation of Flow Cytometry Cell Count and Viability Assay Kit



Graphical representation of Chinook salmon (*Oncorhynchus tshawytscha*) peripheral blood cellular composition and isolated peripheral blood mononuclear cells (PBMCs).

This chapter has been published:

Lulijwa, R., Alfaro, A. C., Merien, F., Burdass, M., Young, T., Meyer, J., Nguyen, T. V., & Trembath, C. (2019). Characterisation of Chinook salmon (*Oncorhynchus tshawytscha*) blood and validation of flow cytometry cell count and viability assay kit. *Fish & shellfish immunology*, *88*, 179-188.

https://www.sciencedirect.com/science/article/pii/S1050464819301330

4.1 Prelude to case study 1

Based on the literature survey on salmonids, we found very limited evidence of PBMC use in studying immune mechanisms. Moreover, most studies involved fish euthanisation to isolate spleen (SP) or head kidney (HK) leucocytes for use in immunological studies. On the other hand, the New Zealand Chinook salmon (*Oncorhynchus tshawytscha*) industry has enjoyed rapid growth and had been free of major salmon pathogens until 2012, when summer-related mortalities occurred due to elevated water temperatures in combination with opportunistic NZ-RLO2. There were limited haematological data on the species and immunological tools to aid health assessment. This chapter thus focussed on characterising New Zealand farmed *O. tshawytscha* selected PB cellular parameters and developed a micro-blood volume method for isolating fish PBMCs by density gradient centrifugation. Subsequently isolated PBMCs were used to validate a rapid and portable flow cytometry assay kit for cell count and viability assessment against the traditional haemocytometer. Results from this study provided an *in vitro* approach for characterising the fish's immune responses to future and emerging pathogens.

4.2 Introduction

Immunity is an integrated physiological and barrier system that enables animals to resist pathogens (Lieschke & Trede, 2009; Sandnes et al., 1988). The vertebrate immune machinery consists of the innate and adaptive immune systems; and fish are the only vertebrate ancestral group that possess both systems (Bassity & Clark, 2012; Rombout et al., 2005). The innate immune response consists of physical barriers (e.g., the skin, scales, gut mucosa and gill epithelia), humoral and cellular components. This system acts as the first line of defence against pathogens. The humoral component includes complement proteins, lysozymes, proteases, esterases, antimicrobial peptides (AMPs) and immunoglobulins, like IgM, IgD and IgT or IgZ (Gomez et al., 2013; Magnadóttir, 2006). The cellular immune constituents include leucocytes, such as T and B lymphocytes, neutrophils, eosinophils, basophils, thrombocytes, cytotoxic cells (natural killer cells), mast cells (MCs), dendritic cells (DCs), macrophages and their precursor monocytes (Castro & Tafalla, 2015; Merien, 2016). Neutrophils, eosinophils and basophils have a granulated cytoplasm, thus called granulocytes (Bruce & Brown, 2017). Macrophages and neutrophils are professional phagocytes (Mathias et al., 2009; Neumann et al., 2001) with a high phagocytic ability and capacity (Neumann et al., 2001). These cells produce antimicrobial nitrogen and oxygen products, cytokines, and ingest a wide range of particle sizes (Rabinovitch, 1995).

When the physical barriers fail to prevent pathogen attacks and entry, other innate immune components become involved through pattern recognition receptors (PRRs) on cell surfaces, which recognise common conserved pathogen-associated molecular patterns (PAMPs) not expressed in multicellular organisms (Castro & Tafalla, 2015; Elward & Gasque, 2003). These PAMPs include viral RNA and bacterial DNA, fungal β1,3-glucans, bacterial cell wall peptidoglycans, polysaccharides and lipopolysaccharides (LPS) (Elward & Gasque, 2003). The recognition of PAMPs initiates cellular responses designed to kill and eliminate microbial pathogens (Rieger & Barreda, 2011) via phagocytosis and exocytosis (Whyte, 2007). Phagocytosis initiates release of cytokines, followed by antigen presentation through the major histocompatibility complex (MHC), resulting in the development of adaptive immunity (Martin & Król, 2017). Overall, the adaptive immune component relies on the humoral (antibody) and cellular responses, and is characterised by specific antigen recognition, which invokes a quick and strong secondary pathogen-specific response (Martin & Król, 2017).

In aquaculture, disease outbreaks caused by viruses, bacteria, and parasites, routinely lead to heavy industry losses [reviewed in Fazio (2019)]. Amidst these challenges, aquaculture has gained significant progress, and is presently recognised as one of the fastest growing food production sectors worldwide (FAO, 2014). To keep up with demand, aquaculture farms aim to produce high quantity, of healthy and fast-growing fish, under optimal husbandry and management practices. Combined with other diagnostic tools, fish haematology can reveal important information on fish physiology and health, with high application potential to investigate

and monitor stress responses, diagnose diseases and identify nutritional problems [reviewed in Fazio (2019)]. For instance, haematology combined with humoral, cellular immune parameters and gene expression have been employed in several fish species to demonstrate immunomodulatory effects of: dietary supplements (Brum et al., 2017; Mansour & Esteban, 2017); immunostimulants and pathogen challenge (Abu-Elala et al., 2013; Biller-Takahashi et al., 2014; Sha et al., 2017; Y. Shen et al., 2018); temperature perturbations (Cheng et al., 2009; Ndong et al., 2007; Wang et al., 2018a); and hypoxia (Gallage et al., 2017). Furthermore, salinity stress (Taylor et al., 2007); crowding stress (Sadhu et al., 2014); heavy metals (El-Boshy et al., 2014; Radhakrishnan, 2010); hormonal components (Xu et al., 2018); and melatonin (Kepka et al., 2015) have been shown to induce dramatic changes in leucocyte counts.

However, most fish immunology studies are conducted *in vivo*, which can be time consuming, costly, and often involves fish euthanasia. Thus, there is a need for alternative models that are less invasive, can be performed under controlled *in vitro* environments, with nominal bio-fluid or tissue requirements, and which are less time consuming and more affordable. To perform *in vitro* immunological studies, fish immune cells are sourced from the thymus, kidney, spleen, or peripheral blood (Trede & Zon, 1998). These cells can be isolated from lymphoid organs and blood by either hypotonic lysis or density gradient centrifugation (Crippen et al., 2001; Pierrard et al., 2012). Indeed, isolated peripheral blood mononuclear cells (PBMCs) composed of suspended lymphocytes and adherent monocytes have been previously used to investigate immunomodulatory effects of: dietary supplements and disease resistance (Brum et al., 2017; Van Doan, Tapingkae, et al., 2016); pathogen challenge (Pietsch et al., 2008; Sha et al., 2017), and vaccine efficacy (Galeottia et al., 2013; Huang et al., 2014; Y. Shen et al., 2018), among others. Thus, fish PBMCs present an important opportunity to model several biological variables via integrated techniques, including microscopy, flow cytometry, RT-qPCR, spectrophotometry, western blotting, among others.

Specifically, flow cytometry has been used to characterize fish PBMCs in rainbow trout (*Oncorhynchus mykiss*) (Crippen et al., 2001), sea bass (*Dicentrarchus labrax*) (Esteban et al., 2000), striped catfish (*Pangasius hypothalamus*), and European eel (*Anguilla anguilla*) (Pierrard et al., 2012). Flow cytometry has also been used to assess PBMCs for: production of reactive oxygen species (ROS) (Samaï et al., 2018; Sha et al., 2017); antibody detection (Hedfors et al., 2012; Pettersen et al., 2003); phagocytosis (Haugland et al., 2012; Haugland et al., 2014; Samaï et al., 2018; Wang et al., 2018a) and natural cytotoxicity (Chilmonczyk & Monge, 1999). Consequently, flow cytometry tools such as the Muse® Cell Analyser, offer accurate and fast quantitative assessment of single cells compared to traditional methods. Fortunately, the Muse® Cell Analyser has been successfully used in our laboratory to characterize haemocytes in black-footed abalone (*Haliotis iris*) (Grandiosa, Bouwman, et al., 2018; Grandiosa et al., 2016); to study immune responses of shellfish to pathogen infections (Grandiosa, Mérien, et al., 2018; Nguyen et al., 2019; Nguyen, Alfaro, Young, et al., 2018) and stressors (Alfaro et al., 2019; Nguyen, Alfaro,

Merien, et al., 2018); and to assess the immune status of spiny lobster (*Jasus edwardsii*) with tail fan necrosis (Zha et al., 2017).

While the Muse® Cell Analyser has been shown to work well with shellfish haemolymph, the platform has not yet been used on fish blood, which first requires isolation of PBMCs to exclude nucleated erythrocytes. Fish PBMCs have previously been isolated for immunological investigations in Atlantic salmon (*Salmo salar*) (Haugland et al., 2012; Hedfors et al., 2012), and in Chinook salmon (*Oncorhynchus tshawytscha*) in Oregon state USA (Milston et al., 2003), but this information is still lacking for cultured *O. tshawytscha* in New Zealand. This presents an opportunity to incorporate the Muse® Cell Analyser as a fish health assessment tool for the aquaculture industry and other research-based applications.

The New Zealand aquaculture sector was valued at over US$ 800 million in 2016 of which *O. tshawytscha* contributed 22% and 12% by value and production volume respectively (FAO, 2019). The species is the second most important aquaculture product after the Greenshell™ mussel (*Perna canaliculus*), making New Zealand, a global leader in production and supply. Despite the economic importance of *O. tshawytscha*, there is a lack of literature on haematological and immunological aspects of this species. Continued growth of the salmon industry will necessitate species-specific immunological information and development of tools to monitor and assess fish health. Thus, the present study characterized the cellular composition of *O. tshawytscha* peripheral blood, and developed a micro-volume method to isolate fish PBMCs. We also characterized isolated PBMCs and used them to validate a commercial cell count and viability assay kit using the Muse® Cell Analyser.

4.3 Materials and methods

4.3.1 Fish samples

Nineteen yearling smolts (weight = 317.2 to 544.5 g; total length = 23.7 to 28.0 cm) were obtained from the Nelson Marlborough Institute of Technology (NMIT) aquaculture facility (Glenduan, Nelson, New Zealand). Fish had been maintained on a saltwater recirculating system (temp = 14.26±0.35°C; DO = 7.30±0.11 mg L^{-1}; pH = 8.12±0.21; NH_4^- = 0.14±0.13 mg L^{-1}; NO_2^- = 0.28±1.12 mg L^{-1}), and they were fed to satiation daily with EWOS Microboost 2.2 mm commercial diet (Cargill) with 50% crude protein and 22% lipid. Ethical approval was obtained from the NMIT Animal Ethics Committee (NMIT-AEC-2017-NMIT-01).

4.3.2 Fish blood sampling

Fish were captured by scoop net, individually anaesthetised with AQUI-S® (25 mg L^{-1} of culture water for 2-3 min), and length/weight measurements quickly recorded. Approximately 1.4 mL of peripheral blood was withdrawn via caudal vein puncture using a non-heparinised 3.0 mL syringe attached to a 20 gauge Terumo needle. Each sample was quickly distributed into three 400 µL BD Microtainer® Lithium Heparin tubes (Becton Dickinson, USA). Blood was mixed by inverting the tube 10 times, as per the manufacturer's recommendations, kept chilled at 4°C, and processed within 5-10 min for PBMC isolation.

4.3.2.1 Peripheral blood total cell count

Serial dilutions of 1 in 20 were made by adding 10 µL of peripheral blood into 190 µL of diluting fluid in an Eppendorf tube to a final dilution of 8000. At each dilution, the suspension was mixed by vortexing for 20 s. Subsequently, a haemocytometer chamber was filled with 10 µL using a micropipette and cells counted at 400x magnification, and recorded. Two replicate counts were conducted for each blood sample, and total cell counts were estimated for 10 individuals according to the formula:

$$Count\ per\ Litre = [(Number\ counted)/(Volume\ (\mu l)]\ x\ Dilution\ Factor\ x\ 1{,}000{,}000$$

4.3.2.2 Isolation of fish PBMCs

Fish PBMCs were isolated using a modified protocol by Pierrard et al. (2012) with minor modifications. Briefly, all buffers and solutions were kept chilled at 4°C during isolations. A micro-heparinised blood volume of 284 µL was quickly diluted 1:1 with sterile filtered (40 µm) phosphate-buffered saline (PBS), pH 7.4. PBMCs were obtained by centrifuging (Heraeus Labofuge 200; Thermo Scientific) the mixture at 3000 rpm (971 g) for 20 min over a layer of 682 µL Lymphoprep™ (1.077 g mL^{-1}) (Stemcell Technologies, Australia) in a 1.5 mL Eppendorf tube. Cells at the interface were aspirated with a pipette and washed twice in 500 µL of sterile filtered PBS by centrifuging at 2500 rpm (674 g) for 7 min. The resulting cell pellet was re-suspended to a final cell concentration of 10^5 - 10^6 cells mL^{-1} in sterile filtered PBS supplemented with 2% fetal calf serum (FCS) and kept at 4°C for further analysis.

4.3.2.3 Peripheral blood and PBMC slide smear preparation

To prepare fresh peripheral blood smears, approximately 10 µL of fresh non-heparinised sample were transferred to glass slides immediately after withdrawal; smears were made, and quickly stained with modified Giemsa Differential Quick Stain (Polysciences, Inc.). Briefly, slides were fixed by dipping in methanol for 10 s, quickly dipped five times in stain 1 (orange) and stain 2 (blue), immediately washed with distilled water and mounted with coverslips. Images were taken with a Nikon Digital Sight DS-Fi1camera (Nikon Corporation, Japan) attached to an inverted Nikon Eclipse TS100 microscope using NIS-Elements software at 400x magnification. Cell and nuclear size parameters (µm) were measured using an ImageJ version 1.52a Java application for cell size characterisation (Wayne, 2018). For differential cell counts, 10 µL of fresh heparinised blood was used to prepare slide smears, while isolated PBMC slides were prepared by adhering a monolayer of 10 µL cell suspension on glass slides. Slides were left to air dry overnight, fixed with methanol, and were subsequently stained with Giemsa. Peripheral blood stained slides were observed under a microscope at1000x magnification with oil immersion for white blood and red blood cell counts. Peripheral blood and PBMC slides were also observed, and targeted photographs taken under a 1000x magnification with oil immersion using an optical microscope (Leica ICC50 HD). In addition, the cellular composition of isolated PBMCs was compared with peripheral blood haematograms, using the Sysmex XT 2000i haematology analyser following the manufacturer's protocol.

4.3.3 Stability of stained PBMCs

The stability of isolated PBMCs stained with the Muse® Count and Viability Assay Kit stain (Merck KGaA, Darmstadt, Germany) and 0.4% Trypan blue (TB) assay stain (Sigma-Aldrich, New Zealand) (Strober, 2001) was assessed over 1 h at room temperature (21°C). Fish PBMC suspensions at 10^6 cells mL^{-1} in sterile filtered PBS with 2% FCS from five biological replicates were separated into paired samples, and repeatedly measured for viability using the Muse® Cell Analyser and the haemocytometer chamber at 5, 15, 30, 45 and 60 min. Duplicate measurements (technical replicates) for each sample were taken. This protocol was repeated to determine the stability of fish PBMCs kept at 4°C using the Muse® Count and Viability Assay Kit only, with five additional PMBC samples. Repeated-measures ANOVA was performed for each time-series dataset to determine approximate limits for PBMC stability using Minitab 16 statistical software at $\alpha = 0.05$.

4.3.4 Positive control for setting Muse® gate parameters

4.3.4.1 Procedure for dead/live cell positive controls

To establish correct Muse® gating parameters, fish PBMCs at $1x10^6$ cells mL^{-1} from one fish were used. Dead cells were prepared via thermal stress induction at 100°C for 5 min, to obtain assay cell suspensions at high and low viability. The high and low viability cell samples were used to set the viability profile and gates for the Muse® Cell Analyser, Cell Count and Viability assay Kit. The haemocytometer chamber was used to verify the cell counts and viability profile of the two sample preparations at high and low viability.

4.3.4.1.1 Estimating method accuracies

Cell viability and cell count data obtained using the Muse® Cell Analyser and haemocytometer chamber were compared to determine congruence. A series of 57 PBMC samples at low, medium and high viability were prepared from 19 fish using proportions (100%, 50% and 0%) of heat-killed cells at 100°C for 5 min from each individual. Duplicate measurements were taken for each method and data for mean cell viability and cell count for the haemocytometer chamber was plotted against data for the Muse® Cell Analyser. A scatter plot was generated with a line of best fit, and the R^2 value was determined; while regression equations were established, and regression were tested for significance at $\alpha = 0.01$.

4.3.4.1.2 Method repeatability (assessing intra-assay precisions)

Cell count and viability data obtained from 57 PBMC suspensions at low, medium and high viability from 19 fish were measured in duplicates using the Muse® Cell Analyser and the Trypan blue haemocytometer chamber technique. To assess intra-assay precisions, scatter plots of individual duplicate measurements for cell viability and live cell counts obtained at low, medium and high viability were produced separately for the two methods. A line of best fit was plotted, linear equations and R^2 values were determined. The Muse® instrument specificity was tested by running blank sterile filtered PBS with 2% FCS. In addition, method repeatability was assessed

by determining the percentage coefficients of variation using the mean cell count and viability data at low, medium, and high viability profiles for both methods.

4.4 Results

4.4.1 Peripheral blood cellular characterization

Five types of cells in Giemsa-stained peripheral blood were identified, comprising erythrocytes, lymphocytes, thrombocytes, monocytes and neutrophils. Neutrophils were observed, but not sufficiently quantifiable. Generally, erythrocytes dominated cell composition (Figure 4.1). The high dominance of erythrocytes was also confirmed via differential cell counts where total leucocytes accounted for less than 3%, and majority were lymphocytes (Table 4.1).

Erythrocytes were the largest cells with ellipsoid, round to oval shape, and had the lowest nuclear to cytoplasmic (N: C) ratio (Table 4.1; Figure 4.1-Figure 4.2). Immature erythrocytes were more rounded with a larger circular nucleus, while mature cells were more ellipsoidal, with a centrally located round to ellipsoid nucleus that stained deep blue (basophilic) against a pinkish (eosinophilic) cytoplasm. Ellipsoid erythrocytes were the most common cell type in salmon peripheral blood.

Table 4.1. Cellular profile of healthy *Oncorhynchus tshawytscha* smolt peripheral blood (mean ± SD; n = 10).

Parameters	Erythrocytes	Thrombocytes	Lymphocytes	Monocytes
Composition (%)	97.18±1.67	0.67±0.92	2.03±1.36	0.09±0.29
Cells μL^{-1}	2,254,132±6,785	15,463±3,762	47,093±5,526	2,109±1,188
Total cell count μL^{-1}	2,319,500±407,041			
Cell length (µm)	11.45±1.01	11.74±0.95	6.48±0.76	7.07±0.50
Cell width (µm)	6.44±0.51	4.39±0.49	6.12±0.86	5.51±0.79
Nuclear length (µm)	5.92±0.67	7.08±0.74	5.01±0.83	5.96±0.60
Nuclear width (µm)	2.80±0.34	2.97±0.36	4.36±0.85	3.50±0.90
Nuclear: Cytoplasm (N:C) ratio	0.21–0.24	0.39–0.42	0.49–0.60	0.45–0.61

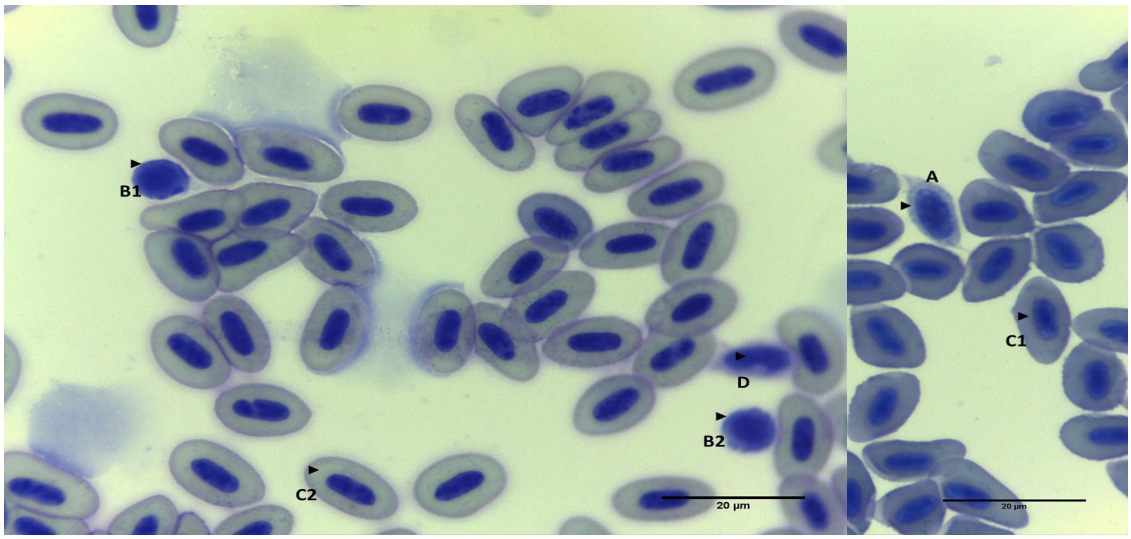


Figure 4.1. Giemsa stained *Oncorhynchus tshawytscha* yearling smolt heparinised peripheral blood cells taken with a Leica ICC50 HD camera attached to a Leica binocular at 1000x magnification. (A) thrombocyte, (B1-B2) lymphocytes, (C1-C2) mature nucleated erythrocytes, and (D) monocyte.

Thrombocytes were the second largest cells. They were ellipsoidal, spindle to oval, usually with a spine-like structure at one end of the cell. Ellipsoid and spike shaped thrombocytes were the most common in salmon blood. They had the second lowest N: C ratio after erythrocytes (Table 4.1). Like erythrocytes, the nucleus varied in shape from ellipsoid to oval, and stained basophilic, while the cytoplasm stained eosinophilic.

Monocytes were the third largest blood cells and possessed an irregular macrophage-like structure and a kidney shaped nucleus that sits on one side of the cell. The nucleus stained basophilic with a violent blue cytoplasm and had a moderate to high N: C ratio after thrombocytes. Small and large monocytes were observed, with cell surfaces exhibiting cytoplasmic extensions or pseudopodia-like structures.

Although lymphocytes were the most numerous of the leucocytes observed in peripheral blood, they were the smallest cells, had a circular or bell shaped structure, with a very thin cytoplasmic rim. Lymphocytes stained deeply basophilic and presented with the highest N: C ratio in most of the cells and commonly stained basophilic with a thin violent blue cytoplasm (Figure 4.1-Figure 4.2). By size, small and large cells were clearly visible in peripheral blood.

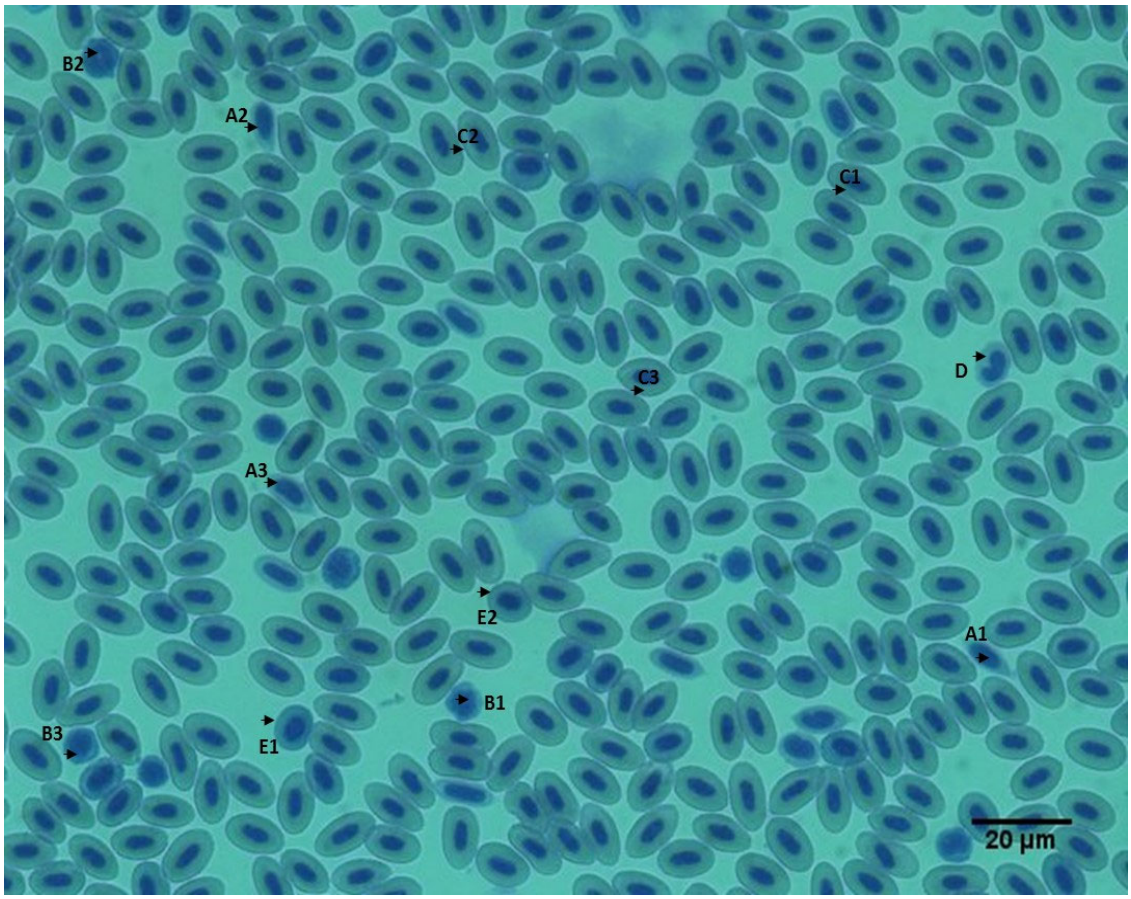


Figure 4.2 Modified Giemsa differential quick stained *Oncorhynchus tshawytscha* yearling smolt fresh un-heparinised blood cells, taken with a Nikon Digital Sight DS-Fi1camera attached to an inverted Nikon Eclipse TS100 microscope at 400x magnification. (A1-A3) thrombocytes, (B1-B3) lymphocytes, (C1-C3) mature erythrocytes, (D) monocyte, and (E1-E2) immature erythrocytes. This picture was specifically used to determine cell size.

Isolated PBMCs were predominantly composed of lymphocytes and monocytes (Figure 4.3), with the composition being confirmed with a Sysmex XT 2000i haematology analyser scattergrams (Figure 4.4). Specifically, nucleated fish peripheral blood cells (Figure 4.4A) presented as a cluster of varying cellular complexity, while isolated PBMCs appeared in scattergrams as two populations composed of smaller and less complex lymphocytes (pink) and more complex monocytes (green) as indicated by side scatter (SSC) (internal organelles and granularity) on the x-axis vs. side fluorescence (SFL) (DNA/RNA) on y-axis (Figure 4.4B). Blue dots on the PBMC scattergram represent cellular debris or ghost cells.

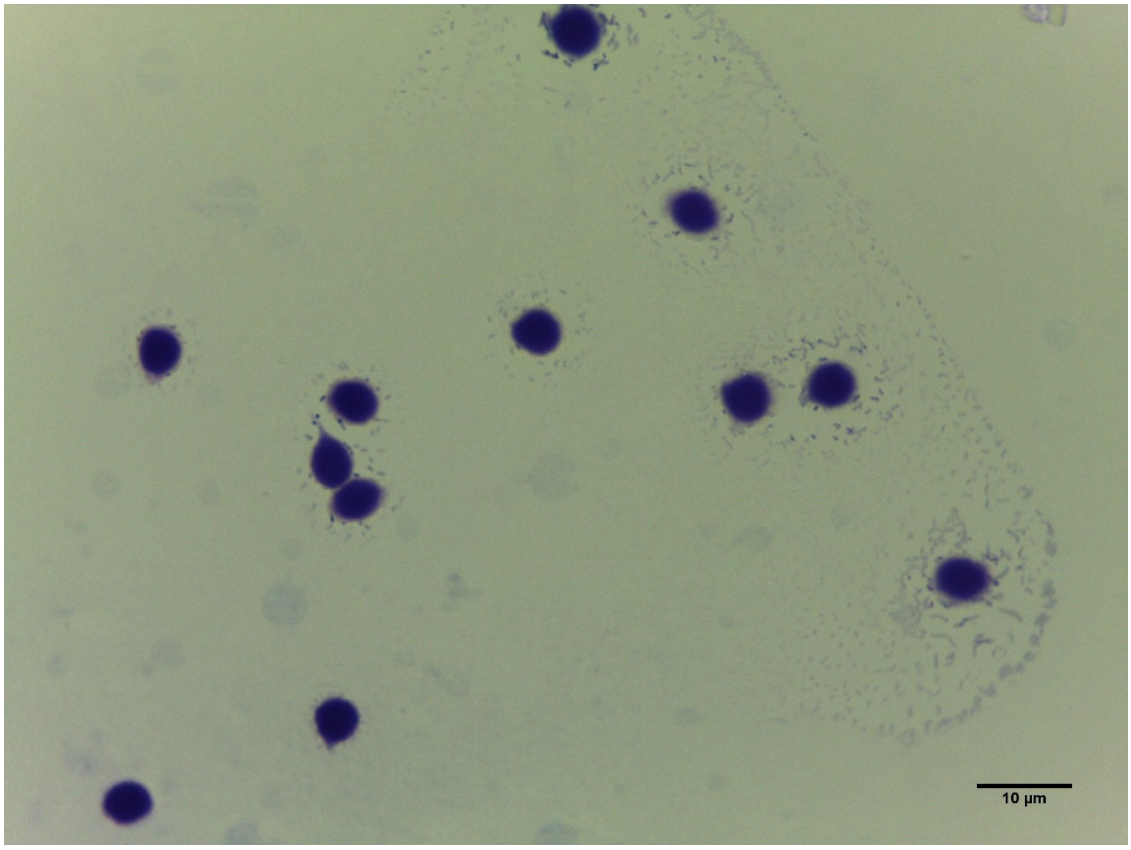


Figure 4.3 Giemsa stained *Oncorhynchus tshawytscha* smolt isolated PBMCs taken with a Leica ICC50 HD camera attached to a Leica binocular microscope at1000x magnification.

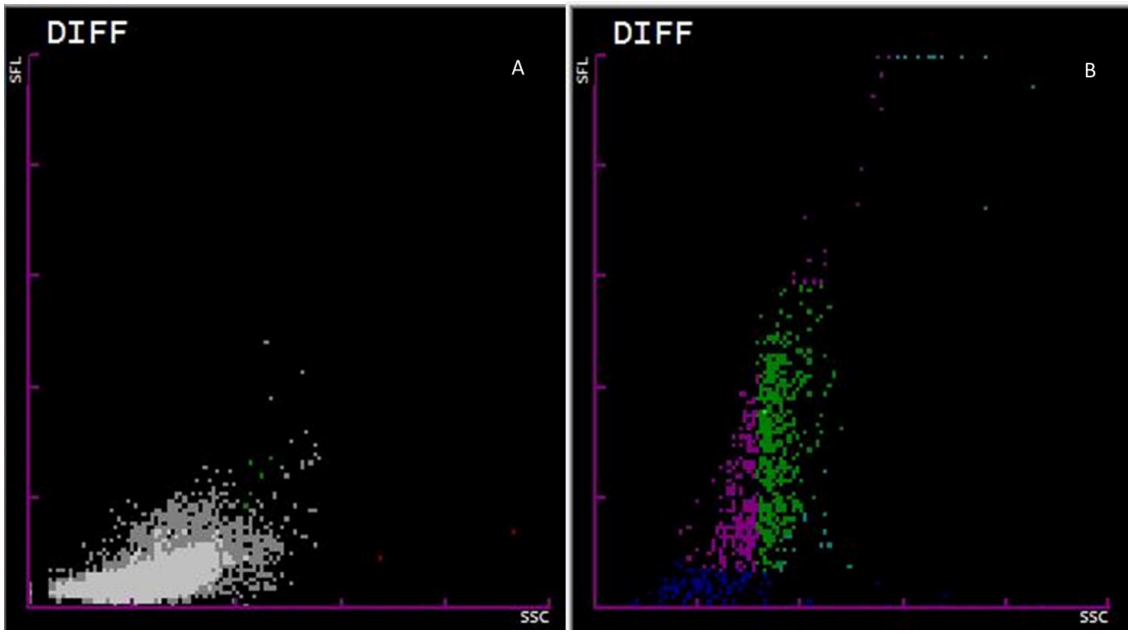


Figure 4.4 Sysmex XT 2000i differential scattergrams of fish peripheral blood, showing whole peripheral blood nucleated cells (A) and differential scattergrams of isolated fish PBMCs (B), lymphocytes are orange, monocytes are green, and blue are cellular debris or ghost cells. SFL: side fluorescence; SSC: side scatter; and DIFF: differential.

4.4.2 Stability of stained PBMCs

Regarding the stability of stained PBMCs, repeated measures ANOVA revealed approximate limits for PBMC stability at 45 min with the Muse® Count and Viability Assay Kit stain at 21°C and 4°C; while the Trypan blue assay stain showed stability limit up to 30 min of incubation at 21°C (Table 4.2).

Table 4.2. Percent viability of stained PBMCs over time at 21°C and 4°C (data points are shown as mean ± SD, n = 5 fish). Repeated measures ANOVA tests were performed at $\alpha = 0.05$. Values with the same superscript in a column are not significantly different. Values are an average of duplicate readings for both viability assessment techniques.

Time (min)	Muse® Count and Viability stain 21°C	Trypan blue stain 21°C	Muse® Count and Viability stain 4°C
5	92.07±4.16^{a}	92.59±3.65^{a}	88.40±5.44^{a}
15	90.66±5.58^{a}	87.16±8.30^{ab}	86.86±5.72^{a}
30	88.99±5.27^{a}	85.54±7.18^{ab}	85.15±4.86^{a}
45	86.50±4.20^{ab}	82.41±7.43^{b}	82.00±4.99^{ab}
60	82.50±5.30^{b}	81.44±8.77^{b}	77.57±6.19^{b}
F-value	5.85	3.69	6.22
p-value	0.001	0.011	0.000

4.4.3 Estimating method accuracies

The accuracy of the Muse® Count and Viability Assay Kit was benchmarked against the traditional haemocytometer chamber with the Trypan blue exclusion assay. A linear regression model across a wide range of measurements, revealed that comparative method accuracies were good for cell viability ($R^2 = 0.87$; $p < 0.01$), and reasonable for cell count ($R^2 = 0.74$; $p < 0.01$) (Figure 4.5).

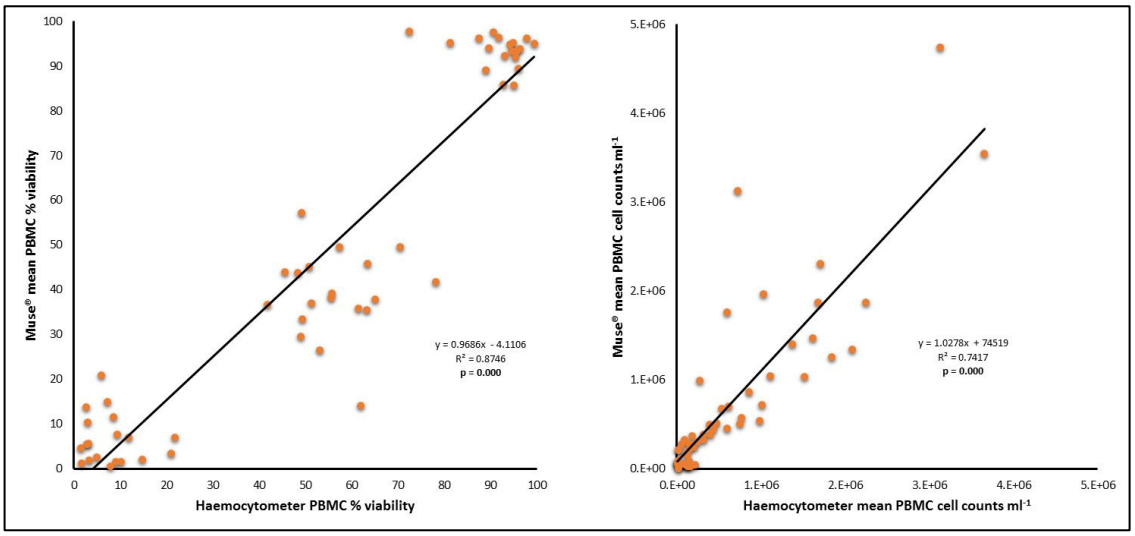


Figure 4.5 Linear regression of mean duplicate cell % viability (left) and viable cell count (right) profiles obtained via the haemocytometer chamber and Muse® Cell Analyser at low, medium and high viability cell suspensions (data comes from 57 samples and n = 19 fish).

4.4.4 Method precision using intra-assay plots

The ability of the Muse® Cell Analyser to consistently produce results was also assessed against the haemocytometer chamber over a wide range of viability profiles. Results of a linear intra assay regression revealed that the Muse® protocol performed with slightly better precision than the haemocytometer method in giving consistent results, as indicated by the higher R^2 range of 0.85 - 0.92 compared to 0.71 - 0.85, respectively (Figure 4.6).

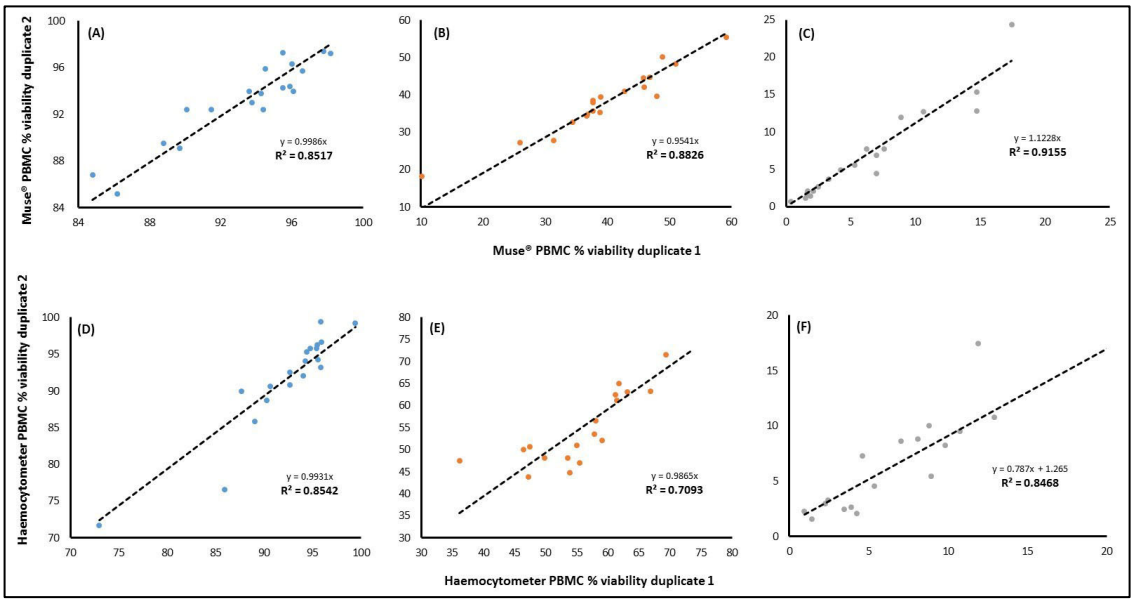


Figure 4.6 Regression profiles of duplicate cell viability assessed by the haemocytometer chamber and Muse® Cell Analyser, at three target viabilities (high, medium and low, n = 19). Panel A-C are viability results with the Muse® Cell Analyser at (A) high viability, (B) medium viability, (C) low viability. Panel D-F are viability results with the haemocytometer chamber at (D) high viability, (E) medium viability, and (F) low viability.

The slightly better precision of the Muse® Cell Analyser in estimating cell viability in comparison to the haemocytometer chamber is further revealed by low % CV among results obtained with the Muse® Cell Analyser in comparison to the haemocytometer chamber (Table 4.3).

Table 4.3. Precision of haemocytometer chamber and Muse® Cell Analyser in assessing PBMC viability at low, medium and high viability samples (data are grand mean from duplicate readings from 19 fish).

Method	Target Viability (%)	Grand mean measured viability (%)	Standard deviation	% CV/RSD
Haemocytometer	100.00	91.87	1.25	1.41
	50.00	56.31	3.09	5.82
	0.00	7.90	1.43	21.24
Average				9.49
Muse®	100.00	93.27	0.81	0.87
	50.00	38.99	1.90	6.14
	0.00	6.53	0.82	12.60
Average				6.53

On the other hand, observations of cell counts at different viability profiles revealed that the Muse® Cell Analyser is comparable to the haemocytometer chamber in consistency as revealed with R^2 range of 0.84 - 0.99 for the Muse® and to 0.88 - 0.98 for the haemocytometer chamber (Figure 4.7).

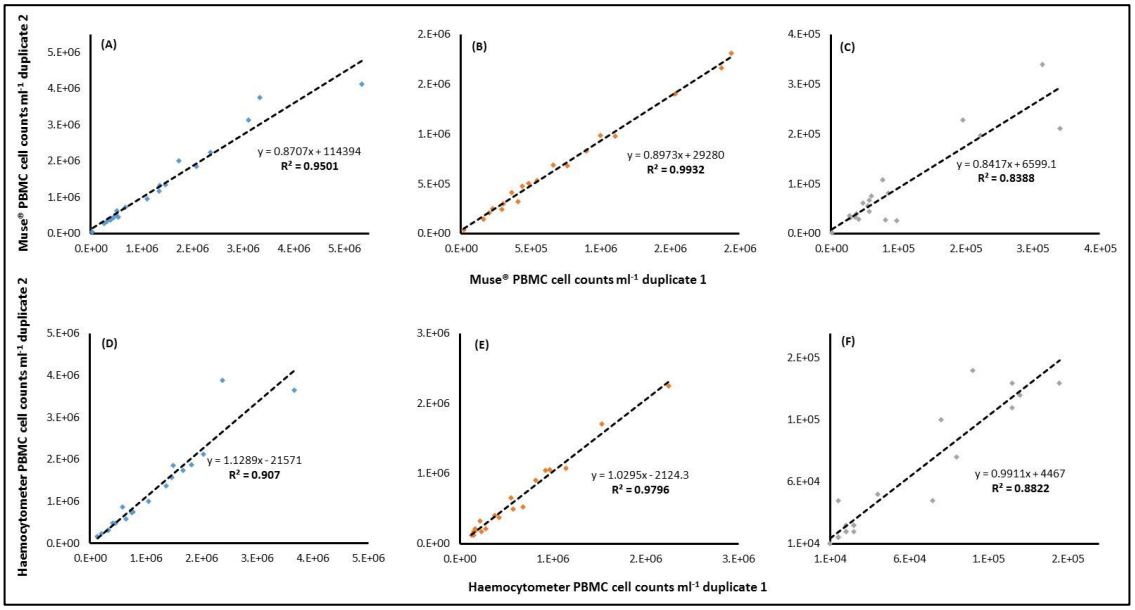


Figure 4.7 Regression profiles of duplicate viable cell counts assessed by haemocytometer chamber and Muse® Cell Analyser, at three target viabilities (high, medium and low, n = 19). Panel A-C are live cell count results with the Muse® at (A) high viability, (B) medium viability, (C) low viability. Panel D-F are count results with the haemocytometer at (D) high viability, (E) medium viability, (F) low viability.

Comparing method repeatability in estimating live cell counts, results obtained with the Muse® Cell Analyser showed closer grand mean % CV to that obtained with the haemocytometer chamber results (Table 4.4).

Table 4.4. Repeatability of haemocytometer chamber and Muse® Cell Analyser in assessing PBMC cell counts (n = 19).

Method	Cells mL^{-1}	Mean cells mL^{-1}	Standard deviation	% CV/RSD
Haemocytometer	1,200,000	1,199,605	104,019	7.69
	600,000	624,868	48,939	10.85
	72,000	60,394	7,629	12.12
Average				10.22
Muse®	1,200,000	1,389,038	116,941	7.69
	600,000	676,804	41,624	7.59
	72,000	93,782	16,845	19.34
Average				11.40

4.5 Discussion

In this study, we characterised peripheral blood cells in *O. tshawytscha* yearling smolts using light microscopy and flow cytometry, we successfully established a micro blood volume (<300 µL) density gradient centrifugation method to isolate and purify PBMCs, and validated an efficient assay kit to assess PBMC count and viability using a portable flow cytometry platform, the Muse® Cell Analyser. Differential cell count revealed five types of peripheral blood cells, consisting of erythrocytes, lymphocytes, thrombocytes, monocytes, and neutrophils, which is consistent with previous reports in juvenile *O. tshawytscha* from British Columbia, Canada (Ackerman et al., 2006). Since unstressed fish were used in our study, neutrophils were observed, but were not quantifiable in peripheral blood as their presence in circulation has been associated with immunological response (Neumann et al., 2001). Overall, erythrocytes were the most abundant cell types in peripheral blood, similar to previous reports in juvenile *O. tshawytscha* (Ackerman et al., 2006) and *S. salar* (Benfey & Sutterlin, 1984; Sandnes et al., 1988). The observed erythrocyte population in this study is within the range of 10^6 cells µL^{-1} for healthy *S. salar* (Sandnes et al., 1988; Vikeså et al., 2017) and *O. mykiss* (Morgan et al., 2008; Řehulka et al., 2004); although lower numbers of erythrocytes were observed in *O. tshawytscha* (Ackerman et al., 2006). Scientific evidence shows that fish erythrocyte counts can be influenced by several extrinsic factors (e.g., temperature, oxygen, salinity, pH, season, culture system, diet) and intrinsic ones like age, sex, maturity, ploidy and metabolic activity [reviewed in Witeska (2013)].

On the other hand, total leucocytes comprised around 3% and mostly consisted of lymphocytes, as previously reported in *O. mykiss* (Crippen et al., 2001) and other teleosts (Pierrard et al., 2012; Sha et al., 2017). Salmonid peripheral blood cellular profile has been reported to vary with species, age, season, photoperiod, sexual maturity and diet (Barnhart, 1969; Conroy, 1972; Härdig & Höglund, 1983; Jamalzadeh & Ghomi, 2009; Lane, 1979; Leonardi & Klempau, 2003; Pettersen et al., 2003; Řehulka, 2000). Lower leucocyte counts may indicate depressed immunity, while variations in circulating leucocyte to erythrocyte levels have been used to assess stress and disease susceptibility (Ackerman et al., 2006). For example, total leucocyte counts have been used to assess immune effects caused by physical (Cheng et al., 2009; Gallage et al., 2017; Ndong et al., 2007; Sadhu et al., 2014; Taylor et al., 2007; Wang et al., 2018a); and chemical (Arkoosh et al., 2018; El-Boshy et al., 2014; Kepka et al., 2015; Radhakrishnan, 2010; Xu et al., 2018) stressors. Furthermore, haematological parameters

combined with humoral, cellular immune responses and molecular techniques are routinely used to assess immunomodulatory effects of dietary supplements and immunostimulants in farmed fish (Biller-Takahashi et al., 2014; Brum et al., 2017; Furne et al., 2013; Mansour & Esteban, 2017; Van Doan, Hoseinifar, et al., 2016; Van Doan, Tapingkae, et al., 2016). This integrated approach has also been applied to investigate immunological effects of pathogen challenges and vaccine efficacy in aquaculture (Galeottia et al., 2013; Huang et al., 2014; Sha et al., 2017; Y. Shen et al., 2018).

For erythrocytes, mature and immature (erythroblasts) cells are observed with varied shapes, lowest N: C ratios, deeply basophilic nuclei and eosinophilic cytoplasm. A deeply basophilic nucleus is suggestive of chromatin richness, while an eosinophilic cytoplasm has been associated with haemoglobin richness in fish, and variations in morphology are associated with extrinsic and intrinsic factors [reviewed in Clauss et al. (2008)]. Although erythrocytes are respiratory cells with a primary gas-transport role, evidence of immunological function is accumulating. For example, expression of immune genes for toll-like receptors and/or interferon regulatory factors have been reported in Nile tilapia (*Oreochromis niloticus*) (Y. Shen et al., 2018), *S. salar* (Workenhe et al., 2008) and *O. mykiss* (Morera et al., 2011). In addition, fish erythrocytes appear to contain high numbers of lysosomes and mitochondria (Y. Shen et al., 2018), which further supports evidence of immunological function.

Thrombocytes facilitate fast blood clotting after injury and control fluid loss (Clauss et al., 2008). Elevated thrombocyte counts in fish have been associated with high clotting capacity and chronic stress (Espelid et al., 1996). Their observed cellular and nuclear shapes have been previously reported in several teleosts [reviewed in Clauss et al. (2008)] and *D. labrax* (Doggett & Harris, 1989; Esteban et al., 2000; Köllner et al., 2004; López-Ruiz et al., 1992). Similar to the current study, thrombocyte cell widths of 3.0 to 4.5µm have been reported in goldfish (*Carassius auratus*) (Watson et al., 1963). Furthermore, spindle and spike shaped cells were the most frequent thrombocytes in the current study as previously reported in *D. labrax* (Esteban et al., 2000), while spherical cells were the most common in *C. auratus* (Watson et al., 1963). The observed thrombocyte shapes in *O.tshawytscha* suggest possession of marginal microtubules and a surface canalicular system previously reported in other fish species (Cannon et al., 1980; Esteban et al., 2000; Savage, 1983), and has been associated with cell shape maintenance (Cannon et al., 1980).

Morphological characteristics of monocytes or macrophages in our study have previously been noted in a review for teleost fishes by Clauss et al. (2008), irregular cell surfaces with protrusions or ridges reported in *D. labrax* (Esteban et al., 2000) and *C. auratus* (Watson et al., 1963). Macrophages are a central component of the innate and adaptive immune responses in teleosts (Chaudhary et al., 2012). Macrophages are professional phagocytes, and have the potential to kill bacteria and protozoans via production of toxic reactive oxygen species and nitrogen intermediaries, lytic enzymes, and antimicrobial peptides (Neumann et al., 2001). The

production of cytotoxic compounds is facilitated via respiratory bursts (Grayfer et al., 2014; Sha et al., 2017) and phagocytosis (Mahmoud et al., 2018; Van Doan, Hoseinifar, et al., 2016; Van Doan, Tapingkae, et al., 2016).

The dominance of the leucocyte population by lymphocytes, and their small size range was previously reported in coho salmon (*Oncorhynchus kisutch*) (Kawahara et al., 1990). A large nucleus that stains basophilic, with a thin cytoplasmic rim and an amoeboid cell shape have also been reported in several fish species (Doggett & Harris, 1989; Esteban et al., 2000). In the current study, we also observed small and large lymphocytes in *O. tshawytscha*, suggesting the need for marker assisted identification of T and B cells. While B lymphocytes with surface immunoglobulins have previously been reported in salmonids (Hedfors et al., 2012; Milston et al., 2003); non-immunoglobulin producing T lymphocytes were reported in *S. salar* (Dannevig et al., 1997). Additionally, some lymphocytes exhibit exterior cytoplasmic protrusions, an indicator of phagocytic capability, while their basophilic cytoplasm and nucleus stain uptake suggests acidic content. Generally, lymphocytes serve immunological functions and can triple their size (plasma cells) following activation in response to antigens (Clauss et al., 2008; Mumford et al., 2007).

Slide smears of purified PBMCs in *O. tshawytscha* confirmed that cells were dominated by lymphocytes, which is consistent with PBMC composition previously reported in several salmonids and other fish species (Blaxhall, 1981; Kalgraff et al., 2011; Kawahara et al., 1990; Sakai, 1981). Isolated PBMC composition was also confirmed by peripheral blood and PBMC scattergram comparisons for which SSC, clearly illustrated two populations, consistent with results reported in several studies (Crippen et al., 2001; Esteban et al., 2000; Pierrard et al., 2012). PBMC haematograms suggested that monocytes are more complex in structure, possibly due to higher nuclear protein content and presence of cytoplasmic organelles, as they emitted more fluorescence compared to the less complex lymphocytes.

In Giemsa-stained PBMCs, monocyte and lymphocyte nuclei stained deeply basophilic, probably due to chromatin richness, which has been associated with innate immune responses in previous studies among salmonids (Dügenci et al., 2003; Gross et al., 2004; Kawahara et al., 1990; Korytář et al., 2013; Pettersen et al., 1995) and other fish species (Huang et al., 2014; Pierrard et al., 2012; Roland et al., 2013; Sha et al., 2017). To the best of our knowledge, the present contribution is the first to report on the isolation of *O. tshawytscha* PBMCs for characterization, which is the first step towards effective *in vitro* immunological and physiological studies.

Despite the successful isolation of PBMCs, we experienced challenges, during preliminary laboratory work (results not shown) on isolating fish PBMCs using anticoagulated blood couriered to our lab and used 24 h after sampling. The aim was to isolate fish PBMCs by density gradient centrifugation following the protocol by (Pierrard et al., 2012) and the SepMate™ tubes (Stemcell Technologies, Australia). Overall, we noted that anticoagulant-treated blood processed 24 h after sampling failed to separate into PBMCs. Furthermore, anticoagulant treated fish

peripheral blood stored for an hour and samples with coagulation particles also failed to isolate into PBMCs. Only freshly taken and quickly heparinised or EDTA preserved blood successfully separated into PBMCs without delay, hence the need to run samples as quickly as possible following draw from fish. Using less than 300 µL of fish blood, the technique enables collection of 1.0 -1.5 mL of PBMCs at 10^5-10^6 cells mL^{-1}, sufficient to conduct common immunological assays, e.g., phagocytosis, ROS and nitric oxide (NO) production. This is a huge sampling advantage as it does not require fish euthanisation, and presents benefits for on-farm individual fish sampling for longitudinal experiments.

With respect to PBMC stability, cells stained with the Muse® Count and Viability Assay Kit stain remained significantly stable within 45 min at 21°C and 4°C; while Trypan blue assay stained cells were significantly stable within 30 min at 21°C. These findings provide important insights into practical handling of fish PBMCs during laboratory assays, and reveal an important time range within which results can be reliably obtained. Overall, reliable measurements of cell viability can be obtained when working with fish PBMCs within the first 45 min following staining, irrespective of storage conditions. However, it is highly recommended that cell viability assessments are made on fresh samples that have been stained for 10 min when possible to minimize the potential for compromising results.

Meanwhile, validation findings showed that the accuracy and precision of the semi-automated Muse® Count and Viability assay Kit was similar or better than the manual Trypan blue and the haemocytometer chamber method for fish PBMC viability and cell count analysis. These results align with other validation studies using this platform and kit for different cell types from humans, hamsters, insects, and microalgae (Gillis et al., 2016; Millipore, 2012). In the current study, lower grand mean % CV values for viability results were obtained with the Muse® Cell Analyser in comparison to the improved haemocytometer, while cell count overall mean % CV values were close for both methods. However, the Muse® Cell Analyser is a portable and cost-effective bench top flow cytometer engineered with a precise microcapillary system that uses fluorescence to accurately, and precisely quantify multiple cell parameters at once, helping to save time. The Muse® Cell Analyser also uses micro sample volumes, requires minimal sample preparation, and works with adherent and non-adherent cells over a wide size range from 2 to 60 µm diameter. The instrument is very robust and has higher sample throughput than using a traditional manual haemocytometer chamber since it provides results and produces cell population profile plots in only 1 - 2 min, compared to 5 - 7 min per sample for haemocytometer. These findings underscore the strength of using the semi-automated Muse® Cell Analyser to quickly process fish PBMC samples using only 20 µL of biofluid.

The use of fish PBMCs and the Muse® Cell Analyser provides a highly improved method to elucidate immune responses in fish, and can be easily deployed to the field for monitoring and performing health assessments in farmed fish without killing stocks. The Muse® Cell Analyser can be used with a range of cell health assessment kits, including the Muse® Oxidative Stress Kit

for quantification of ROS, the Muse® Nitric Oxide Kit for intracellular NO activity, the Muse® MultiCaspase Assay Kit for rapid and quantitative measurements of cell death, and the Muse® MitoPotential Assay Kit for simultaneous measurement of changes in mitochondrial potential and cell death. Other kits include the Muse® Caspase-3/7 Kit to quantify cellular apoptosis and cellular plasma membrane permeabilization, and the Muse® Annexin V and Dead Cell Assay to quantify live cells, early and late apoptosis and cell death. Some of these kits have been earmarked for further immunological studies with *O. tshawytscha*.

4.6 Conclusions and recommendations

In this study, we demonstrated that *O. tshawytscha* yearling smolts peripheral blood is dominated by erythrocytes, while lymphocytes were the most common leucocytes. In addition, differential cell count revealed five cell types including erythrocytes, thrombocytes, lymphocytes, monocytes and unquantifiable neutrophils. The study also provides a micro-volume method to successfully isolate fish PBMCs through density gradient centrifugation. Using the isolated PBMCs, we validated a microcapillary flow cytometry Muse® Cell Count & Viability Assay Kit against the traditional manual haemocytometer chamber Trypan blue assay method for cell viability. We conclude that we successfully characterised fish peripheral blood cellular composition; and that the portable Muse® Cell Analyser can accurately and precisely be used to assess *O. tshawytscha* PBMC health in field or laboratory conditions. The protocol is easy-to-use, fast, cheap, and may be used to monitor fish health or assess physiological responses *in vitro* using a high throughput Muse® Cell Analyser.

5 Chapter 5. Case study 2: *In vitro* Immune Response of Chinook salmon (*Oncorhynchus tshawytscha*) Peripheral Blood Mononuclear Cells Stimulated by Bacterial Lipopolysaccharides.

This chapter has been published:

Lulijwa, R., Alfaro, A. C., Merien, F., Burdass, M., Venter, L., & Young, T. (2019). In vitro immune response of chinook salmon (*Oncorhynchus tshawytscha*) peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide. *Fish & shellfish immunology*, *94*, 190-198. https://www.sciencedirect.com/science/article/pii/S1050464819308800

5.1 Prelude to case study 2

A method for isolating Chinook salmon (*Oncorhynchus tshawytscha*) PBMCs by density gradient centrifugation was successfully developed from chapter 4. Employing the developed PBMC isolation technique in chapter 4, chapter 5 focussed on using the obtained cellular suspensions to model *O. tshawytscha* cellular functional and targeted immune cytokine mRNA transcript responses *in vitro* to Gram-negative bacterial LPS products from *Escherichia coli* (*E. coli*) serotypes O111: B4 and O55: B5 over a 24 h period. LPS is a well-conserved Gram-negative bacterial PAMP that forms a major part of the outer cell wall membrane, which is recognised by the vertebrate immune system to orchestrate a defence response. Consequently, LPS is commonly used to modulate teleost immunity in aquaculture. With limited literature on New Zealand farmed *O. tshawytscha* immunology, this was the first study to unravel LPS induced immunomodulatory mechanisms in PBMCs *in vitro*. Results will aid future studies aimed at understanding emerging and future *O. tshawytscha* bacterial pathogens *in vitro*.

5.2 Introduction

Salmonids are the third most farmed finfish species after cyprinids and tilapines, and they accounted for 6% of global finfish production in 2016, mostly from Atlantic salmon (*Salmo salar*), and rainbow trout (*Oncorhynchus mykiss*) (FAO, 2018c). Norway, Chile, Scotland, Canada, and the Faroe Islands are the major *S. salar* producers (Asche et al., 2013; Irarrázaval & Bustos-Gallardo, 2019). Norway and Scotland farm *S. salar* and *O. mykiss* (Munro & Wallace, 2018; Norwegian Veterinary Institute, 2016), and the Faroe Islands only farm *S. salar* (Faroe Fish Farmers Association, 2012). In Chile, production comes from S. *salar*, *O. mykiss*, Coho salmon (*Oncorhynchus kisutch*) [reviewed in Miranda et al. (2018)] and *O. tshawytscha* (Lozano et al., 2018) in order of production volume. Canada has the most diverse salmon industry composed of *S. salar, O. kisutch*, *O. tshawytscha*, brown trout (*Salmo trutta*), Lake trout (*Salvelinus namaycush*), brook trout (*Salvelinus fontinalis*) and *O. mykiss* (Statistics Canada, 2017). New Zealand leads in global *O. tshawytscha* farming, and produced up to 13,000 ton in 2016 worth over US$ 176 million (FAO, 2018b). Fifty percent of this production is marketed internationally under the trade name King salmon, and constitutes about half of the global supply chain.

New Zealand's current *O. tshawytscha* stock was successfully established in 1904 as Chinook salmon, after initial introductions in 1870s failed (McDowall, 1994). The species is currently New Zealand's main farmed finfish after the Greenshell™ mussels (*Perna canaliculus*) (FAO, 2018b). The New Zealand aquaculture industry is also known for its sustainable practices, partly making salmon farming relatively disease free. However, we understand that farmed fish co-exist with bacteria, viruses, fungi, and parasites, which rapidly proliferate following deviations from optimal culture conditions and compromise the innate immune system. Consequently, disease outbreaks routinely lead to heavy industry losses in aquaculture [reviewed in Fazio (2019)]. Indeed, there have been recent reports of *Tenacibaculum maritimum* associated with summer mortality (Brosnahan et al., 2019b; Brosnahan et al., 2019a) and New Zealand rickettsia-like organism (NZ-RLO) (Brosnahan et al., 2017). The situation may be complicated with challenges posed by climate change (e.g., rising seawater temperatures and ocean acidification). The above scenarios, thus make fish immunology understanding an indispensable asset to allow routine detection and monitoring of health problems [reviewed in Uribe et al. (2011)].

The fish immune system consists of the innate or nonspecific (physical, cellular and humoral) components, and acts as the first line of defence [reviewed in Ángeles Esteban (2012); Martin and Król (2017)]. The adaptive or specific immune components rely on humoral and cellular responses, and is characterized by specific antigen recognition, which invokes a quick secondary pathogen specific response (Martin & Król, 2017). Fish rely on the innate and adaptive immune components for defence (Sahoo et al., 2021; Whyte, 2007). Evolutionally, pathogenic Gram-negative bacteria and viruses have common conserved pathogen associated molecular patterns (PAMPs) (Castro & Tafalla, 2015). They include viral double stranded ribonucleic acid (dsRNA) and bacterial deoxyribonucleic acid (DNA), fungal β1, 3-glucans (β-glucan), bacterial cell wall

peptidoglycans, polysaccharides and Gram-negative bacterial endotoxin or lipopolysaccharides (LPS) (Elward & Gasque, 2003).

These PAMPs can be recognised by the vertebrate immune system through pattern recognition receptors (PRRs) on cell surfaces, following breach of physical barriers (e.g. cellular epithelia and the skin). The binding of the host cellular PRRs and the PAMPs initiates cellular responses specifically designed to kill and eliminate the microbial pathogen (Rieger & Barreda, 2011). Endotoxic bacterial LPS, a cell wall component of gram-negative bacteria, is widely used in aquaculture immunological investigations (Anderson, 1992; Kadowaki et al., 2009; Magnadottir, 2010; Nya & Austin, 2010; Salati et al., 1987). Structurally, LPS consists of lipid A, a polysaccharide core, and an O-polysaccharide of variable length. Lipid A confers the endotoxic effects that lead to innate immune responses [reviewed in Sepulcre et al. (2009)].

Bacterial LPS induces reactive oxygen species (ROS) production via activation and induction of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and the suppression of anti-oxidative enzymes involved in ROS clearance (L. Li et al., 2010). The enzyme NADPH oxidase facilitates transfer of electrons to molecular oxygen resulting in superoxide anion production (Briggs et al., 1975). In teleosts, the process of ROS production has been reviewed thoroughly by several authors (Grayfer et al., 2014; Neumann et al., 2001), and ROS progression explained in a review by Grayfer et al. (2014). Also, genes for the enzyme NADPH oxidase have been linked with ROS production in *O. mykiss*, *S. salar*; have been cloned and sequenced in *O. mykiss* (Grayfer et al., 2014; Grayfer et al., 2018). Earlier work showed that *O. mykiss* leucocytes isolated from head kidney (HK), thymus (TH), peripheral blood (PB) and spleen (S), responded to LPS and concanavalin A (con A) *in vitro* (Warr & Simon, 1983).

At the molecular level, LPS induced expression of interleukin-1 (*il-1*) like compounds in channel catfish (*Ictalurus punctatus*) (Clem et al., 1985), and upregulated interleukin-1β (*il-1β*) and tumor necrosis factor alpha (*tnf-α*) in HK leucocytes (Holen et al., 2011). LPS also induced expression of macrophage stimulating factor 1 receptor (M-CSFR), major histocompatibility complexity II (MHC-II), and interleukin-6 (*il-6*) in half smooth tongue sole (*Cynoglossus semilaevis*) PB leucocytes (Sha et al., 2017). In established *S. salar* TO cell line, LPS induced significant upregulation of cluster of differentiation 88 (CD83) gene transcript, indicative of dendritic cell (DC) origin (Pettersen et al., 2008). Symbiotically, β-glucan and bacterial LPS administration enhanced accumulation of lysozyme gene transcripts in *S. salar* HK macrophages (Paulsen et al., 2001). In addition, *S. salar* HK macrophage *in vitro* stimulation with bacterial LPS and β-glucan highly induced gene expression for arginase-1 and IL-1β (Ulvestad et al., 2018).

In previous immunological studies on *O. tshawytscha*, researchers mostly used LPS *in vitro* to assess HK and SP immunocompetence following fish exposure to environmental chemical stressors (Arkoosh et al., 1991; Arkoosh et al., 1994; Misumi et al., 2005; Misumi et al., 2009). Done *in vitro*, Milston et al. (2003) investigated the effect of cortisol on *O. tshawytscha* HK and SP leucocytes immunocompetence via blastogenesis and surface IgM positive cells activation

using LPS. In addition, Slater and Schreck (1993) used HK leucocytes to study effects of steroid hormones on fish immunocompetence via plaque forming cells, following LPS activation. In this study, we isolated peripheral blood mononuclear cells (PBMCs) from health animals to demonstrate immunocompetence against Gram-negative bacterial LPS products *in vitro*. The study characterised the cellular functional and molecular immunomodulatory responses of farmed *O. tshawytscha* PBMCs *in vitro* using LPS from *Escherichia coli* (*E. coli*) serotypes O111: B4 and O55: B5 plus PMA. These findings contribute towards the understanding of immunocompetence in *O. tshawytscha* under different conditions.

5.3 Materials and methods

5.3.1 Fish husbandry and blood sampling

Captive two year old *O. tshawytscha* postsmolt (weight = 480.00±119.11 g; total length = 36.67±2.02 cm) were obtained from the Nelson Marlborough Institute of Technology (NMIT) aquaculture facility (Glenduan, Nelson, New Zealand). Fish had been maintained in a saltwater recirculating system (temp = 16.02±0.94°C; DO = 7.34±0.32 mg L^{-1}; pH = 8.18±0.17; NH_4^+ = 0.17±0.12 mg L^{-1}; NO_2^- = <0.01 mg L^{-1}) at NMIT aquaculture facility at 16°C and fed to satiation with Orient 3.0 mm commercial diet (Skretting, Australia) containing 44% crude protein and 28% lipid. Fish were randomly taken by scoop net from the culture tank and individually euthanized by Ikijime for isolation of PBMCs. All fish included in the study were free of physical abnormality, had intact skin, without lesions and tail rub. Approximately 2.5 mL of blood were collected from each fish via caudal vein puncture using a chilled non-heparinised 3.0 mL syringe attached to a 20 gauge Terumo needle. Blood was quickly distributed into six 400 µL BD Microtainer® Lithium Heparin tubes (Becton Dickinson, USA) and used for PBMC processing as illustrated below. Ethical approval was obtained from the NMIT Animal Ethics Committee (NMIT-AEC-2018-NMIT-03).

5.3.2 PBMC isolation

Fish PBMCs were isolated from blood following a protocol from our previous work (Lulijwa et al., 2019a) using Ficoll-Paque Premium density gradient medium (1.077 g mL^{-1}) (Sigma-Aldrich, New Zealand). Briefly, 284 µL of heparinized blood were quickly diluted 1:1 with sterile filtered (40 µm) phosphate-buffered saline (PBS) (pH 7.4) in triplicates. PBMCs were obtained by centrifuging the diluted blood samples at 971 g for 20 min over a layer of 682 µL of Ficoll-Paque. Cells at the interface were aspirated with a precision pipette, pooled together and gently washed twice with 1 mL of sterile PBS at 674 g for 7 min. The cell pellet was resuspended to a final concentration of 1×10^5 cells mL^{-1} in 2 mL of PBS supplemented with 2% fetal calf serum (FCS). This working cell suspension was kept chilled at 4°C for further analysis. Cell viability was determined using the Muse® Count and Viability Assay Kit as previously described (Lulijwa et al., 2019a).

5.3.3 Preparation of bacterial LPS and PMA

Bacterial LPS products from *E. coli* 0111: B4, *E. coli* serotype O 55:B5 previously used on *O. tshawytscha* (Arkoosh et al., 1991; Milston et al., 2003; Misumi et al., 2009) and PMA were obtained from Sigma Aldrich, New Zealand. These LPS products were prepared by suspending the solute in sterile filtered PBS (pH 7.4) to a concentration of 1 mg mL^{-1} and stored in small aliquots at −20°C. Similarly, a PMA solution was prepared in DMSO (1mg mL^{-1}) and brought to a final concentration of 200 ng mL^{-1} in sterile PBS (pH 7.4) and kept at −20°C until further use.

5.3.4 *In vitro* LPS and PMA challenge

Fish PBMCs isolated from 3 fish were divided into four 450 µL aliquots, centrifuged at (16,250 g; 4 min), and resuspended in an equivalent volume of PBS containing 10 µg mL^{-1} of LPS *E. coli* serotype 0111: B4, *E. coli* serotype O 55:B5 and 200 ng mL^{-1} of PMA according to Solem et al. (1995). Cells were incubated at room temperature (18°C) and monitored for ROS production, and phagocytosis activity at 0, 0.5, 1, 2, 3, 6, 12 and 24 h. After 24 h, the remaining cells were centrifuged in cryo-vials at (16,250 g; 4 min), the pellet snap frozen in liquid nitrogen and stored at −80°C for gene expression analysis.

5.3.5 Reactive oxygen species (ROS) assay

Intracellular ROS production in PBMCs was measured using the Muse® Oxidative Stress Kit protocol (Merck KGaA, Darmstadt, Germany), with the Muse® Cell Analyzer at 0, 0.5, 1, 2, 3, 6, 12 and 24 h. At each time point, 20 µL of PBMC suspension were incubated with 180 µL of Muse® Oxidative Stress working solution following the manufacturer's procedures. Subsequently, the samples were mixed thoroughly and run on the Muse® Cell Analyzer and monitored for ROS production using the Muse® Oxidative Stress Kit (Merck KGaA, Darmstadt, Germany) at the specified time points.

5.3.6 Phagocytic activity and phagocytic index determination

Isolated PBMCs were also monitored for phagocytosis activity at 0, 0.5, 1, 2, 3, 6, 12 and 24 h. Ten microliters of PBMC suspension (1×10^5 cells mL^{-1}) were pipetted onto sterile glass microscope slides and incubated at room temperature for 1 h. Non-adherent cells were washed off using sterile filtered PBS with 2% FCS. Then, 10 µL of fluorescent latex beads (1×10^5 mL^{-1}) amine-modified polystyrene suspension (1.0–1.3 µm in diameter, Sigma-Aldrich, New Zealand) were added and incubated for another 2 h at room temperature. Non engulfed latex beads were washed off using sterile filtered PBS with 2% FCS. The slides were subsequently fixed with absolute methanol and later Giemsa stained. Exactly 100 cells were counted microscopically at 400x magnification and the percent phagocytic activity (PA) and phagocytic index determined according to the following equations:

$$\text{Phagocytosis} = (\text{Number of phagocytic leucocytes})/(\text{Number of total leucocytes}) \times 100$$

While the phagocytic index was determined by the following equation:

$$\text{Phagocytic index} = (\text{Number of latex beads per cell})/(\text{Number of phagocytic cells})$$

5.3.7 Molecular assessment of immune genes

5.3.7.1 Analysis of mRNA expression in PBMCs

Total RNA was extracted from each PBMC sample using the Roche MagNA Pure LC 2 instrument with the MagNA Pure LC RNA Isolation Kit – High Performance (Roche Life Sciences, New Zealand). Subsequently, 100 µL of eluted mRNA were collected and stored at −80°C prior to gene expression analysis. Relative quantification of gene expression by one step reverse transcriptase polymerase chain reaction (RT-PCR) was performed (LightCycler 480 instrument II [Roche Diagnostics, Auckland, New Zealand]).

Table 5.1. Primer sequences used for PBMC cytokine salmon gene expression.

Genes	Sequence (5'–3')	Product size (bp)	Gene function	Species & GenBank Accession no.	Reference
Actb	(F)- GTCACCAACTGGGACGACAT (R)- GTACATGGCAGGGGTGTTGA	175	ATP binding, determines cell shape and controls motility	*O. tshawytscha* FJ890357.1	(Monjo et al., 2017)
Ifnγ	(F)- CAACATAGACAAACTGAAAGTCCA (R)- ACATCCAGAACCACACTCATCA	129	Triggers cellular response to viral and microbial infections	*O. tshawytscha* GT897806	(Bjork et al., 2014)
il-1β	(F)- ACCGAGTTCAAGGACAAGGA (R)- CATTCATCAGGACCCAGCAC	181	Potent pro-inflammatory cytokine	*O. tshawytscha* DQ778946	(Bjork et al., 2014)
il-6	(F)- CAGTTTGTGGAGGAGTTTCAGA (R)- TGTTGTAGTTTGAGGTGGAGCA	118	Potent pleiotropic cytokine	*O. mykiss* NM_001124657	(Bjork et al., 2014)
tnf-α	(F)- ACCAAGAGCCAAGAGTTTGAAC (R)- CCACACAGCCTCCATAGCCA	154	Multifunctional pro-inflammatory cytokine	*O. tshawytscha* DQ778945	(Bjork et al., 2014)
il-10	(F)- CTACGAGGCTAATGACGAGC (R)- GATGCTGTCCATAGCGTGAC	97	Anti-inflammatory roles; limits excessive tissue damage caused by inflammation	*O. mykiss* AB118099	(Bjork et al., 2014)

F: forward primer, and R: reverse primer.

Primers (Integrated DNA Technologies, Inc., Singapore) at a working concentration of 0.9 µmol L^{-1} and RNA in a final volume of 20 µL were used. The reactions were performed using the following thermocycling conditions: 60°C for 15 min (reverse transcription/cDNA synthesis), 95°C for 10 min (transcriptase inactivation and initial denaturation step) and 40 cycles of amplification (95°C for 15 s for denaturation and 60°C for 1 min for annealing and extension). Upon completion, dissociation/melting curve analyses were performed to reveal and exclude nonspecific amplification or primer-dimer issues (all melting analyses in this study presented single reproducible peaks for each target gene suggesting amplification of a single product). The housekeeping gene used for mRNA expression analysis was β-actin (*actb*) due to its stability within mitogen-stimulated PBMCs. Primer sequences used in this study are provided in Table 5.1. Relative gene expression levels were calculated using the comparative Ct (△△Ct) equation (Schmittgen & Livak, 2008). Gene expression was calculated as $2^{-\triangle\triangle Ct}$ and expressed as a fold-change. The mRNA transcripts for all target genes were normalised to the reference gene (*actb*) within the same sample, condition and time point, and to a calibrator (control with PBS).

5.3.8 Data processing and statistical analysis

Data from fish PBMC cellular functional characterisation for ROS production, phagocytosis and phagocytic index were processed to assess the effect of *in vitro* PMA and LPS from *E. coli* serotype 0111: B4 and *E. coli* serotype 055: B5 on fish immune system by one-way time series

ANOVA using Minitab 17 statistical software at 95% confidence interval. Data for immune genes were expressed as relative gene expression using the "delta-delta method"(Schmittgen & Livak, 2008), and graphical presentation in Microsoft Office Excel 2016. One-way ANOVA was used to detect differences induced by PAMPs and PMA using Minitab 17 statistical software at 95% confidence interval.

5.4 Results

5.4.1 Fish PBMC cellular functional characterisation

In this study, we assessed the immunological response of primary *O. tshawytscha* PBMCs to LPS from *E. coli* serotype 0111: B4 and *E. coli* serotype 055: B5 and PMA *in vitro* 24 h post challenge at 18 °C. Cellular functional parameters of ROS production, phagocytosis and phagocytic index, and targeted immune gene expression were determined.

5.4.1.1 Reactive oxygen species (ROS) production

Following *in vitro* stimulation with PMA, *O. tshawytscha* primary PBMCs exhibited significantly ($p < 0.05$) high peak ROS production 30 min post induction. Twenty-four hours post stimulation, ROS production reduced below initial levels, with a general biphasic trend (Figure 5.1).

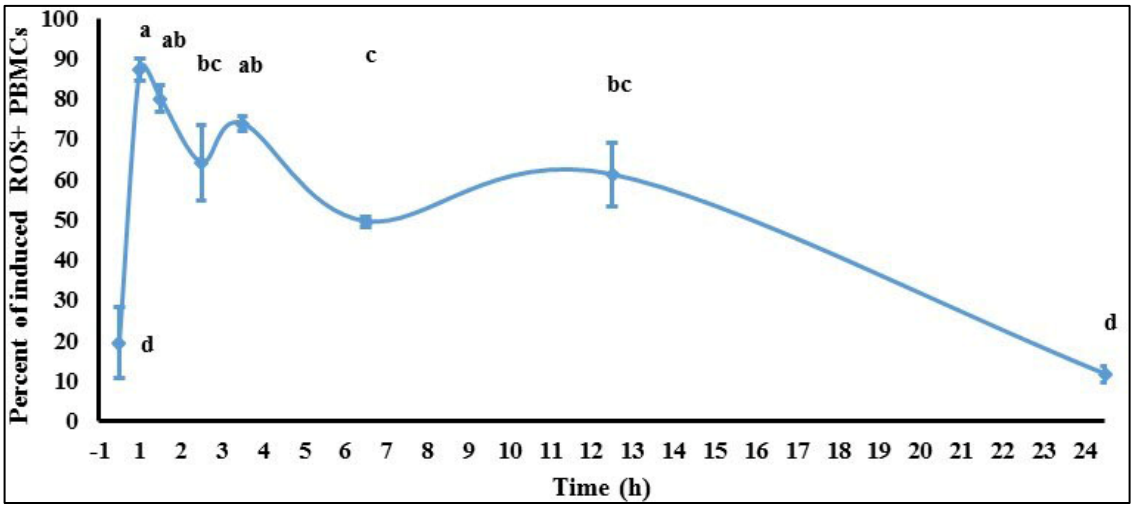


Figure 5.1 *In vitro* induction of ROS production in *Oncorhynchus tshawytscha* PBMCs by 200 ng mL^{-1} of PMA over 24 h incubation at 18°C. Data points are mean±SEM from duplicate technical readings of three biological triplicates at each time point. Data points with different superscripts are significantly different, One-way time series ANOVA at $p = 0.05$.

Similarly, bacterial LPS from *E. coli* serotype 0111:B4, induced a biphasic ROS response that significantly ($p < 0.05$) peaked after 2 h, followed by a second major but nonsignificant peak ($p > 0.05$) after 12 h and remained elevated above initial levels 24 h later (Figure 5.2).

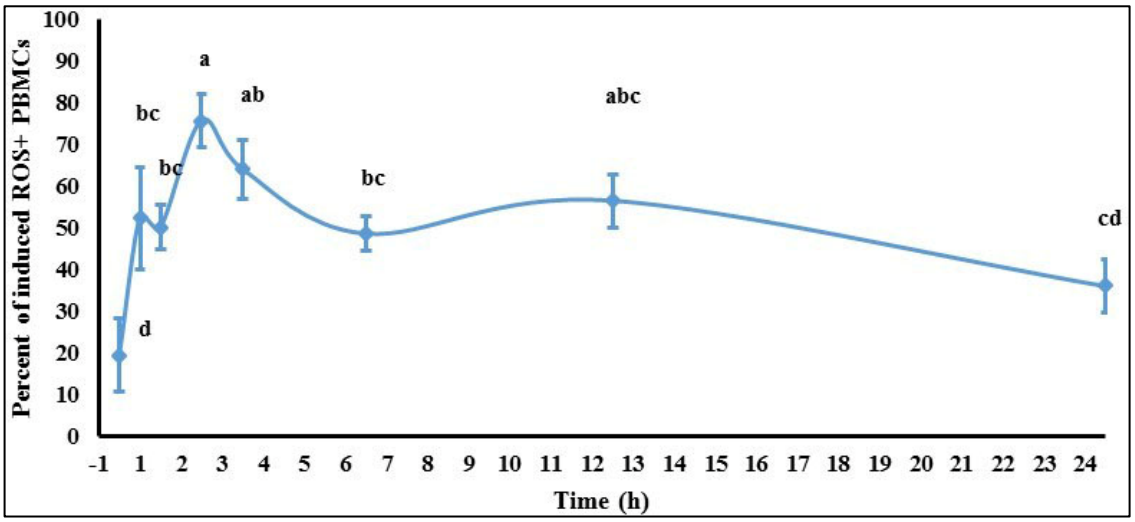


Figure 5.2 *In vitro* induction of ROS production in *Oncorhynchus tshawytscha* PBMCs by 10 µg mL^{-1} of LPS from *E. coli* serotype 0111: B4 over 24 h incubation at 18°C. Data points are mean±SEM from duplicate technical readings of three biological triplicates at each time point. Data points with different superscripts are significantly different, One-way time series ANOVA at p = 0.05.

Likewise, bacterial LPS from *E. coli* serotype 055:B5, induced low bimodal ROS production in *O. tshawytscha* primary PBMCs that significantly ($p < 0.05$) peaked after 2 h following induction, followed by a second peak ($p > 0.05$) at 12 h, and remained above initial levels 24 h later (Figure 5.3).

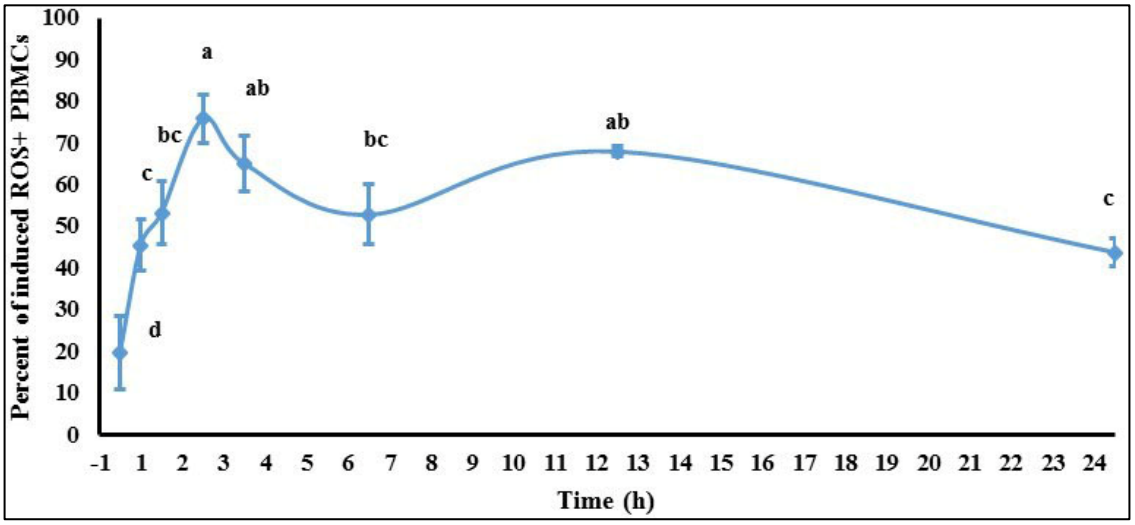


Figure 5.3 *In vitro* induction of ROS production in *Oncorhynchus tshawytscha* PBMCs by 10 µg mL^{-1} of LPS from *E. coli* serotype 055: B5 over 24 h incubation at 18°C. Data points are mean±SEM from duplicate technical readings of three biological triplicates at each time point. Data points with different superscripts are significantly different, One-way time series ANOVA at p = 0.05.

5.4.1.2 Phagocytosis activity

Also, bacterial LPS and PMA significantly ($p < 0.05$) enhanced *O. tshawytscha* primary PBMCs latex bead phagocytosis; particularly at 1 h post PMA incubation and at 2 h following incubation with LPS from *E. coli* serotype 0111:B4. Conversely, LPS from *E. coli* serotype

055:B5 did not significantly ($p > 0.05$) induce phagocytosis in the fish PBMCs for the entire experimental period (Table 5.2).

Table 5.2. *In vitro* phagocytosis (%) of latex beds in *Oncorhynchus tshawytscha* PBMCs using PMA and two bacterial PAMPs over a 24 h incubation period at 18°C. Data are mean±StDev from duplicate readings of three fish at each time point. Values in a column with different superscripts are significantly different, One-way time series ANOVA at $p = 0.05$.

Time (h)	PMA	LPS serotype 0111:B4	LPS serotype 055:B5
0	9.00 ± 6.00^{b}	9.00 ± 6.00^{c}	9.00 ± 6.00^{a}
0.5	18.33 ± 6.35^{ab}	18.33 ± 2.08^{abc}	13.00 ± 3.00^{a}
1.0	22.67 ± 2.52^{a}	22.00 ± 2.65^{ab}	13.00 ± 5.00^{a}
2.0	15.67 ± 2.08^{ab}	25.33 ± 9.07^{a}	12.67 ± 0.57^{a}
3.0	16.67 ± 0.58^{ab}	17.00 ± 1.73^{abc}	17.67 ± 2.08^{a}
6.0	-	16.67 ± 0.58^{abc}	16.67 ± 2.52^{a}
12.0	-	12.67 ± 1.53^{bc}	16.00 ± 3.46^{a}
24.0	-	13.33 ± 3.06^{abc}	16.00 ± 6.56^{a}
F-value	4.22	4.56	1.43
P-value	0.029	0.006	0.260

5.4.1.3 Phagocytic index

Although PMA and LPS significantly ($p < 0.05$) enhanced *O. tshawytscha* primary PBMCs latex bead phagocytosis, the phagocytic index was not significantly ($p > 0.05$) affected by the LPS and PMA in fish PBMCs for the entire experimental period (Table 5.3).

Table 5.3. *In vitro* phagocytic index in *Oncorhynchus tshawytscha* PBMCs following challenge with a macrophage activating factor PMA and two bacterial PAMPs over a 24 h incubation period at 18°C. Data are mean±StDev from duplicate readings of three fish at each time point. Values in a column with different superscripts are significantly different, One-way time series ANOVA at $p = 0.05$.

Time (h)	PMA	LPS serotype 0111:B4	LPS serotype 055:B5
0.0	1.80 ± 1.06^{a}	1.80 ± 1.06^{a}	1.80 ± 1.06^{a}
0.5	2.59 ± 0.43^{a}	2.13 ± 0.17^{a}	2.25 ± 0.80^{a}
1.0	2.93 ± 0.06^{a}	2.11 ± 0.40^{a}	2.21 ± 0.30^{a}
2.0	2.60 ± 0.33^{a}	1.92 ± 0.68^{a}	2.14 ± 0.34^{a}
3.0	2.73 ± 0.46^{a}	1.96 ± 0.34^{a}	2.16 ± 0.21^{a}
6.0	-	2.07 ± 0.45^{a}	2.19 ± 0.22^{a}
12.0	-	2.05 ± 0.39^{a}	2.07 ± 0.16^{a}
24.0	-	2.49 ± 0.05^{a}	1.80 ± 0.17^{a}
F-value	1.71	0.44	0.37
P-value	0.225	0.865	0.908

5.4.2 Fish PBMC immune gene expression

When challenged with bacterial LPS from *E. coli* serotype 055:B5 and *E. coli* serotype 0111:B4, the expression levels for pro-inflammatory cytokines *ifnγ* and *tnf-α*, plus anti-inflammatory *il-10* were significantly upregulated in *O. tshawytscha* primary PBMCs 24 h post-challenge (Figure 5.4).

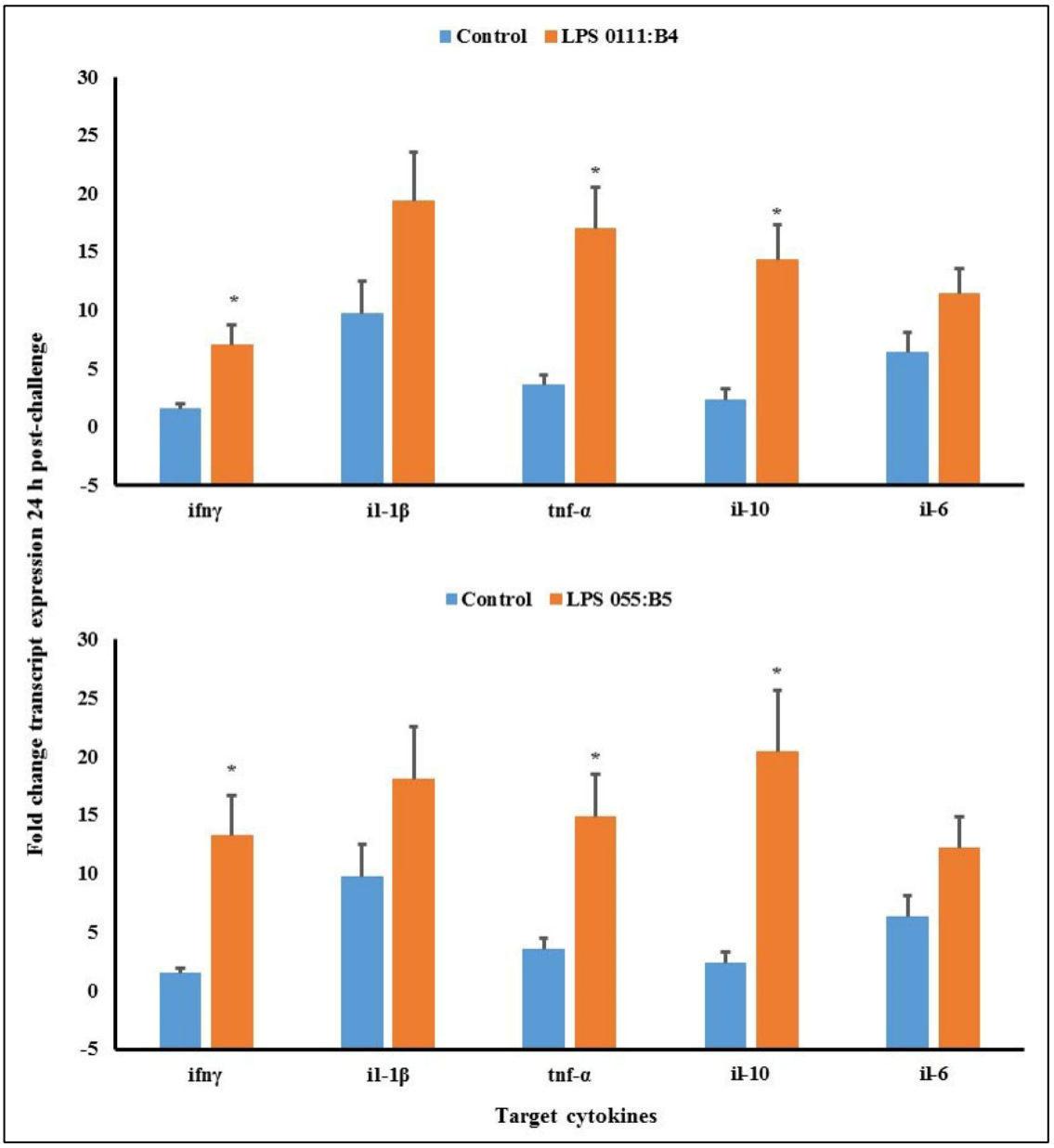


Figure 5.4 Relative expression of mRNA PBMC cytokines by RT-qPCR following *in vitro* challenge of *Oncorhynchus tshawytscha* PBMCs by 10 µg mL^{-1} of LPS from *E. coli* serotype 055: B5, LPS from *E. coli* serotype 0111: B4 over 24 h incubation at 18°C. Data are mean±SEM of three technical replicates of three fish 24 h post-challenge, One-way time series ANOVA at p = 0.05.

Comparatively, PBMCs challenged with PMA significantly ($p < 0.05$) induced over 90, 30 and 70 fold increase in pro-inflammatory *ifnγ*, *tnf-α* and anti-inflammatory *il-10* expression. Cytokine levels for *il-1β* were significantly ($P < 0.05$) downregulated in treated fish PBMCs while *il-6* was not affected in *O. tshawytscha* primary PBMCs 24 h post challenge (Figure 5.5).

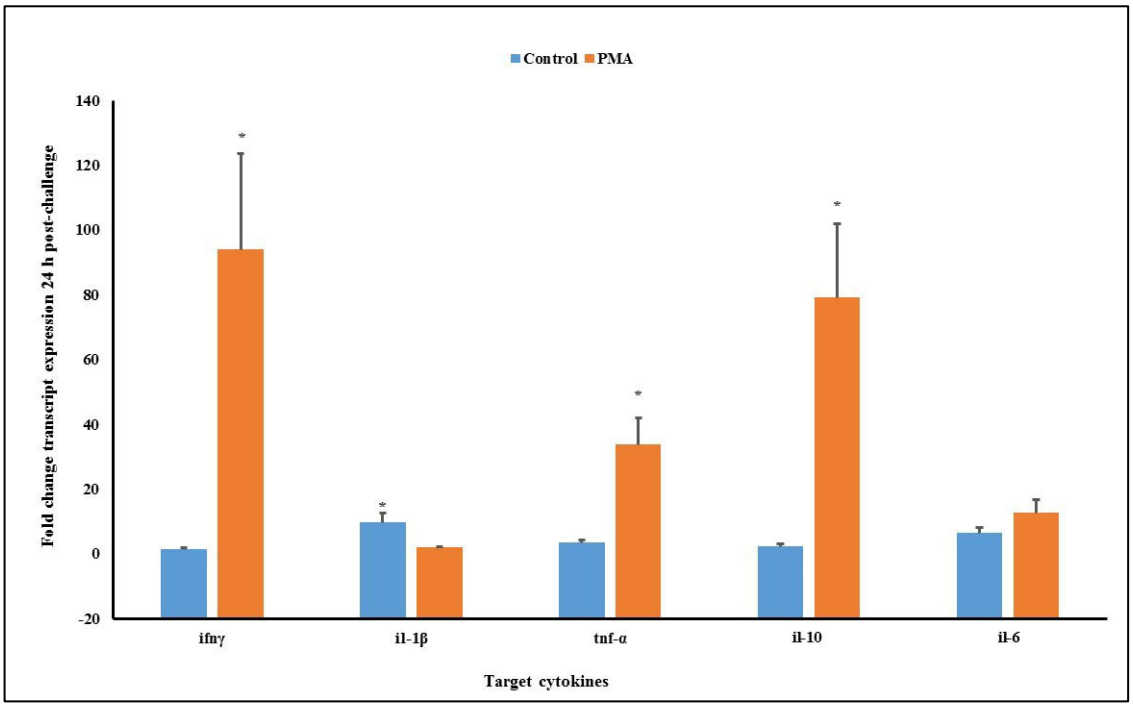


Figure 5.5 Relative expression of mRNA PBMC cytokines by RT-qPCR following *in vitro* challenge of *Oncorhynchus tshawytscha* PBMCs by 200 ng mL^{-1} of PMA over 24 h incubation at 18°C. Data are mean ± SEM of three technical replications from three fish 24 h post-challenge, One-way time series ANOVA at p = 0.05.

5.5 Discussion

In the current study, we investigated *in vitro* immunomodulatory effects of two bacterial LPS products and PMA on captive *O. tshawytscha* primary PBMCs via cellular functional parameters of ROS production, phagocytosis and phagocytic index, plus targeted mRNA immune gene expression.

Phorbol ester PMA mimics diacyl glycerol (DAG) to activate the enzyme protein kinase C (PKC) (Garcia et al., 2012) in cells initiating, protein biosynthesis, DNA, polyamine, cell differentiation, and gene expression activities (Goel et al., 2007). Also, PMA induces cellular differentiation via enhanced cellular surface adherence, phagocytosis and superoxide production (Garcia et al., 2012). Data in the current study revealed that *in vitro* administration of PMA, significantly ($p < 0.05$) induced a rapid and substantial increase in ROS production within 30 min of stimulation in primary PBMCs (Figure 5.1). Similarly, previous studies have demonstrated in *O. mykiss* HK macrophages that PMA induces ROS (Chung & Secombes, 1988) and a greater respiratory burst activity compared to stimulation with PAMPs (Novoa et al., 1996). PMA targets cellular membrane phospholipid receptors for attachment and interaction to augment uptake of 2-deoxyglucose, induces arachidonic acid (ARA) release and prostaglandin production, alters cellular receptor activities, obstructs cellular surface receptor binding of epidermal growth factor (EGF), and affects lipid metabolism [reviewed in Goel et al. (2007)]. The rapid induction of ROS production by PMA as observed in this study could be attributed to surface binding and damage that PMA induces during interaction with cellular membranes (Weinstein et al., 1979; Weissmann

et al., 1968). This process is facilitated by enhanced release of proteases, cytokines, and activation of NADPH oxidase (Goel et al., 2007), which damages cells. This theory is supported by the elevated expression of *ifnγ* and *tnf-α* cytokines in PMA treated PBMCs. In particular, *tnf-α* has been linked with cellular necrosis and/or apoptosis (Idriss & Naismith, 2000).

LPS from *E. coli* serotype 0111:B4, and *E. coli* serotype 055:B5 also induced high ROS production 2 h post stimulation. Similar findings have been reported previously among salmonids as LPS *in vitro* enhanced cellular functions and ability to kill an avirulent A-layer lacking strain of *Aeromonas salmonicida* in *S. salar* HK macrophages (Solem et al., 1995) and activated respiratory burst in *O. mykiss* HK macrophages (Jang et al., 1995). In addition, supplementary LPS *in vivo* enhanced cellular functions, and bactericidal effects against pathogenic *Aeromonas hydrophila* in *O. mykiss* (Nya & Austin, 2010). Overall, *O. tshawytscha* PBMCs exhibited the ability to recognise bacterial LPS as in other vertebrates. These results provide invaluable information as to the mechanisms by which the host interacts with bacterial pathogens (Rieger & Barreda, 2011), via strong respiratory burst activity facilitated by the NADPH oxidase complex (Boltana et al., 2009).

In addition, a biphasic ROS production trend was observed following stimulation with PMA and bacterial LPS with a second, but nonsignificant ($p > 0.05$) ROS peak after 12 h. The initial ROS peak is due to direct cellular LPS and PMA response facilitated by the NADPH oxidase complex, followed by a temporal ROS clearance after 6 h, probably due to cellular anti-inflammatory cytokine production to abate damage. However, the second strong ROS peak is due to PMA and LPS induced pro-inflammatory *tnf-α* and IFN-inducible *ifnγ* cytokine production (Bussolati et al., 1997). While ROS levels remained elevated above basal levels 24 h later for LPS (Figure 5.2 and Figure 5.3), lower than initial levels were observed with PMA after 24 h (Figure 5.1), probably due to cellular damage (Repine et al., 1974; Weissmann et al., 1968). This is evidenced by the over expression of pro-inflammatory *ifnγ* and *tnf-α* in PMA challenged PBCs, which probably resulted into cellular necrosis and/or apoptosis (Idriss & Naismith, 2000). In addition, PMA as a DAG analogue reportedly hyper activates PKC, and unlike DAG, PMA is hardly metabolised by cells, which induces cellular proliferation, and carcinogenic effects. Similarly, longer PMA incubation results into PKC down regulation (Goel et al., 2007), corresponding with the observed results.

Phagocytosis is the most important innate immune defence mechanisms in fish due to its limited disturbance by temperature [reviewed in Uribe et al. (2011)]. The phagocytic process involves uptake of pathogens by engulfment into phagosomes (Øverland et al., 2010). In this study, PAMPs and PMA significantly ($p < 0.05$) enhanced *O. tshawytscha* primary PBMCs latex bead phagocytosis; 1 h post PMA incubation and at 2 h after LPS from *E. coli* serotype 0111:B4 stimulation (Table 5.2). Similarly *in vitro* LPS exposure enhanced phagocytic activity in *S. salar* HK macrophages (Solem et al., 1995). *In vivo*, supplementary LPS enhanced HK leucocyte phagocytosis, and bactericidal effects against pathogenic *A. hydrophila* in *O. mykiss* (Nya &

Austin, 2010). In PMA challenged PBMCs, cellular damage (Repine et al., 1974; Weissmann et al., 1968), observed as aggregations resulted into phagocytosis and phagocytic index determination up to only 3 h post stimulation. This is further suggestive that PMA may have induced *O. tshawytscha* primary PBMC apoptosis and or necrosis (Idriss & Naismith, 2000).

The above findings are supported by the significant ($p < 0.05$) enhancement of pro-inflammatory cytokines *ifnγ* and *tnf-α* by PAMPs and PMA (Figure 5.4), which are quick responders to infection (Bjork et al., 2014). Comparably, the magnitude of pro-inflammatory cytokine expression was considerably higher with PMA than in both PAMPs probably due to PMA's strong inflammatory action in PBMCs. This is supported by the rapid PMA induced ROS production, making this agent extremely potent. Previous findings show that PMA binds and damages cellular surfaces during membrane interaction (Weinstein et al., 1979; Weissmann et al., 1968), while LPS binds cell surfaces via specific Toll-like receptors (TLRs) including TLR4, TLR1 and TLR2, which are not universal to all fish [reviewed in Zhang et al. (2014)]. Several studies have demonstrated that teleost fish TLRs can immunologically detect LPS (Han et al., 2018; Palti et al., 2010; Wu et al., 2018), and bacterial PAMPs (Gong et al., 2017; Lai et al., 2016; Wu et al., 2018; Zhang et al., 2014; Zhang et al., 2017). As strong upregulation of pro-inflammatory cytokines were observed with the PAMPs and PMA in this study, the associated TLRs could probably be involved in the myeloid differentiation primary response gene 88 (MyD88) and the Toll-interleukin-1 receptor (TIR) domain containing adaptor inducing interferon-β (TRIF) signalling pathways (Basu et al., 2013). The MyD88 pathway activates nuclear factor kappa (NF-κB) to produce pro-inflammatory genes observed in this study. The TIR domain pathway activates IFN-regulatory factor 3 (IRF3) and the expression of IFN-β and IFN-inducible genes [reviewed in (Basu et al., 2013), also detected in the current study. Since TLR4 orthologues were reported for salmonids during an in-depth blast survey using catfish and zebrafish TLR4 as baits (Quiniou et al., 2013), the discovery helps to elucidate the salmonid immune system recognition mechanism of LPS, which has been previously unclear (Palti et al., 2010).

Also, *ifnγ* and *tnf-α* expression were concurrently upregulated following stimulation with bacterial LPS and PMA, and remained relatively high 24 h post stimulation. This is suggestive of a synergistic action of the two cytokines, as demonstrated by their concurrent expression with *il-10*. Concurrently, the high expression of anti-inflammatory *il-10* observed in challenged O. *tshawytscha* primary PBMCs with PAMPs and PMA 24 h post incubation confirms the suppressive effect of this cytokine against cellular damage by the highly expressed anti-inflammatory cytokines previously reported in *S. salar* and *O. mykiss* HK and SP leucocytes (Sangrador-Vegas et al., 2000; Sugamata et al., 2009). The extremely low levels of pro-inflammatory *il-6* and *il-1β*, 24 h post LPS and PMA stimulation confirms the anti-inflammatory effect of *il-10* and suggestive of possible earlier upregulation of *il-6* and *il-1β*, as previously reported in S. *salar* HK macrophages (Ulvestad et al., 2018). This is in line with previous studies

with mrigal (*Cirrhinus mrigala*) and Indian major carp (*Catla catla*), where LPS induced *il-10* expression to suppress *il-1β* through TLR4 signalling and activation of NF-κB via the MyD88 pathway (Basu et al., 2013; Swain et al., 2012).

Administration of LPS *in vitro* has been reported to induce expression of immune related genes in *O. mykiss* HK leucocytes (Goetz et al., 2004; Zou et al., 1999). Also, LPS *in vitro* induced increased trout (t*nf-α*) expression in *S. trutta* HK leucocytes (MacKenzie et al., 2003), *tnf-α* in *O. mykiss* HK leucocytes (Qin et al., 2001; Zou et al., 2002), and *il-1β* and *tnf-α* in *S. salar* HK leucocytes (Holen et al., 2011). Thus, we postulate that LPS induced immune stimulation in O. *tshawytscha* could be regulated via TLR4, or synergistic TLR1 and TLR2 most probably via the MyD88 and TRIF pathways. However, further studies will have to be conducted to confirm the exact bacterial LPS TLRs in this species. These findings improve our understanding of LPS signalling in O. *tshawytscha* immune cells and provide the foundation for further investigations on immunomodulatory effects that may be induced due to environmental influences, farm-related stresses, pathogenic exposures and dietary manipulations. The research also contribute towards development of sustainable strategies for improving farmed fish health.

5.5.1 Conclusions and recommendations

The response of *O. tshawytscha* primary PBMCs to two *E. coli* LPS products were investigated via cellular functional ROS production and phagocytosis, plus targeted immune gene expression. Findings demonstrate significant induction of cellular ROS production and phagocytic ability, in O. *tshawytscha* primary PBMCs *in vitro*. The results also suggest that LPS significantly ($p < 0.05$) induced upregulation of pro-inflammatory *ifnγ*, *tnf-α* and anti-inflammatory *il-10 in vitro*. We suggest that immune related cytokine release could be regulated via candidate TLR4, or synergistic TLR1 and TLR2 ligands most probably via the MyD88 and TRIF pathways. This is the first report to illustrate *in vitro* LPS effects in farmed O. *tshawytscha* PBMCs. The findings provide valuable information for studying physiological and immune related effects in farmed fish stocks. Further long term *in vitro* and *in vivo* studies are recommended to model bacterial pathogenic effects.

6 Chapter 6. Case Study 3: Metabolic and Immune Responses of Chinook salmon (*Oncorhynchus tshawytscha*) Smolts to a Short-term Poly (I: C) Challenge.

This chapter has been published:

Lulijwa, R., Alfaro, A. C., Merien, F., Burdass, M., Meyer, J., Venter, L., & Young, T. (2020). Metabolic and immune responses of Chinook salmon (*Oncorhynchus tshawytscha*) smolts to a short-term poly (I: C) challenge. *Journal of Fish Biology*, *96*(3), 731-746. https://onlinelibrary.wiley.com/doi/full/10.1111/jfb.14266

6.1 Prelude to case study 3

Currently, the New Zealand Chinook salmon (*Oncorhynchus tshawytscha*) industry remains free from major viral pathogens that have characterised salmon production in the northern and some southern hemisphere countries. However, a viral infection remains a priority threat to the country's niche primary industry, driven by climate change, confinement stress, and other anthropogenic variables. Like bacterial challenges, viral-related immuno-metabolic literature in New Zealand farmed *O. tshawytscha* remains scanty. This chapter used an integrated approach using PB haematological microscopic characterisation, PBMC isolation in chapter 4, molecular techniques employed in chapter 5, and Gas chromatography-mass spectrometry (GC-MS) based metabolomics. The chapter applied an integrated immuno-metabolic approach to unravel *O. tshawytscha* response mechanisms to *in vivo* poly (I:C) 24 h post-intraperitoneal (i.p) administration at haematological, metabolic, and molecular levels. Poly (I:C) is a synthetic double-stranded ribonucleic acid (dsRNA) analogue. It is commonly used in immunological studies to mimic a viral infection to induce antiviral immune responses *in vivo* and *in vitro*. Results from this experiment were discussed in the manuscript and may potentially aid the development of amelioration strategies against potential future viral pathogens.

6.2 Introduction

Aquaculture is considered to be one of the fastest growing food production sectors worldwide and contributed 11.79% of global animal-sourced food in 2015 (Edwards et al., 2019; Joffre & Verdegem, 2019). This has been partly fuelled by the decline in global fisheries and an increasing demand for seafood. To meet demands, aquaculture farms aim to produce high quantities of healthy and fast-growing fish, under optimal husbandry and management practices in limited water volumes (Martinez et al., 2016). However, the practice of intensive aquaculture leads to water quality deterioration, which provides a conducive environment for disease outbreaks by viruses, bacteria, and parasites. Health challenges from such pathogens have been associated with heavy industry losses [reviewed in Fazio (2019)]. Farmed chinook salmon (*Oncorhynchus tshawytscha*) is New Zealand's main farmed finfish species, making the country a major global supplier. Although salmon farming in New Zealand is currently considered 'virus free', continued industry growth combined with climate change challenges necessitate that species-specific immunological information is acquired to develop mitigation strategies against future pathogen threats. Consequently, studying antiviral mechanisms in this species provides critical information that contributes to the sustainable development of this important sector.

Various strategies aimed at boosting host immunity have been used to counter disease-related economic losses in aquaculture (Magnadottir, 2010). In some instances, immunostimulants such as nucleotides, β-glucans (Magnadottir, 2010; Uribe et al., 2011), lipopolysaccharide (LPS) (Anderson, 1992), and polyinosinic: polycytidylic acid (poly [I:C]) (Jensen et al., 2002a; Zhou et al., 2014) are administered to stimulate the immune system. Poly (I:C) is a synthetic double-stranded ribonucleic acid (dsRNA) analogue, which mimics a viral infection to induce antiviral immune responses. When administered, the innate defence system acts as if challenged with a viral pathogen and initiates molecular mechanisms designed to fight the aetiological agent. Poly (I:C) effects its antiviral properties through a broadly expressed and well-conserved toll-like receptor 3 (*tlr3*) (Kitao et al., 2009; Tanekhy, 2016; Zhou et al., 2014), retinoic acid-inducible gene I (*rig-I*) and melanoma differentiation-associated protein 5 (Mda5), both termed RIG-I-like receptors (RLRs), and class A scavenger receptors (SR-As) (Brisse & Ly, 2019; Semple et al., 2018; Zhou et al., 2014).

After poly (I:C) stimulation, RLRs transmit signals through the interferon-β (*ifn-β*) promotor stimulator 1 and *tlr3* uses the toll-interleukin 1 receptor domain-containing adapter-inducing *ifn-β* (Zhou et al., 2014). In mammals, dsRNA binding to both adaptors signal downstream development of an antiviral state via activation of interferon (IFN) regulatory transcription factors (IRF3 and IRF7), production of type I interferons (I IFN), pro-inflammatory cytokines and IFN stimulated genes (ISGs) (Monjo et al., 2017; Zhou et al., 2014). Majority of ISGs enhance host cellular antiviral defence (Zhang & Gui, 2012). The antiviral ISGs are mostly stimulated by type I [interferon alpha (*ifn-α*) and interferon beta (*ifn-β*)], type III interferon lambda (*ifnλ*) and partially by type II interferon gamma (*ifnγ*) which collaboratively target viral replication

(Schoggins & Rice, 2011). The response also generates pro-inflammatory reactions via activation of pro-inflammatory and pro-survival transcription factors, nuclear factor kappa (NF-κB), activator protein 1 and induction of apoptosis in a caspase-8-dependent fashion via receptor-interacting protein 1 (Zhou et al., 2014). In teleosts, mammalian *tlr3* orthologues have been profiled in fugu (*Takifugu rubripes*) (Oshiumi et al., 2003), zebrafish (*Danio rerio*) (Phelan et al., 2005), channel catfish (*Ictalurus punctatus*) (Baoprasertkul et al., 2006), Japanese flounder (*Paralichthys olivaceus*) (Hwang et al., 2012), rainbow trout (*Oncorhynchus mykiss*) (Rebl et al., 2007; Rodriguez et al., 2005), and Atlantic salmon (*Salmo salar*) (Arnemo et al., 2014; Lee et al., 2013; Svingerud et al., 2012).

Poly (I:C) administration in teleosts induced type 1IFNs and subsequent production of antiviral Mx protein in *S. salar* and *O. mykiss in vivo* (Das et al., 2009; Lockhart et al., 2004a; Purcell et al., 2004), and *S. salar in vitro* (Arnemo et al., 2014). Teleostian immune cells cloned with Mx protein inhibited infectious pancreatic necrosis virus (IPNV) replication in *S. salar* (Larsen et al., 2004) and rhabdoviruses replication in *P. olivaceus in vitro* (Caipang et al., 2003). Administered *in vivo*, poly (I: C) induced antiviral Mx protein against infectious salmon anaemia virus (ISAV) in *S. salar* (Jensen et al., 2002a), infectious haematopoietic necrosis virus (IHNV) in *O. mykiss* (Kim et al., 2009), and channel catfish virus infection in *I. punctatus* (Plant et al., 2005). Poly (I:C) *in vivo* also induced antiviral protection against red-spotted grouper nervous necrosis virus (RGNNV) in sevenband grouper (*Epinephelus septemfasciatus*) (Nishizawa et al., 2009), viral haemorrhagic septicaemia (VHS) and megalocytivirus in *P. olivaceus* (Takami et al., 2010; Zhou et al., 2014). Stabilised in chitosan, poly (I:C) induced antiviral state against VHS in *D. rerio* (Kavaliauskis et al., 2015). Conversely, poly (I:C) induced protection was temporary in IPNV carrier *S. salar* adults (Lockhart et al., 2004b). Using viral vaccines, antiviral activity was activated in IPNV injected *O. mykiss* (Kim et al., 2009), in *S. salar* post-smolts, but not carrier adults (Lockhart et al., 2004b), and in IHNV injection of *O. mykiss* (Purcell et al., 2004).

At the molecular level, poly (I:C) acts on *tlr3* to induce the expression of cytokine producing genes in common carp (*Cyprinus carpio*) (Kitao et al., 2009; Tanekhy, 2016), *ifnγ* in goldfish (*Carassius auratus*) (Grayfer & Belosevic, 2009), type I IFNs and *ifnγ* in *S. salar* (in synergy with CpG oligodeoxynucleotides) (Strandskog et al., 2011; Thim et al., 2012), interferon regulatory factors (IRF 1-5, 9) and *tlr3* in Nile tilapia (*Oreochromis niloticus*) (Y. Shen et al., 2018), and *tlr3* and Mda5 in *P. olivaceus* (Zhou et al., 2014). Furthermore, virally infected fish (Lockhart et al., 2007; McBeath et al., 2007) and poly (I:C) treated fish expressed gene transcripts for type 1 IFNs and Mx protein expression (Arnemo et al., 2014; Das et al., 2009; Lockhart et al., 2004a; McBeath et al., 2007). At cellular level, repeated poly (I:C) stimulation enhanced reactive oxygen species (ROS) production in *O. mykiss* HK macrophages (Boltana et al., 2009). Furthermore, *ifnγ* induced nitric oxide (NO) production, to facilitate intracellular pathogen destruction (Stafford et al., 1999), and *ifnγ* enhanced respiratory burst, phagocytosis and intracellular NO production *in vitro* (Grayfer & Belosevic, 2009) and *in vivo* (Zhou et al., 2014).

In addition, metabolomics is one of the omic techniques which has been identified as vital in the near future improvement of aquaculture (Alfaro & Young, 2018), and can be employed to for instance assess farmed fish immunology (Browdy et al., 2012). Metabolomics, involves the study of chemical processes mediated by metabolites, and with the help of analytical techniques, statistical differences can be observed among samples and metabolic signatures identified as biomarkers for specific challenges (Alfaro & Young, 2018). Among salmonids, past metabolomic studies have focussed on nutrition (Berntssen et al., 2016; Jasour et al., 2017; Roques et al., 2018a; Wagner et al., 2014) and providing metabolic effects of contaminants (Olsvik et al., 2015; Olsvik et al., 2017a; Olsvik & Søfteland, 2018; Softeland et al., 2016; Softeland et al., 2014), among others. Of recent, metabolomics has been used to demonstrate effects of pre-sampling handling and sedation practices on *O. tshawytscha* biochemistry (Young et al., 2019).

Now, limited information exists on *O. tshawytscha* immunology following *in vivo* poly (I:C) administration. The only available studies are *in vitro*, and demonstrated protective benefits against viral infections in poly (I:C) transfected *O. tshawytscha* embryonic cells CHSE-214 (Jensen et al., 2002b; Monjo et al., 2017) and triploid *O. tshawytscha* spleen epithelia derived cells CHSS (Semple et al., 2018). Nonetheless, untransfected *O. tshawytscha* embryonic cells CHSE-214 lack cell surface receptors for dsRNA, while CHSS cells do (Semple et al., 2018). Furthermore, the effects of poly (I:C) on haematological parameters, peripheral blood mononuclear cells (PBMC) functional parameters of phagocytosis, ROS and NO production, and tissue-specific biochemical profiles remain entirely unknown in *O. tshawytscha*. Thus, the present study aims to provide insights into the mechanisms by which *O. tshawytscha* responds to *in vivo* poly (I:C) 24 h post exposure. A multidisciplinary approach was used to measure fish responses, including: classical haematology; PBMC latex bead phagocytosis; flow cytometry (PBMC viability, ROS and NO production); gas chromatography-mass spectrometry (GC-MS) metabolomics (serum biochemical profiles); and qRT-PCR targeted immune cytokine expression in fish red blood cells (RBCs), spleen (SP) and head kidney (HK) samples.

6.3 Materials and methods

6.3.1 Fish husbandry

Two-year-old *O. tshawytscha* post-smolts (weight = 363.29±66.21 g; total length = 30.11±1.68 cm) were obtained from the Nelson Marlborough Institute of Technology (NMIT) aquaculture facility (Glenduan, Nelson, New Zealand). Fish had been cultured in a saltwater recirculating system (temp = 16.02±0.94°C; DO = 7.34±0.32 mg L^{-1}; pH = 8.18±0.17; NH_4^+ = 0.17±0.12 mg L^{-1}; NO_2^- ≤ 0.01 mg L^{-1}), being fed to satiation daily with Orient 3.0 mm commercial diet (Skretting) (44% crude protein; 28% lipid). Ethical approval was obtained from the NMIT Animal Ethics Committee (NMIT-AEC-2018-NMIT-03).

6.3.2 Poly (I:C) challenge and sampling

Fish were captured by scoop net and individually anaesthetised with AQUI-S® (25 mg L^{-1} of culture water for 2-3 min). Fish total length (cm) and wet weight (g) were recorded quickly

and individual weights used to calculate the required poly (I:C) injection volumes. Five control fish were intraperitoneally (i.p.) injected with 5 µL g^{-1} fish of sterile filtered (40 µm) phosphate-buffered saline (PBS), and five fish i.p. injected with 10 µg g^{-1} fish body weight poly (I:C) (P1530-100MG, Sigma-Aldrich, New Zealand) dissolved in PBS following previous procedures in *S. salar* (Jensen et al., 2002a; Robertsen et al., 2003).

Twenty-four hours post-injection, fish were individually taken by scoop net from the culture tank and immediately euthanised by Ikijime without sedation to minimise pre-sampling influences on plasma biochemistry (Young et al., 2019). Approximately 4 mL of blood were quickly taken via caudal vein puncture using non-heparinised 3.0 mL syringes attached to 20-gauge Terumo needles (SH177, Amtech Medical Limited, New Zealand). For metabolomics, 1 mL of unheparinised blood was quickly taken and 600 µL of serum were obtained by centrifuging at 16,250 g; 8 min. Serum and the resultant blood pellet (400 µL) were transferred to 2 mL cryovials (BS20NA-PS, BioStor™, Interlab, New Zealand), snap frozen in liquid nitrogen and stored at –80°C. Unheparinised blood was also used for differential cell counts. The remaining 3 mL of blood were immediately preserved in BD lithium heparin microtainer (BD 365966, Phoenix Pharm Distributors, New Zealand) and used for haematocrit value and processing PBMC. Fish were quickly dissected and ~ 500 mg of HK and SP transferred separately to 2 mL cryovials, snap frozen in liquid nitrogen, and stored at –80°C until serum metabolite profiling and targeted immune gene expression analysis (RBC, HK, and SP).

6.3.3 Peripheral blood differential cell counts

For differential cell counts, 10 µL of fresh unheparinised blood was used to prepare slide smears. Slides were left to air dry overnight, fixed with methanol, and stained with Giemsa (51811826, Sigma-Aldrich, New Zealand). Peripheral blood stained slides were observed under a microscope at 400x magnification for white and red blood cell counts. A total of 200 cells were randomly counted in duplicate and the cellular composition recorded for erythrocytes, thrombocytes, monocytes, lymphocytes, and neutrophils. For differential leucocyte counts, 200 white blood cells were counted in triplicate and composition recorded for thrombocytes, lymphocytes, monocytes, and neutrophils.

6.3.4 Haematocrit value assessment

Lithium heparin preserved blood was drawn into unheparinised capillary tubes and centrifuged in a Haematokrit 210 Hettich Zentrifugen at 6081 g for 10 min (Kortet et al., 2003) to obtain the packed cell volume.

6.3.5 Isolation of fish PBMCs

Fish PBMCs were isolated from 284 µL of blood as previously described (Lulijwa et al., 2019a) using Histopaque sterile filtered density gradient medium (1.077 g mL^{-1}) (10771-6X100ML, Sigma-Aldrich, New Zealand). The 284 µL of heparinised blood were quickly diluted 1:1 with sterile filtered (40 µm) PBS (pH 7.4) in triplicate. PBMCs were obtained from blood samples by centrifuging at 971 g for 20 min over a layer of 682 µL Histopaque. Cells at the

interface were aspirated with a pipette, pooled together, washed twice into 1 mL of sterile filtered PBS at 674 g for 7 min. The resultant cell pellet was re-suspended to a final cell concentration of 1×10^6 cells mL^{-1} in 1.5 mL PBS supplemented with 2% fetal bovine serum (FBS) (MG-FBS0820-500ML, Mediray, New Zealand). This working cell suspension was kept chilled at 4°C for further analysis.

6.3.6 PBMC cell count and viability assessment

A sub-sample of 20 µL of the working PBMC cell suspension at 1×10^6 mL^{-1} was taken and transferred into microcentrifuge tubes. Exactly 380 µL of the Muse® Count and Viability Assay Kit (MPMCH100102, Abacus Dx, New Zealand) reagents were added, and vortexed briefly. The mixture was incubated for 5 min at room temperature (18°C). The viability profile of the isolated mononuclear cells was determined with the Muse® Count & Viability Assay Kit on the Muse® Cell Analyzer in duplicate readings as previously described (Lulijwa et al., 2019a). For the positive control, dead cells were prepared via thermal treatment at 100°C for 5 min and viability determined with the Muse® Count & Viability Assay protocols to set the gates for live and dead cells prior to sample analysis.

6.3.7 ROS production assay

Intracellular PBMC ROS production was measured using the Muse® Oxidative Stress Assay Kit (MPMCH100111, Abacus Dx, New Zealand) protocol, with the Muse® Cell Analyzer. Approximately 10 µL of PBMC suspension was incubated with 190 µL of Muse® Oxidative Stress working solution following the manufacturer protocols. The sample was mixed thoroughly and run on the Muse® Cell Analyzer to determine basal ROS production in control and treatment fish. A positive control (Figure 6.2b) sample was prepared to validate correct Muse® flow cytometer gate and threshold settings: ROS production was primed by concentrating 100 µL of PBMCs (1×10^6 mL^{-1}) and re-suspending the pellet in 100 µL of LPS from *E. coli* serotype O 111: B4 (10 µg mL^{-1}) in L-15 Medium (L1518-500ML, Leibovitz, Sigma-Aldrich, New Zealand) supplemented with 10% FBS and incubated at room temperature (18°C) for 20 min, and ROS production analysed according to previous work (Lulijwa et al., 2019b).

6.3.8 NO production assay

Isolated PBMC nitric oxide (NO) production was determined following a standard Muse® intracellular NO production protocol (MPMCH100112, Abacus Dx, New Zealand) and run on the Muse® Cell Analyzer. For the positive control (Figure 6.2d), NO production was primed by concentrating 100 µL of PBMCs (1×10^6 cells mL^{-1}) and re-suspending the pellet in 100 µL of 200 ng mL^{-1} of PMA in L-15 Medium (Leibovitz) (Jørgensen & Robertsen, 1995; Solem et al., 1995) supplemented with 10% FBS and incubated at room temperature for 20 min.

6.3.9 Phagocytic activity determination

This was accomplished following a modified protocol by Sakai et al. (1995) and previous work (Lulijwa et al., 2019b). Ten microliters of PBMC suspension (1×10^6 mL^{-1}) were pipetted into sterile glass microscope slides and incubated at room temperature for 1 h. Non-adherent cells

were washed off using PBS with 2% FBS. Then, 10 µL of Fluorescent latex beads (1×10^6 mL^{-1}) amine-modified polystyrene suspension (L9654-1ML, 1.0–1.3 µm, Sigma-Aldrich, New Zealand) were added and incubated for 2 h at room temperature. Non phagocytosed latex beads were washed off using PBS with 2% FBS. The slides were fixed with absolute methanol and stained with Giemsa. A total of 100 cells were counted at 400x magnification and percentage phagocytic activity (PA) determined according to the following equation:

$$Phagocytosis = (Number\ of\ phagocytic\ PBMCs)/(Number\ of\ total\ PBMCs)\ x\ 100$$

The phagocytic index was determined by the following equation:

$$Phagocytic\ Index = (Number\ of\ latex\ beads\ per\ cell)/(Number\ of\ phagocytic\ cells)$$

6.3.10 Metabolite extractions

Fish serum metabolites were extracted following standard protocols (Young et al., 2019). Briefly, ice thawed serum (100 µL) samples were co-extracted with 20 µL L-alanine-2,3,3,3-d4 (d_4 alanine 10 mM for methyl chloroformate [MCF] derivatisation) and ribitol (10 mM for trimethylsilyl [TMS] derivatisation) as internal standards in a cold methanol: water (MeOH:H_2O) solution. Keeping samples on dry ice, metabolites within serum, and pooled quality control (QC) samples were extracted first with 500 µL of 50% cold methanol: water (MeOH:H_2O) solution, vortexed vigorously in 1.5 mL microcentrifuge tubes on ice. The resulting slurry was centrifuged in a –9°C pre-cooled centrifuge at 3500 g for 5 min. Supernatants (0.4–0.5 mL) were transferred to 2 mL microcentrifuge tubes and kept on dry ice. The pellets were taken through a second extraction process with 500 µL of 80% cold MeOH:H_2O, vortexed, centrifuged as above, and the supernatants transferred to the respective 2 mL microcentrifuge tubes on dry ice. Sample extracts were freeze dried in a speedvac for 4 h at 0.8 HPa, ramp 3-5, prior to alkylation (MCF) and silylation (TMS) derivatisation.

6.3.10.1 Methyl chloroformate derivatisation

Extracted metabolites were derivatised following standard protocols (Villas-Boas et al., 2011). Sample extracts, blanks and a standard amino acid (AA) mix were dissolved in 400 µL of 1M sodium hydroxide (NaOH). The mixture was vortexed until resuspended. Methanol (334 µL) and pyridine (68 µL) were added to a series of 5 mL salinised tubes, then samples added. Two 40 µL MCF aliquots were sequentially added into the reagent mixture at 30 s intervals with vortexing in between.

A positive displacement pipette was used to saturate chloroform in the head space, 400 µL of chloroform were pipetted into the mixture and vortexed for 10 sec. Then 800 µL of sodium bicarbonate ($NaHCO_3$) (50 mM) were added and the mixture vortexed for another 10 secs. The mixture was centrifuged at 2500 g for 5 min. The upper aqueous layer was removed and discarded with the help of a glass Pasteur pipette and bung. Anhydrous sodium sulfate (Na_2SO_4) (100 mg) was added to remove residual moisture. Derivatised extracts were transferred to GC vials for GC-MS analysis.

6.3.10.2 Trimethylsilyl derivatisation

Extracted metabolites were TMS derivatised following standard protocols (Villas-Boas et al., 2011). Extracted QC, serum samples, blank and standard AA mix were kept in a desiccator overnight prior to TMS derivatisation. To each sample vial, 80 µL of methoxyamine hydrochloride in pyridine (2 g 100 mL^{-1}) were added to re-suspend extracts and vortexed for 60 sec. The resulting suspension was pipetted into GC vial inserts, and incubated at 28°C for 90 min. Subsequently, 80 µL of MSTFA (N-methyl -N-(trimethylsilyltrifluoroacetamide) were added and the mixture incubated for a further 30 min at 37°C. After the second incubation, samples were analysed by GC-MS.

6.3.11 GC-MS analysis

Serum metabolite extracts were analysed using a GC7890-MS5970 system (Agilent Technologies, California, United States) equipped with a ZB-1701 GC capillary column (Phenomenex) and a quadrupole analyser with an electron impact (EI) ion source operated at 70 eV. Metabolite spectra data were deconvoluted using Automated Mass Spectral Deconvolution and Identification System (AMDIS) v2.66 software integrated with the MassOmics R-based script to interrogate an in-house library of derivatised compounds (Aggio et al., 2011). The intensities of the internal standards were used to normalise data and compensate for technical variation prior to statistical analysis.

6.3.12 Fish head kidney and spleen mRNA immune gene expression analysis

For total RNA isolation, 10 mg of RNAlater-ICE (AM7030, Life Technologies, New Zealand) stabilised HK, SP samples, and RBC pellet were processed in 600 µL and 900 µL of tissue lysis buffer (3604721001, MagNA Pure LC RNA Isolation Tissue Lysis Buffer, Roche Diagnostics NZ Ltd) respectively. The lysates were homogenised in a microcentrifuge tube for several times and vortexed. Lysates were incubated at room temperature for 30 min and centrifuged for 2 min at 21130 g in a 5424R Eppendorf centrifuge. Total RNA was extracted from 200 µL of lysate supernatant using the Roche MagNA Pure LC2 instrument with the MagNA Pure LC RNA isolation Kit – High Performance (03542394001, Roche Life Sciences, New Zealand).

After total RNA purification, 100 µL of eluted RNA were collected. Cytokine transcript expression was quantified by one step reverse-transcription polymerase chain reaction (RT-PCR) on the LightCycler 480 Instrument II (Roche Diagnostics, New Zealand) using the LightCycler® EvoScript RNA SYBR® Green I Master Kit (07800134001, Roche Life Sciences, New Zealand). Reactions were performed using primers (Integrated DNA Technologies, Inc., Singapore) at a concentration of 0.9 µmol l^{-1} and RNA in a final volume of 20 µL. The reactions were performed under the following thermocycling conditions: 60°C for 15 min (reverse transcription/cDNA synthesis), 95°C for 10 min (transcriptase inactivation and initial denaturation step) and 40 cycles of amplification (95°C for 15 s for denaturation and 60°C for 1 min for annealing and extension).

Upon completion, melting curve analyses were performed to reveal and exclude nonspecific amplification or primer-dimer issues (all melting analyses in this study presented single reproducible peaks for each target gene suggesting amplification of a single product). The housekeeping gene used for mRNA expression analysis was β-actin (*actb*). All primer sequences (Table 6.1) were acquired from previously published sources (Bjork et al., 2014; Monjo et al., 2017). Cytokine transcript expression levels were calculated using the comparative Ct (△△Ct) equation (Schmittgen & Livak, 2008). Cytokine transcript expression was calculated as $2^{-\triangle\triangle Ct}$ and expressed as a fold-change. The mRNA transcripts for all target genes were normalised to the reference gene (*actb*) within the same sample, condition and time point, and presented as fold change with respect to the control group.

Table 6.1. Primers used to amplify *Oncorhynchus tshawytscha* genes.

Genes	Sequence (5'–3')	Product size (bp)	Gene function	Reference, gene bank accession number
Actb	(F)- GTCACCAACTGGGACGACAT (R)- GTACATGGCAGGGGTGTTGA	175	ATP binding, determines cell shape and controls motility	*O. tshawytscha* FJ890357.1 (Monjo et al., 2017)
Type I interferon 1 (*ifn1)*	(F)- AAACTGTTTGATGGGAATATGAAA (R)- CGTTTCAGTCTCCTCTCAGGTT	140	Activate intracellular antimicrobial programmes and elicit development of innate and adaptive immunity	*O. mykiss* AJ580911 (Monjo et al., 2017)
Mx1 protein (*mx1*)	(F)- CGGAGTTCGTCTCAACGTCT (R)- CCCTTCCACGGTACGTCTTC	140	Interferon-induced dynamin-like GTPase with antiviral activity against a wide range of RNA viruses and some DNA viruses	*O. mykiss* U30253.1 (Monjo et al., 2017)
Ifnγ	(F)- CAACATAGACAAACTGAAAGTCCA (R)- ACATCCAGAACCACACTCATCA	129	Triggers cellular response to viral and microbial infections	*O. tshawytscha* GT897806 (Bjork et al., 2014)
Interleukin-1β (*il-1β*)	(F)- ACCGAGTTCAAGGACAAGGA (R)- CATTCATCAGGACCCAGCAC	181	Potent pro-inflammatory cytokine	*O. tshawytscha* DQ778946 (Bjork et al., 2014)
Interleukin-6 (*il-6*)	(F)- CAGTTTGTGGAGGAGTTTCAGA (R)- TGTTGTAGTTTGAGGTGGAGCA	118	Potent pleiotropic cytokine	*O. mykiss* NM_001124657 (Bjork et al., 2014)
Tumor necrosis factor-alpha (*tnf-α*)	(F)- ACCAAGAGCCAAGAGTTTGAAC (R)- CCACACAGCCTCCATAGCCA	154	Multifunctional pro-inflammatory cytokine	*O. tshawytscha* DQ778945 (Bjork et al., 2014)
Interleukin-10 (*il-10*)	(F)- CTACGAGGCTAATGACGAGC (R)- GATGCTGTCCATAGCGTGAC	97	Plays anti-inflammatory roles, helping to limit excessive tissue damage caused by inflammation	*O. mykiss* AB118099 (Bjork et al., 2014)

Note: F: forward primer; R: reverse primer; bp: base pair.

6.3.13 Statistical analyses

Data from peripheral blood cellular counts, differential leucocyte counts and fish PBMC cellular functional characterisation were processed to assess the effect of *in vivo* poly (I:C) i.p. injection on fish immune system using an independent two sample student t-test with Minitab 17 statistical software at $p = 0.05$. Cytokine transcript expression data were analysed via independent two sample t-*test* at $p = 0.05$.

Metabolomics data were auto scaled and processed using MetaboAnalyst 4.0, a web-based tool for metabolomics data analysis (Chong et al., 2018) (MetaboAnalyst, https://www.metaboanalyst.ca/). Significance analysis of microarray (and metabolites) (SAM) was used to detect differences between control and treated fish at a Delta value of 0.1 to set the False Discovery Rate cut-off at <10%. Univariate fold change (FC) analysis was used to acquire

absolute change in metabolites between the treated and control group at a threshold of 1.0, and data presented as $\log_2$ (FC). Heat map with combined hierarchical cluster analysis of metabolites was used to visualise metabolites identified as being significantly altered by SAM. For functional assessment, pathway enrichment and topology analysis (PETA) was used to extract biological information within relevant networks of metabolic pathways contained within the Kyoto Encyclopaedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/) database, and identify pathways most likely affected by poly (I:C) treatment. Excluding unidentified or without matching KEGG ID metabolic features, PETA was performed using a global test and a relative betweenness centrality measure, and the *D. rerio* model fish pathway library. Criteria for considering pathways as being affected by the treatment required identification of two or more metabolites within the pathway set, that pathways had a global test p-value < 0.05 with combined FDR ≤ 0.2 and a pathway impact score of ≥ 0.05.

6.4 Results

6.4.1 Peripheral blood haematological parameters

Short term poly (I:C) administration did not significantly ($p > 0.05$) alter peripheral blood cellular parameters (Figure 6.1a), although slight increases were observed in haematocrit value in the treated fish group compared to the control (Table 6.2).

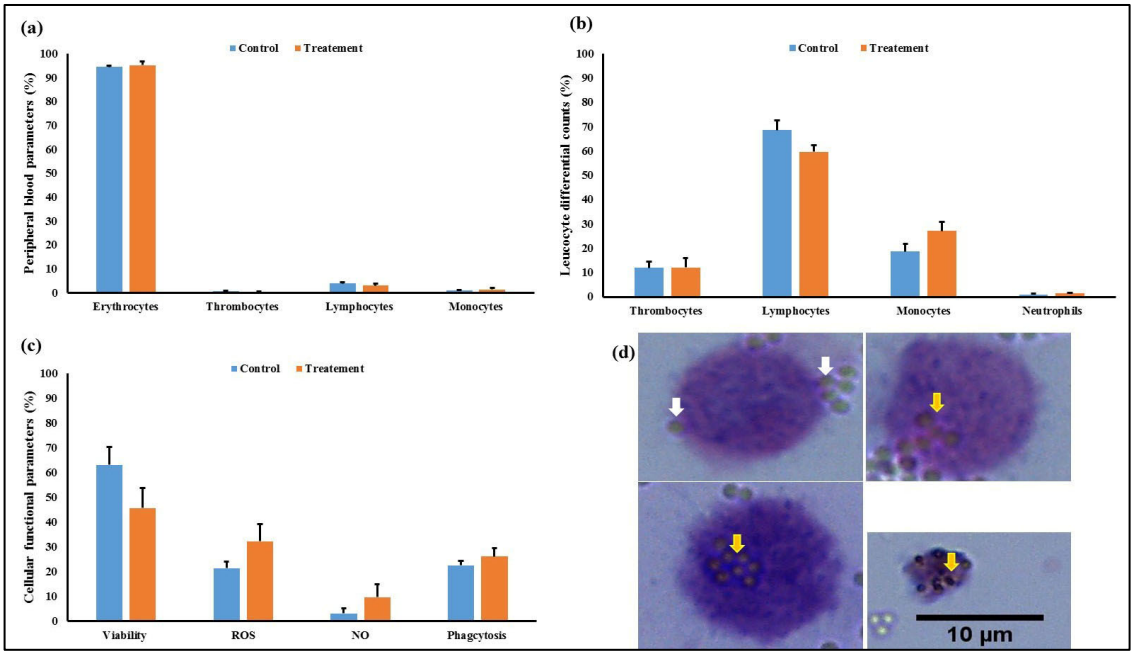


Figure 6.1 (a) *Oncorhynchus tshawytscha* peripheral blood cell counts, (b) leucocyte differential cell counts, (c) peripheral blood mononuclear cell (PBMC) functional characteristics, and (d) PBMC latex bead phagocytosis, following *in vivo* intraperitoneal injection with 10 µg g^{-1} fish of poly (I:C). Peripheral blood cell count percentage data are mean±SEM of duplicate count of 200 cells, and leucocyte differential percentage data are mean±SEM of triplicate counts of 200 cells on Giemsa-stained slides. Data are obtained from five control and five treatment fish. Bars without an asterisk are not significantly different, two independent sample t-test at $p = 0.05$ and 8 degrees of freedom. Yellow and white arrows indicate phagocytosed and opsonised beads, respectively.

6.4.2 Differential leucocyte counts

Poly (I:C) administration did not significantly ($p > 0.05$) affect fish differential leucocyte counts between the control and treated groups (Figure 6.1b).

Table 6.2. Peripheral blood haematocrit value and peripheral blood mononuclear cellular phagocytic index.

Parameter	Haematocrit value (%)	Phagocytic Index
Control	36.70±2.50	2.44±0.15
Treatment	39.70±1.70	2.56±0.19
t-value	-0.98	-0.51
p-value	0.357	0.622

Note: Data are mean± SEM of duplicate readings from five control and five treatment fish for haematocrit value and from phagocytic cells for phagocytic index. Values in a column without asterisks are not significantly different, two independent sample t-test at $p = 0.05$ and 8 degrees of freedom.

6.4.3 Fish PBMC cellular functional characterisation

With reference to the immunological effects of short term *in vivo* i.p. injection administration of poly (I:C) in *O. tshawytscha*, cellular functional assessment of isolated PBMCs showed no significant (p>0.05) differences between the control and treated fish. No significant differences were observed for cellular viability, ROS and NO production, phagocytosis (Figure 6.2c and Figure 6.2d) and phagocytic index (Table 6.2) 24 h post *in vivo* poly (I:C) administration.

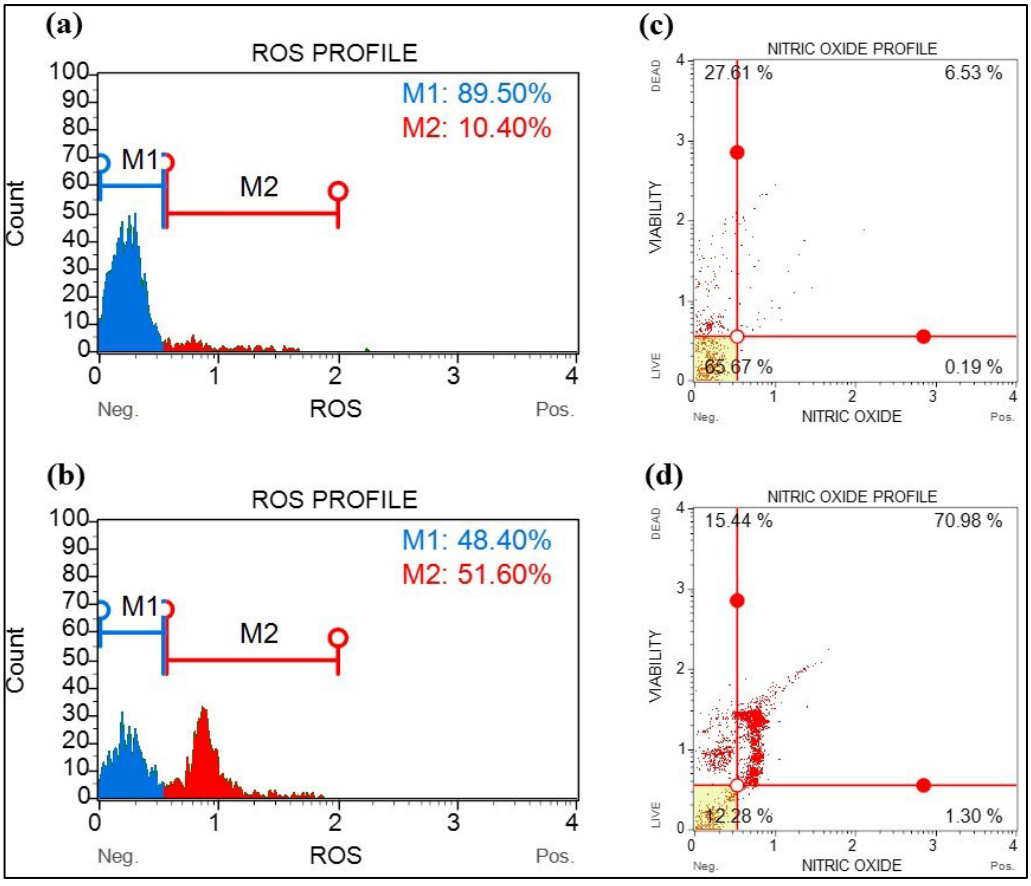


Figure 6.2 Histograms showing (a) Muse flow cytometer gate and threshold settings for ROS negative control, (b) lipopolysaccharide (LPS)-induced ROS positive peripheral blood mononuclear cells (PBMCs), (c) NO negative control and (d) PMA-induced NO positive PBMCs (d). LPS induced ROS production was primed by incubating PBMCs in 100 µL of LPS (10 µg mL^{-1}) in L-15 medium, and NO production primed by incubating PBMCs in 100 µL of 200 ng mL^{-1} of PMA in L-15 medium.

6.4.4 Fish serum univariate metabolite analysis

A total of 186 metabolites were detected in fish serum samples, respectively, following GC-MS analysis. Of these, 141 metabolites were reliably identified, while 45 metabolites did not match with the in-house mass spectral database (currently listed as unknowns).

Table 6.3. List of serum-altered metabolites identified by significance analysis of microarray (and metabolites (Delta = 0.1, FDR = 0.0757, $p < 0.05$ and $q < 0.1$) and fold change (threshold: 1) following *in vivo* intraperitoneal injection of *Oncorhynchus tshawytscha* with 10 µg g^{-1} fish of poly (I:C).

		SAM			Log_2 Fold change
Metabolites	**d.value**	**Up/Dw**	***p*-value**	***q*-value**	**(FC)**
2-Oxobutyric acid	1.621	+	0.001	0.022	1.227
D-Glucose	-1.621	-	0.001	0.022	-1.005
Arabinose	1.510	+	0.003	0.041	0.708
1-Aminocyclopropanecarboxylic acid	1.493	+	0.003	0.041	0.835
2-Hydroxyisobutyric acid	1.410	+	0.005	0.053	0.811
Beta-D-Glucopyranose	-1.371	-	0.006	0.055	-0.878
2-Aminobutyric acid	1.267	+	0.011	0.082	0.735
S-Adenosylhomocysteine	1.255	+	0.012	0.082	2.520
Malonic acid	1.202	+	0.015	0.089	0.736
Ethanolamine	1.180	+	0.017	0.089	1.370
Methyl 6-deoxy-beta-L-Galactopyranoside	-1.172	-	0.018	0.089	-0.987
1H-Indole-1-acetic acid	1.144	+	0.021	0.093	0.605
Lignoceric acid	1.136	+	0.022	0.093	0.681
Aspartic acid	1.111	+	0.025	0.094	0.755
Caproic acid	1.106	+	0.026	0.094	1.370
Unknown 119100 14796.0 10153.9	1.087	+	0.028	0.094	0.732
Cysteine	1.076	+	0.030	0.094	0.530
2-Amino-3-phenylpropanamide	-1.066	-	0.031	0.094	-0.473
Unknown 059100 8140.5 9134.4	-1.060	-	0.033	0.094	-0.583
4-Methyl-2-oxopentanoic acid	1.035	+	0.038	0.094	0.963
Methionine	1.025	+	0.040	0.094	0.662
n-Pentadecane	1.024	+	0.040	0.094	0.669
3-Methyl-2-oxopentanoic acid	0.992	+	0.048	0.094	0.745
Arachidic acid	0.990	+	0.049	0.094	0.536
Stearic acid	0.990	+	0.049	0.094	0.551

Note: Data are from five control and five treatment fish 24 h post-challenge. SAM: significance analysis of microarray (and metabolites); FC: fold change; while +: upregulated (Up) and -: downregulated (Dw) metabolites in the treated group.

SAM identified 25 metabolites as being significantly altered in fish serum 24 h post poly (I:C) treatment with a global FDR of 7.6% (Table 6.3, Table 6.5S and Table 6.6S). Heat map analysis of these metabolites show sample groupings between the treated and control fish (Figure 6.3a). Of the 25 metabolites altered, five metabolites were downregulated in the poly (I:C) treated group, and 20 metabolites were upregulated in the poly (I:C) treated group (Table 6.3 and Figure 6.3).

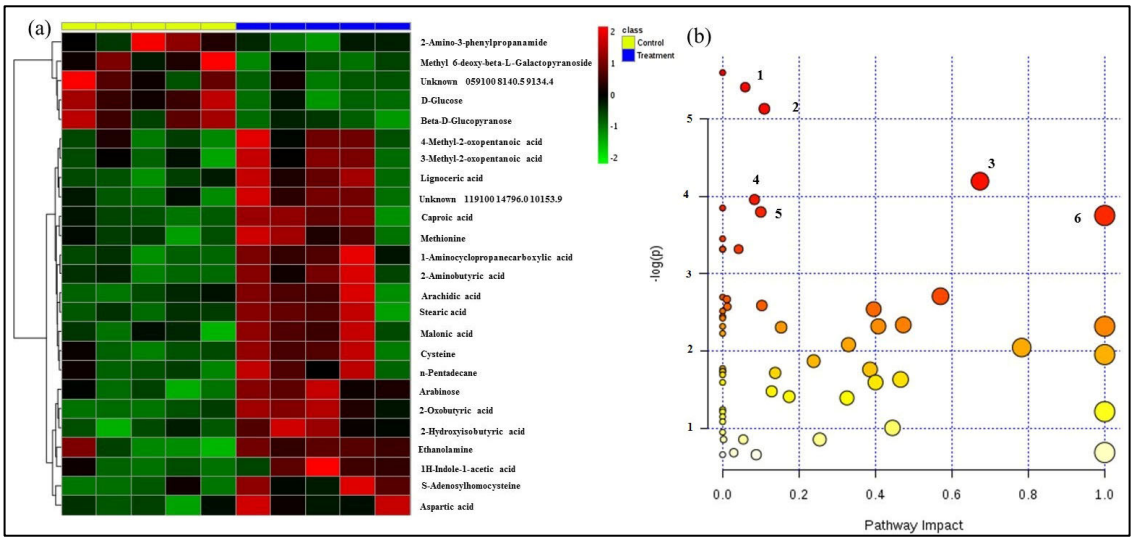


Figure 6.3 Serum metabolite analysis following *in vivo* intraperitoneal injection of *Oncorhynchus tshawytscha* with 10 µg g^{-1} fish of poly (I:C). Heat map (a) of the top 25 altered metabolites detected by significance analysis of microarray (and metabolites). Secondary bioinformatics (b) of the profiled metabolites indicating topology-based pathway analysis of detected networks in fish plasma following poly (I:C) *in vivo* challenge. The most affected pathways are indicated in colour with yellow as least relevant and red most relevant, grouped by their statistical relevance. Six pathways: 1. starch and sucrose metabolism, 2. galactose metabolism, 3. cysteine and methionine metabolism, 4. pentose and glucuronate interconversions, 5. glycolysis or gluconeogenesis, 6. valine, leucine and isoleucine biosynthesis contain some of the highest relevant metabolite coverage.

6.4.5 Functional analysis

Pathway enrichment and topology analysis identified 57 biochemical pathways relevant to the serum metabolite profiles. Six primary pathways of interest which passed the analysis selection criteria were screened as being likely affected by poly (I:C) administration (Table 6.4, Table 6.7S and Figure 6.7S).

Table 6.4. List of serum metabolic pathways altered ($p < 0.05$ and FDR ≤ 0.2) following *in vivo* intraperitoneal injection of *Oncorhychus tshawytscha* with 10 µg g^{-1} fish of poly (I:C).

Altered pathways	Total metabolites	Hits	Raw *p*	FDR	Impact
Starch and sucrose metabolism	22	4	0.004	0.112	0.059
Galactose metabolism	26	6	0.006	0.112	0.109
Cysteine and methionine metabolism	29	8	0.015	0.166	0.674
Pentose and glucuronate interconversions	15	2	0.019	0.166	0.083
Glycolysis or Gluconeogenesis	26	3	0.022	0.166	0.100
Valine, leucine, and isoleucine biosynthesis	13	7	0.023	0.166	1.000

Note: Data comes from five control and five treatment fish 24 h post-challenge. Abbreviations: FDR, false discovery rate.

6.4.6 Fish immune gene expression

The expression levels for the target cytokine transcripts in HK, SP and RBC pellets of *O. tshawytscha* are illustrated (Figure 6.4). Expression levels for pro-inflammatory *ifnγ* and anti-inflammatory *il-10* were significantly ($p < 0.05$) up-regulated in *O. tshawytscha* HK samples 24 h post-poly (I:C) challenge up to 8-fold and 11-fold, respectively, compared to controls (Figure

6.4a). In SP tissue, only anti-inflammatory *il-10* was significantly ($p < 0.05$) up-regulated (Figure 6.4c), and none in fish RBC pellet, despite a boost in some cytokines (Figure 6.4b).

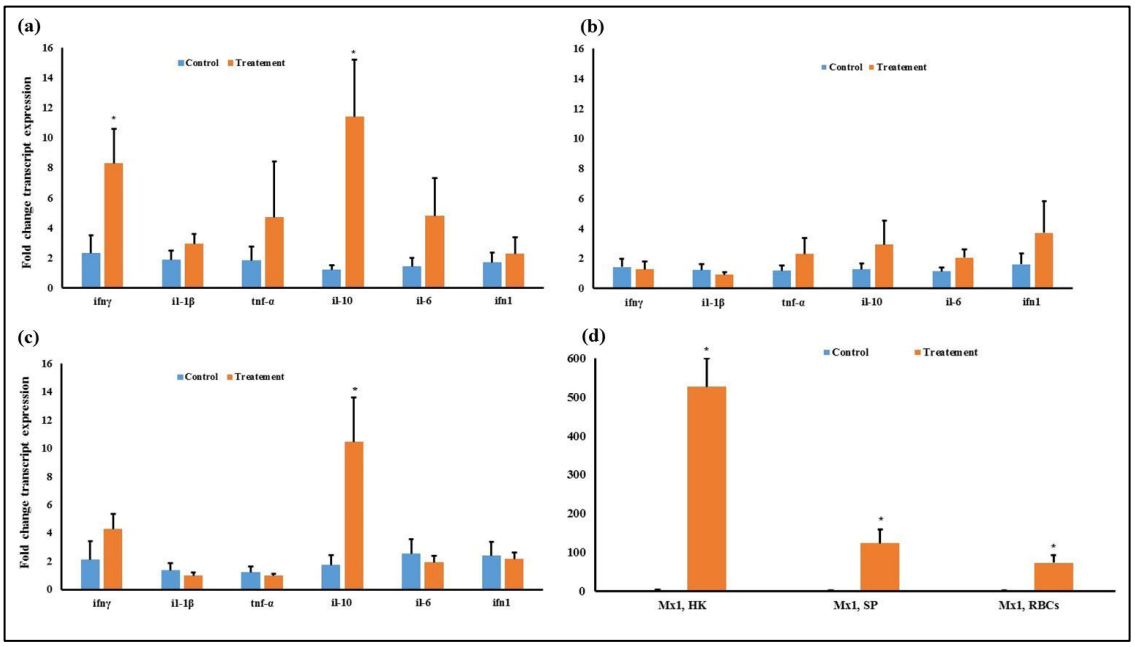


Figure 6.4 Expression mRNA sample cytokines by reverse-transcriptase polymerase chain reaction following *in vivo* intraperitoneal injection of *Oncorhynchus tshawytscha* with 10 µg g^{-1} fish of poly (I:C). Data are obtained from mean±SEM of five fish 24 h post-challenge. (a) Head Kidney, (b) RBC pellet, (c) spleen and (d) indicates mRNA Mx1 protein transcripts in control and treated fish samples (Mx1, HK = head kidney; Mx1, SP = spleen; and Mx1, RBCs = RBC pellet). Data bars with * are significantly different at $p = 0.05$, two independent sample *t*-test, 8 degrees of freedom.

Expression levels for antiviral Mx1 protein 24 h post-stimulation were significantly ($p < 0.05$) and highly up-regulated in all sample types, with fold-changes for HK, SP and RBC of 527, 123, and 73-fold, respectively, in the poly (I:C) challenged fish (Figure 6.4d).

6.5 Discussion

The present study investigated the immunomodulatory effects of *in vivo* poly (I:C) i.p. injection administration on farmed *O. tshawytscha*. Data were collected on peripheral blood haematological parameters, PBMC ROS and NO production, phagocytosis and phagocytic index. In addition, the effects of poly (I:C) on HK, SP and RBC pellet mRNA cytokine transcript expression, and serum metabolomics were assessed.

Since, poly (I:C) administration did not induce significant alterations in fish peripheral blood haematological parameters, the current findings may suggest that a longer incubation period is required to elicit cellular population alterations, as evidenced by a general increase in haematocrit value and leucocyte differential monocyte counts. The observed increase in leucocyte differential monocyte counts, is suggestive of induced cellular proliferation to enhance fish capacity to mount an immune response. However, a larger sample size would benefit detection of statistical differences.

At the cellular functional level, poly (I:C) *in vivo* administration did not significantly affect PBMC cellular viability, ROS and NO production, phagocytosis and phagocytic index, despite a

general increase in ROS, NO and phagocytosis in the treated fish. The observed delay in significant ROS production in treated fish could be linked with individual variations in responses, as *P. olivaceus* HK macrophages, *in vivo* treated with poly (I:C) at 10 µg g^{-1} fish body weight induced ROS production after 24 h (Zhou et al., 2014). Interestingly, the general increase in NO and ROS production is suggestive of cellular functional activation, as evidenced by upregulation of metabolites indicative of oxidative stress and induction of pro-inflammatory *ifnγ* in fish HK and SP samples. At the cellular level, *ifnγ* has been reported to facilitate ROS and NO production, to enhance defence against intracellular infections (Stafford et al., 1999). In line with the present study, work in teleosts demonstrated that poly (I:C) induced *ifnγ* to stimulate respiratory burst activity *in vitro* (Grayfer & Belosevic, 2009) and *in vivo* (Zhou et al., 2014; Zhu et al., 2019).

Metabolite profiling and characterisation following poly (I:C) *in vivo* administration is a critical step towards the elucidation of mechanisms by which poly (I:C) mediates fish innate immune defences. Fish serum is an important biofluid as it can reveal cellular physiological processes and immunity. In this study, the immunomodulatory effects of poly (I:C) in *O. tshawytscha* serum included the alteration in levels of 25 metabolites. Metabolites associated with the valine, leucine, and isoleucine biosynthesis pathway were upregulated in fish serum (Figure 6.5). The three branched chain amino acids (BCAA) are essential amino acids (AAs) for fish (Halver et al., 1957; National Research Council, 2011; Wilson, 2003), and are involved in energy metabolism (Roques et al., 2018c; Wagner et al., 2014).

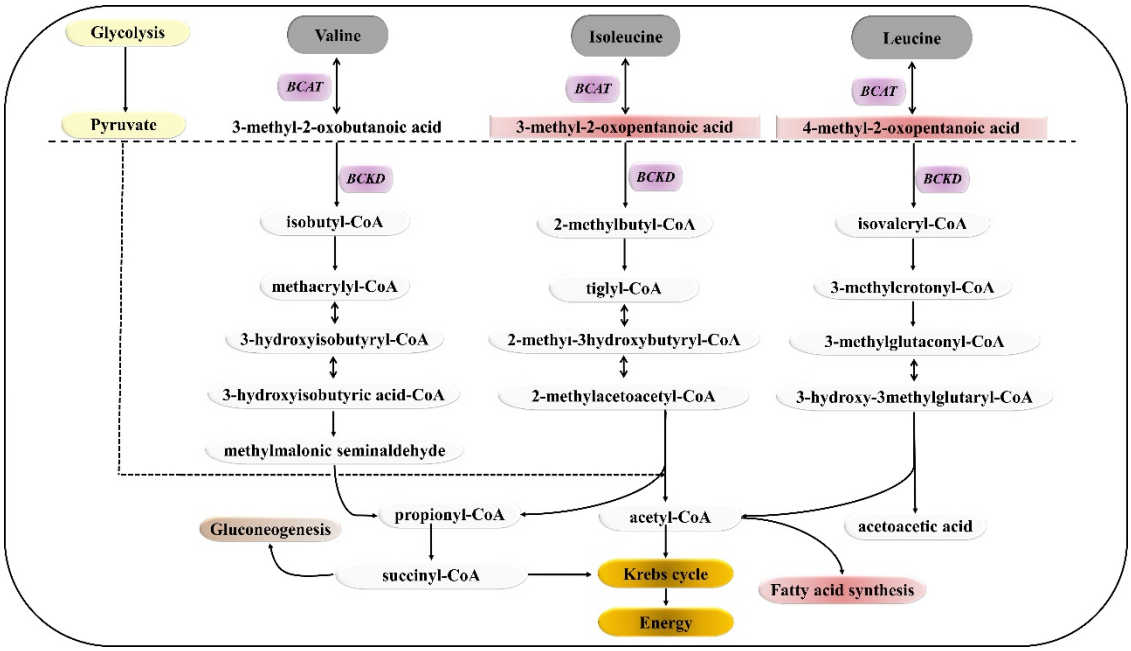


Figure 6.5 Schematic of serum-altered metabolites involved in branched-chain amino acid metabolism following *in vivo* intraperitoneal injection of *Oncorhynchus tshawytscha* with 10 µg g^{-1} fish of poly (I:C). Upregulated metabolites or pathways are indicated in red and downregulated pathways are indicated in light yellow. For enzymes: BCAT is branched-chain amino acid aminotransferase; BCKD is branched-chain alpha-keto acid dehydrogenase.

Under the BCAA pathway, 4-methyl-2-oxopentanoic acid and 3-methyl-2-oxopentanoic acid are direct precursor intermediates of leucine and isoleucine biosynthesis, respectively. Serum

levels of both intermediates were elevated by poly (I:C) treatment. The degradation of leucine and isoleucine has been linked with elevation of branched chain by-products (ketoacids) in blood plasma, with a series of negative effects on energy metabolism and protein synthesis (Yudkoff et al., 2005). Observed upregulation of 2-aminobutyric acid is a non-proteinogenic AA which results from transamination of 2-oxobutyric acid, (Wishart et al., 2018) an important isoleucine biosynthesis intermediate. In addition, isoleucine and valine metabolites were observed in fish serum, but these were not significantly altered by poly (I:C) administration, suggesting that BCAA catabolism in fish may have been activated to probably support enhanced energy requirements or increased protein synthesis (Brestenský et al., 2015).

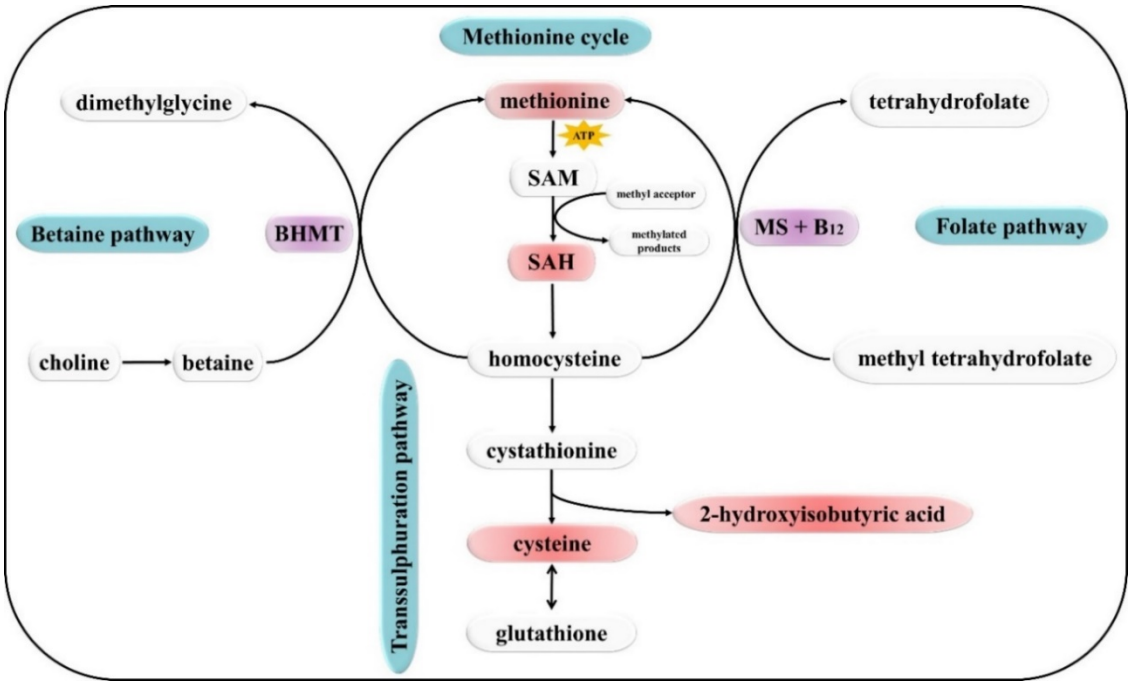


Figure 6.6 Schematic of serum-altered metabolite profile for methionine and cysteine metabolism following *in vivo* intraperitoneal injection of *Oncorhynchus tshawytscha* with 10 µg g^{-1} fish of poly (I:C). Upregulated metabolites are indicated in red. For enzymes: BHMT is betaine-homocysteine S-methyltransferase; MS is methionine synthase, and vitamin B12. SAM is S-adenosyl-L-methionine and SAH is S-adenosylhomocysteine.

Furthermore, poly (I:C) influenced methionine metabolism and cysteine (the transsulphuration pathway) (Figure 6.6), which supplies cysteine for glutathione synthesis during mild stress (Elmada et al., 2016; Séité et al., 2018). In this pathway, methionine is a methyl donor following conversion to S-adenosyl-L-methionine (SAM), a precursor for S-adenosylhomocysteine (SAH), which may be hydrolysed to homocysteine, needed for cysteine formation (D'Mello, 2003). Upregulated levels of SAH in poly (I:C)-treated fish were observed. This is suggestive of reduced SAH use to form glutathione, and consequent conversion back to methionine via methylation by betaine or methylated folic acid pathways (Olsvik et al., 2017a). Also, upregulation of SAH potentially suggests that poly (I:C) together with DNA, proteins, phospholipids, neurotransmitters and hormones, compete for the methyl moiety from SAM, a precursor for SAH, post methyl donation (Cavallaro et al., 2016). Indeed, levels of cysteine and

methionine were upregulated in the poly (I:C) treated fish. This suggests enhanced immune response to supply these metabolites for the transsulphuration and glutathione pathways in response to increases in ROS production in the treated fish, as previously demonstrated elsewhere (Levine et al., 1999; Séité et al., 2018; Wu et al., 2012). Although this study did not report methylation data, poly (I:C) stimulation in a vertebrate model induced methylation to favour expression of immune related genes (Wang et al., 2017). It is probable that poly (I:C) induced DNA methylation in this study, a hypothesis for future investigations.

Levels of 2-hydroxyisobutyric acid, a metabolite of methionine catabolism and glutathione anabolism (Gall et al., 2010) were also elevated in poly (I:C) treated fish (Gall et al., 2010). Enhanced biosynthesis of 2-hydroxyisobutyric acid (Figure 6.6) is suggestive of increased ROS induction to detoxify xenobiotics (Wishart et al., 2018) such as poly (I:C). In higher vertebrates 2-hydroxyisobutyric acid is a biomarker for impaired glucose regulation resulting from increased lipid oxidation and oxidative stress (Gall et al., 2010). Although the glutathione pathway itself was seemingly not perturbed by poly (I:C) after 24 h, these findings are suggestive of an early mild stress response to poly (I:C) administration, affecting important immune related metabolic pathways in fish. In addition, poly (I:C) upregulated aspartic acid levels in fish serum, a non-essential AA, important in asparagine, arginine, methionine, lysine and isoleucine biosynthesis; and also used in the citric acid and urea cycles (Johnson, 2017). Aspartic acid serves a glucogenic role in fish (Ballantyne, 2001; Costas et al., 2011). Accumulation of metabolites indicative of mild oxidative stress induction is supported by upregulation of pro-inflammatory cytokine expression at the cytokine transcript level. In particular, *ifnγ* is a well-documented antiviral cytokine in teleosts, which also promotes immune cells differentiation to enhance immunity (Langevin et al., 2019) and known to induce ROS production (Stafford et al., 1999).

Other metabolites involved in energy metabolism were significantly downregulated 24 h post-poly (I:C) *in vivo* challenge included D-glucose, beta-D-glucose, and methyl 6-deoxy-beta-L-galactopyranoside. These metabolites are important in the pathway topologies of galactose metabolism, starch and sucrose metabolism, and the glycolysis/gluconeogenesis pathway. Overall, poly (I:C) administration appears to enhance energy requirements 24 h post-stimulation, as has been reported in proliferating cells (Fritsch & Weichhart, 2016). This could be due to increased energy requirements by enzymes involved in BCAA/glutathione synthesis and transsulphuration pathways, glycolysis, and the cellular nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex immune response. In aquatic invertebrates, poly (I:C) and viral infections enhanced energy requirements (Li et al., 2014; Saray et al., 2018). Based on these results, it appears that poly (I:C) induces Warburg-like metabolic effects in treated fish, similar to conditions observed during intracellular viral replication (Eisenreich et al., 2019). These results are in line with those observed at transcript level, as pro-inflammatory *ifnγ*-driven immune cells differentiation to enhance immunity (Langevin et al., 2019) require elevated energy (Fritsch &

Weichhart, 2016) for several biochemical processes. In agreement, monocyte counts increased in treated fish, suggestive of enhanced cellular proliferation.

Furthermore, saturated free fatty acid metabolites were upregulated in poly (I:C) treated fish. These include long chain fatty acids such as, arachidic acid, stearic acid, and lignoceric acid. Upregulation of these metabolites may suggest changes in hydrolysis of triglycerides (Olsvik et al., 2015) in poly (I:C) treated fish. In addition, proliferating cells synthesise new phospholipids from diacylglycerols and polar groups like choline or ethanolamine (van Meer et al., 2008). In this respect, the observed upregulation of ethanolamine in poly (I:C) treated fish may suggest lower levels of diacylglycerols, and alterations in phospholipid membrane composition. Indeed, upregulation of ethanolamine in fish plasma is a precursor and a metabolite associated with phospholipid membrane catabolism in mammals (Podo, 1999; Swanson et al., 2008). In addition, upregulation was observed for organic acid metabolites such as, 1H-Indole-1-acetic acid, malonic acid, caproic acid; acyclic hydrocarbon n-pentadecane; a non-proteinogenic 1-aminocyclopropanecarboxylic acid; a sugar arabinose and downregulation observed for 2-amino-3-phenylpropanamide in treated fish. However, the immunoregulatory mechanisms associated with these metabolites remain unclear at the moment, and thus deserve future targeted investigations.

In support of the observed poly (I:C) induced mild oxidative stress, enhanced energy requirements at a metabolic level, and increased monocyte proliferation, cytokine transcript results show that poly (I:C) upregulated antiviral *ifnγ* in HK and SP and Mx1 protein in all fish samples 24 h post-challenge. However, *ifnγ* transcript expression was only significantly upregulated in HK of poly (I:C) treated fish, and the same organ exhibited higher Mx1 protein expression levels. Observed differential cytokine transcript expression could probably be explained by the fact that induction of IFN initially occur in HK and SP, followed by a systemic spread (Jørgensen et al., 2003). Cytokine transcript expression differences could also be due to the fact that HK compared to SP and RBC pellet is the main source of immune cells and hence the center for antigen processing (Rauta et al., 2012). In addition, HK are richer in natural killer (NK) cells and $CD4^+$ or T-helper (Th1) lymphocytes, responsible for *ifnγ* production during the innate immune response (McNab et al., 2015; Shtrichman & Samuel, 2001) and antiviral activities (Zou et al., 2005). *ifnγ* is also produced by immune cells and is more important in later adaptive immunity (Schroder et al., 2004). During adaptive immune responses, *ifnγ* is produced by T-helper lymphocytes and $CD8^+$ or cytotoxic T lymphocytes (Biron & Sen, 2001). The current findings on poly (I:C) induced effects are in agreement with existing studies in which poly (I:C) stimulated antiviral state via *ifnγ* induction following infection with for example ISAV in *S. salar* (Arnemo et al., 2014), and Mx protein expression in *S. salar* (Salinas et al., 2004). Poly (I:C) induced antiviral effects *in vitro* in *O. tshawytscha* transfected embryonic cells CHSE-214 (Jensen et al., 2002b; Monjo et al., 2017), and in triploid *O. tshawytscha* spleen epithelia derived cells CHSS (Semple et al., 2018). Mechanistically, poly (I:C) is a TLR3, SR-As and RLRs ligand and

binds to the receptors to stimulate IFN production and Mx proteins to elicit antiviral defence in salmonids and other teleosts (Arnemo et al., 2014; Das et al., 2009; Semple et al., 2018; Zhu et al., 2019). Similar results have been reported in salmonids following viral infections (Kim et al., 2009; Lockhart et al., 2004a; Lockhart et al., 2007; McBeath et al., 2007). Nonetheless, elevated Mx1 protein expression, and lower *ifn1* transcript levels observed, suggest earlier *ifn1* induction post poly (I:C) treatment. Indeed, *ifn1* was highly expressed in *O. tshawytscha* CHSS cells, at 6 h (Semple et al., 2018), and in *S. salar* HK at 14 hr, HK leucocytes at 12 h, diminishing 24 and 48 h afterwards (Robertsen et al., 2003). Working with S. *salar in vitro*, *ifnγ* partially stimulated Mx protein (Sun et al., 2011) suggesting that *ifnγ* probably synergised with earlier effects of *ifn1* activity to maintain the antiviral Mx1 protein levels observed.

The strong up-regulation of anti-inflammatory *il-10* in the treated fish HK and SP is commensurate with the need to suppress the production of pro-inflammatory *il-1β*, *tnf-α*, and *il-6*, which remained at low levels similar to those in the control fish samples to avoid cellular damage, particularly in the HK and SP (Aste-Amezaga et al., 1998). Although in the RBC pellet, *il-10* levels were not significantly upregulated, *il-10* expression levels increased in the treated fish corresponding with the trend for *tnf-α*, *il-6*, and *ifn1*. These findings add to the growing body of knowledge indicating that erythrocytes may be respiratory cells, but also supplement vertebrate immune function since they expressed immune cytokine transcripts for toll-like receptors and/or interferon regulatory factors in Nile tilapia (*Oreochromis niloticus*) (Y. Shen et al., 2018), *S. salar* (Workenhe et al., 2008) and *O. mykiss* (Morera et al., 2011).

6.5.1 Conclusions and recommendations

Poly (I:C) did not significantly alter fish haematological and cellular PBMC functional parameters 24 h post-stimulation, although increases were observed in leucocyte monocyte counts and cellular functional phagocytosis, ROS and NO production. At a metabolic level, poly (I:C) downregulated metabolites involved in the glycolytic and energy metabolism pathways. Poly (I:C) also upregulated biosynthesis of intermediate metabolites within the BCAA/glutathione and transsulphuration pathways and induced accumulation of metabolites involved in phospholipid metabolism. Genomically, poly (I:C) induced *ifnγ* transcripts in fish HK and SP and anti-viral Mx1 protein in HK, SP and RBC pellet. These results provide insights into biomarkers for poly (I:C) induced immune related stimulation in farmed *O. tshawytscha in vivo* at haematological, innate immunity and metabolic levels, essential in future studies.

6.6 Appendices

Table 6.5S List of serum fold change metabolites

Number	Metabolites	Fold Change	log2(FC)	Number	Metabolites	Fold Change	log2(FC)
1.	S-Adenosylhomocysteine	0.174	-2.520	54.	1H-Indole-1-acetic acid	0.658	-0.605
2.	2-Butyne-1,4-diol	0.242	-2.049	55.	Unknown 115100 11895.4 8669.3	0.663	-0.593
3.	Caproic acid	0.387	-1.370	56.	E-heptadec-10-enoic acid	0.664	-0.591
4.	Ethanolamine	0.393	-1.347	57.	Vitamin E	0.666	-0.586
5.	2-Oxobutyric acid	0.427	-1.227	58.	Citraconic acid	0.666	-0.585
6.	Scyllo-Inositol	0.440	-1.185	59.	Unknown 059100 8140.5 9134.4	1.498	0.583
7.	2-Oxovaleric acid	0.448	-1.160	60.	Glycerol	0.669	-0.580
8.	4-Hydroxybutanoic acid	0.459	-1.125	61.	Heptadecanoic acid	0.669	-0.580
9.	D-Xylose	0.478	-1.065	62.	Unknown 102100 10326.6 4419.0	0.670	-0.578
10.	Unknown 135100 18028.0 7719.9	0.483	-1.050	63.	Guanine	0.673	-0.572
11.	Glutamine	0.494	-1.019	64.	n-Tricosane	0.674	-0.569
12.	Glutaric acid	2.022	1.016	65.	Proline	0.679	-0.559
13.	D-Glucose	2.007	1.005	66.	2-Aminobutanoic acid	0.682	-0.552
14.	Methyl 6-deoxy-beta-L-Galactopyranoside	1.982	0.987	67.	alpha-Hydroxyglutarate	0.682	-0.551
15.	Myo-Inositol	0.510	-0.970	68.	Stearic acid	0.682	-0.551
16.	4-Methyl-2-oxopentanoic acid	0.513	-0.963	69.	Unknown 091100 10654.7 10521.8	0.684	-0.548
17.	Malic acid	0.524	-0.931	70.	Docosapentaenoic acid	0.684	-0.547
18.	DL-3-Aminoisobutyric acid	0.532	-0.912	71.	Unknown 081100 13651.6 15242.5	0.686	-0.545
19.	Unknown 201100 11789.6 11986.2	0.542	-0.884	72.	Adrenic acid	0.686	-0.544
20.	Beta-D-Glucopyranose	1.838	0.878	73.	Unknown 043100 5916.5 7415.0	0.687	-0.542
21.	Caprylic acid	0.555	-0.850	74.	Arachidic acid	0.690	-0.536
22.	1-Aminocyclopropanecarboxylic acid	0.560	-0.835	75.	D-Fructose	0.690	-0.536
23.	2-Hydroxyisobutyric acid	0.570	-0.811	76.	Unknown 113100 8548.1 5921.2	0.690	-0.534
24.	2-2-hydroxyethyl-1-isoquinolinone	1.753	0.810	77.	Z-Docos-13-enamide	0.691	-0.534
25.	Raffinose	0.575	-0.799	78.	Unknown 102100 580.86 590.75	1.444	0.530
26.	Threonine	0.576	-0.796	79.	Cysteine	0.692	-0.530
27.	Itaconic acid	0.592	-0.757	80.	Homocysteine	0.698	-0.518
28.	Aspartic acid	0.593	-0.755	81.	Lysine	0.699	-0.517
29.	3-Methyl-2-oxopentanoic acid	0.597	-0.745	82.	Unknown 149100 16732.8 5717.4	0.701	-0.513
30.	Unknown 071100 5794.4 8572.9	0.598	-0.741	83.	Unknown 071100 5792.3 4375.3	0.702	-0.510
31.	Urea	1.666	0.736	84.	Gamma-Linolenic acid	0.710	-0.495
32.	Malonic acid	0.600	-0.736	85.	Glycine	0.711	-0.493
33.	2-Aminobutyric acid	0.601	-0.735	86.	Unknown 082100 15390.1 5665.2	0.714	-0.486
34.	Unknown 119100 14796.0 10153.9	0.602	-0.732	87.	Linoleic acid	0.719	-0.475
35.	Asparagine	0.609	-0.715	88.	2-Amino-3-phenylpropanamide	1.388	0.473
36.	Arabinose	0.612	-0.708	89.	Nervonic acid	0.723	-0.469
37.	Unknown 198100 13861.7 16640.8	0.615	-0.701	90.	Unknown 114100 14731.9 11527.2	0.726	-0.463
38.	Lignoceric acid	0.624	-0.681	91.	Conjugated linoleic acid	0.727	-0.460
39.	Pyroglutamic acid	0.625	-0.679	92.	Citramalic acid	0.727	-0.460
40.	D-Ribose	0.628	-0.671	93.	Unknown 059100 14844.7 4333.8	0.730	-0.454
41.	N-PENTADECANE	0.629	-0.669	94.	D-Mannose	1.366	0.450
42.	Methionine	0.632	-0.662	95.	Unknown 074100 8768.8 5533.1	0.732	-0.450
43.	Dodecanoic acid	0.634	-0.658	96.	Tryptophan	0.733	-0.448
44.	Beta-Alanine	0.643	-0.637	97.	Benzoic acid	0.736	-0.442
45.	Unknown 056100 5924.8 11510.8	0.643	-0.637	98.	Fumaric acid	0.741	-0.432
46.	Ornithine	0.648	-0.626	99.	Arachidonic acid	0.741	-0.432
47.	Dihomo-gamma-Linolenic acid	0.649	-0.625	100.	Putrescine	1.348	0.431
48.	Cis-4-Hydroxyproline	0.649	-0.625	101.	Unknown 115100 10070.4 5660.1	0.744	-0.427
49.	Nonadecanoic acid	0.649	-0.624	102.	Myristic acid	0.746	-0.423
50.	NADPH	0.649	-0.623	103.	Dodecane	0.747	-0.420
51.	11,14-Eicosadienoic acid	0.650	-0.622	104.	Palmitelaidic acid	0.749	-0.417
52.	Oxalic acid	0.657	-0.606	105.	Unknown 114100 5961.6 14633.7	0.749	-0.417
53.	11Z,14Z,17Z-Eicosa-11,14,17-trienoic acid	0.658	-0.605	106.	Pentadecanoic acid	0.752	-0.411

Number	Metabolites	Fold Change	log2(FC)
107.	Isocitric acid	0.755	-0.405
108.	9E-heptadecenoic acid	0.757	-0.402
109.	Unknown 074100 4621.3 590.97	1.318	0.399
110.	Dehydroascorbic acid	0.760	-0.397
111.	dimethyl 2-aminopropanedioatehydrochloride	0.761	-0.394
112.	Unknown 115100 5965.5 18950.1	0.763	-0.391
113.	Pyruvic acid	0.766	-0.384
114.	Unknown 249100 5731.8 26422.9	0.767	-0.383
115.	Isopropanolamine	0.769	-0.380
116.	Cis-Aconitic acid	0.769	-0.379
117.	Unknown 142100 5919.9 14117.0	0.769	-0.378
118.	Unknown 045100 5989.0 7467.8	0.770	-0.377
119.	Beta-Citryl-L-glutamic acid	0.771	-0.375
120.	Alpha-Linolenic acid	0.772	-0.374
121.	Serine	0.777	-0.364
122.	Valine	0.780	-0.358
123.	Citric acid	0.781	-0.357
124.	Adipic acid	1.281	0.357
125.	Tyrosine	0.787	-0.346
126.	Unknown 128100 13921.1 4219.1	0.788	-0.344
127.	Sucrose	1.267	0.342
128.	4-Hydroxyphenylacetic acid	0.792	-0.337
129.	Decanoic acid	0.793	-0.334
130.	Tert-Butylpentamethyldisiloxane	0.794	-0.333
131.	2-Oxoglutaric acid	1.254	0.326
132.	Eicosapentaenoic acid	0.803	-0.317
133.	Phenylalanine	0.807	-0.309
134.	Isoleucine	0.812	-0.301
135.	beta-D-Arabinopyranose	0.812	-0.300
136.	Docosahexaenoic acid	0.813	-0.298
137.	1,2,7,8-Tetrahydro-thiino2,3-f-1-benzopyran-2,8-dione	0.813	-0.298
138.	Leucine	0.818	-0.290
139.	Palmitic Acid	0.818	-0.290
140.	Alanine	0.821	-0.284
141.	Unknown 128100 4210.5 1296.8	0.826	-0.277
142.	Unknown 086100 5956.6 12839.3	1.205	0.269
143.	Unknown 101100 11980.5 7435.3	0.834	-0.263
144.	2-Aminoadipic acid	0.835	-0.261
145.	Cholesterol	0.838	-0.255
146.	2-Methyloctadecanoic acid	1.186	0.246
147.	Unknown 160100 5943.4 11622.7	0.846	-0.241
148.	Glyoxylic acid	0.850	-0.235
149.	Histidine	0.850	-0.234
150.	L-5-Oxoproline	0.851	-0.234
151.	Glyceric acid	0.860	-0.218
152.	Creatinine	0.862	-0.215
153.	Phosphate	0.864	-0.212
154.	4-Aminobutyric acid	0.866	-0.208
155.	Tridecane	0.870	-0.202
156.	Nicotinamide	1.149	0.200
157.	Unknown 059100 7369.3 7745.8	0.879	-0.185
158.	Unknown 088100 5753.2 5945.8	1.136	0.184
159.	Unknown 088100 4430.3 5919.6	1.135	0.183
160.	Trans-Vaccenic acid	0.882	-0.182
161.	2-Hydroxybutyric acid	0.882	-0.182
162.	Unknown 156100 9636.6 12831.0	0.892	-0.165
163.	D-Galactose	0.895	-0.159
164.	Succinic acid	0.896	-0.158
165.	Unknown 070100 4228.5 698.0	0.897	-0.157
166.	Trans-4-Hydroxyproline	0.902	-0.150
167.	Unknown 125100 18490.5 9654.3	0.903	-0.148
168.	Glutathione	0.906	-0.142
169.	Azelaic acid	0.914	-0.130
170.	Unknown 112100 4360.3 11549.2	1.094	0.130
171.	n-Heptadecane	0.919	-0.122
172.	Propanoic acid	0.921	-0.119
173.	Glutamic acid	0.925	-0.113
174.	Unknown 114100 5915.3 8214.4	0.935	-0.097
175.	Cystathionine	1.066	0.092
176.	Nicotinic acid	1.052	0.073
177.	Unknown 126100 12746.7 5933.2	1.048	0.067
178.	N-Acetylglutamic acid	1.047	0.067
179.	Lactic acid	0.961	-0.057
180.	Salicylic acid	0.964	-0.053
181.	2,2,8-Trimethyl-4,5-dihydro-2H-cyclopentadechromene	0.973	-0.040
182.	Unknown 142100 15351.8 5922.0	1.026	0.037
183.	Unknown 232100 12872.0 15651.0	1.025	0.035
184.	Unknown 082100 14256.8 11042.6	0.987	-0.019
185.	Unknown 131100 7189.6 10388.8	0.992	-0.011
186.	Pyrimidin-2-yl trifluoromethanesulfonate	1.007	0.010

Table 6.6S Serum significant metabolites identified by significant analysis of microarray (and metabolites) (SAM).

Number	Metabolite	d.value	Stdev	Rawp	Q.value	Number	Metabolite	d.value	Stdev	Rawp	Q.value
1.	2-Oxobutyric acid	1.621	0.339	0.001	0.022	48.	Benzoic acid	0.868	0.555	0.088	0.097
2.	D-Glucose	-1.621	0.339	0.001	0.022	49.	Nonadecanoic acid	0.864	0.556	0.089	0.097
3.	Arabinose	1.51	0.373	0.003	0.041	50.	Pyruvic acid	0.863	0.556	0.09	0.097
4.	1-Aminocyclopropanecarboxylic acid	1.493	0.379	0.003	0.041	51.	Adrenic acid	0.861	0.557	0.09	0.097
5.	2-Hydroxyisobutyric acid	1.41	0.404	0.005	0.053	52.	Dodecanoic acid	0.851	0.56	0.094	0.097
6.	Beta-D-Glucopyranose	-1.371	0.416	0.006	0.055	53.	Unknown 056100 5924.8 11510.8	0.85	0.56	0.094	0.097
7.	2-Aminobutyric acid	1.267	0.447	0.011	0.082	54.	Tryptophan	0.846	0.561	0.095	0.097
8.	S-Adenosylhomocysteine	1.255	0.45	0.012	0.082	55.	Ornithine	0.836	0.563	0.1	0.098
9.	Malonic acid	1.202	0.466	0.015	0.089	56.	Fumaric acid	0.831	0.564	0.102	0.098
10.	Ethanolamine	1.18	0.472	0.017	0.089	57.	Asparagine	0.824	0.566	0.105	0.098
11.	Methyl 6-deoxy-beta-L-Galactopyranoside	-1.172	0.474	0.018	0.089	58.	Oxalic acid	0.823	0.566	0.105	0.098
12.	1H-Indole-1-acetic acid	1.144	0.482	0.021	0.093	59.	Beta-Citryl-L-glutamic acid	0.821	0.567	0.107	0.098
13.	Lignoceric acid	1.136	0.484	0.022	0.093	60.	Unknown 198100 13861.7 16640.8	0.802	0.571	0.115	0.102
14.	Aspartic acid	1.111	0.492	0.025	0.094	61.	Unknown 102100 10326.6 4419.0	0.801	0.571	0.116	0.102
15.	Caproic acid	1.106	0.493	0.026	0.094	62.	Unknown 059100 14844.7 4333.8	0.79	0.574	0.121	0.103
16.	Unknown 119100 14796.0 10153.9	1.087	0.498	0.028	0.094	63.	Arachidonic acid	0.789	0.574	0.121	0.103
17.	Cysteine	1.076	0.501	0.03	0.094	64.	D-Xylose	0.774	0.577	0.128	0.108
18.	2-Amino-3-phenylpropanamide	-1.066	0.504	0.031	0.094	65.	DL-3-Aminoisobutyric acid	0.767	0.579	0.131	0.108
19.	Unknown 059100 8140.5 9134.4	-1.06	0.506	0.033	0.094	66.	Docosapentaenoic acid	0.766	0.579	0.132	0.108
20.	4-Methyl-2-oxopentanoic acid	1.035	0.512	0.038	0.094	67.	Citraconic acid	0.762	0.58	0.134	0.108
21.	Methionine	1.025	0.515	0.04	0.094	68.	Linoleic acid	0.756	0.581	0.138	0.109
22.	N-PENTADECANE	1.024	0.515	0.04	0.094	69.	Unknown 082100 15390.1 5665.2	0.754	0.582	0.138	0.109
23.	3-Methyl-2-oxopentanoic acid	0.992	0.524	0.048	0.094	70.	Unknown 149100 16732.8 5717.4	0.749	0.583	0.141	0.109
24.	Arachidic acid	0.99	0.524	0.049	0.094	71.	Dodecane	0.74	0.585	0.146	0.109
25.	Stearic acid	0.99	0.524	0.049	0.094	72.	Unknown 081100 13651.6 15242.5	0.733	0.586	0.149	0.109
26.	Nervonic acid	0.984	0.526	0.05	0.094	73.	Glycine	0.733	0.586	0.15	0.109
27.	Unknown 045100 5989.0 7467.8	0.975	0.528	0.052	0.094	74.	2-Butyne-1,4-diol	0.728	0.587	0.152	0.109
28.	Unknown 135100 18028.0 7719.9	0.968	0.53	0.055	0.094	75.	Leucine	0.724	0.588	0.154	0.109
29.	Z-Docos-13-enamide	0.962	0.532	0.056	0.094	76.	Unknown 115100 11895.4 8669.3	0.724	0.588	0.154	0.109
30.	Unknown 201100 11789.6 11986.2	0.956	0.533	0.057	0.094	77.	Trans-Vaccenic acid	0.716	0.59	0.159	0.109
31.	Glutamine	0.955	0.534	0.057	0.094	78.	Pentadecanoic acid	0.712	0.591	0.162	0.109
32.	Phenylalanine	0.95	0.535	0.059	0.094	79.	Dehydroascorbic acid	0.712	0.591	0.162	0.109
33.	Heptadecanoic acid	0.945	0.536	0.06	0.094	80.	Isoleucine	0.711	0.591	0.162	0.109
34.	Glycerol	0.94	0.537	0.061	0.094	81.	Docosahexaenoic acid	0.71	0.591	0.163	0.109
35.	D-Mannose	-0.939	0.538	0.062	0.094	82.	Unknown 114100 5961.6 14633.7	0.706	0.592	0.166	0.109
36.	Threonine	0.933	0.539	0.064	0.094	83.	Myristic acid	0.7	0.593	0.169	0.109
37.	11Z,14Z,17Z-Eicosa-11,14,17-trienoic acid	0.933	0.539	0.064	0.094	84.	Unknown 114100 14731.9 11527.2	0.697	0.594	0.171	0.109
38.	2-2-hydroxyethyl-1-isoquinolinone	-0.925	0.541	0.065	0.094	85.	Palmitic Acid	0.696	0.594	0.172	0.109
39.	2-Oxovaleric acid	0.922	0.542	0.067	0.094	86.	Proline	0.695	0.594	0.173	0.109
40.	Dihomo-gamma-Linolenic acid	0.917	0.543	0.069	0.095	87.	Gamma-Linolenic acid	0.685	0.597	0.178	0.11
41.	Valine	0.909	0.545	0.072	0.095	88.	Lysine	0.683	0.597	0.179	0.11
42.	2-Aminoadipic acid	0.898	0.548	0.076	0.095	89.	Itaconic acid	0.657	0.602	0.197	0.119
43.	Malic acid	0.896	0.548	0.077	0.095	90.	Citramalic acid	0.655	0.603	0.198	0.119
44.	11,14-Eicosadienoic acid	0.896	0.549	0.077	0.095	91.	Vitamin E	0.649	0.604	0.203	0.12
45.	Pyroglutamic acid	0.894	0.549	0.078	0.095	92.	dimethyl 2-aminopropanedioatehydrochloride	0.648	0.604	0.204	0.12
46.	E-heptadec-10-enoic acid	0.884	0.551	0.082	0.097	93.	D-Ribose	0.646	0.604	0.205	0.12
47.	2-Aminobutanoic acid	0.874	0.554	0.085	0.097						

Table 6.7S. Serum pathway results

Number	Pathways	Total Compound	Hits	Raw p	#NAME?	Holm adjust	FDR	Impact
1.	Pentose phosphate pathway	19	2	0.004	5.596	0.212	0.112	0.000
2.	Starch and sucrose metabolism	22	4	0.004	5.409	0.251	0.112	0.059
3.	Galactose metabolism	26	6	0.006	5.131	0.325	0.112	0.109
4.	Cysteine and methionine metabolism	29	8	0.015	4.198	0.811	0.166	0.674
5.	Pentose and glucuronate interconversions	15	2	0.019	3.962	1.000	0.166	0.083
6.	Glycerophospholipid metabolism	28	1	0.021	3.854	1.000	0.166	0.000
7.	Glycolysis or Gluconeogenesis	26	3	0.022	3.803	1.000	0.166	0.100
8.	Valine, leucine and isoleucine biosynthesis	13	7	0.023	3.757	1.000	0.166	1.000
9.	Pantothenate and CoA biosynthesis	15	3	0.032	3.458	1.000	0.171	0.000
10.	Sulfur metabolism	9	1	0.036	3.324	1.000	0.171	0.042
11.	Taurine and hypotaurine metabolism	7	1	0.036	3.324	1.000	0.171	0.000
12.	Thiamine metabolism	7	1	0.036	3.324	1.000	0.171	0.000
13.	Glycine, serine and threonine metabolism	31	9	0.067	2.703	1.000	0.219	0.570
14.	Fructose and mannose metabolism	21	1	0.068	2.692	1.000	0.219	0.000
15.	Purine metabolism	66	3	0.070	2.665	1.000	0.219	0.011
16.	Aminoacyl-tRNA biosynthesis	67	19	0.075	2.584	1.000	0.219	0.103
17.	Valine, leucine and isoleucine degradation	38	4	0.077	2.568	1.000	0.219	0.012
18.	beta-Alanine metabolism	16	2	0.079	2.537	1.000	0.219	0.395
19.	Lysine degradation	18	1	0.081	2.513	1.000	0.219	0.000
20.	Biosynthesis of unsaturated fatty acids	42	15	0.087	2.443	1.000	0.219	0.000
21.	Pyrimidine metabolism	41	3	0.089	2.419	1.000	0.219	0.000
22.	Arginine and proline metabolism	43	11	0.097	2.333	1.000	0.219	0.473
23.	Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	0.099	2.315	1.000	0.219	1.000
24.	Phenylalanine metabolism	11	2	0.099	2.315	1.000	0.219	0.407
25.	Propanoate metabolism	20	5	0.099	2.314	1.000	0.219	0.000
26.	Tryptophan metabolism	39	1	0.100	2.302	1.000	0.219	0.153
27.	Amino sugar and nucleotide sugar metabolism	37	2	0.108	2.223	1.000	0.229	0.000
28.	Arachidonic acid metabolism	31	1	0.125	2.078	1.000	0.255	0.329
29.	Alanine, aspartate and glutamate metabolism	24	10	0.130	2.040	1.000	0.255	0.783
30.	Linoleic acid metabolism	7	1	0.142	1.949	1.000	0.271	1.000
31.	Histidine metabolism	14	3	0.155	1.865	1.000	0.285	0.238
32.	Fatty acid biosynthesis	38	2	0.170	1.769	1.000	0.285	0.000
33.	Glycerolipid metabolism	18	2	0.173	1.757	1.000	0.285	0.386
34.	Fatty acid elongation in mitochondria	27	1	0.177	1.733	1.000	0.285	0.000
35.	Fatty acid metabolism	38	1	0.177	1.733	1.000	0.285	0.000
36.	Tyrosine metabolism	44	3	0.180	1.713	1.000	0.285	0.137
37.	Biotin metabolism	5	1	0.185	1.689	1.000	0.285	0.000
38.	Glutathione metabolism	26	8	0.196	1.628	1.000	0.291	0.466
39.	Methane metabolism	9	2	0.204	1.590	1.000	0.291	0.400
40.	Cyanoamino acid metabolism	6	2	0.204	1.590	1.000	0.291	0.000
41.	Nicotinate and nicotinamide metabolism	14	3	0.229	1.475	1.000	0.318	0.128
42.	Pyruvate metabolism	22	2	0.245	1.406	1.000	0.331	0.174
43.	Citrate cycle (TCA cycle)	20	7	0.249	1.389	1.000	0.331	0.325
44.	Sphingolipid metabolism	21	1	0.290	1.239	1.000	0.370	0.000
45.	D-Glutamine and D-glutamate metabolism	5	3	0.297	1.213	1.000	0.370	1.000
46.	Nitrogen metabolism	9	5	0.298	1.209	1.000	0.370	0.000
47.	Ubiquinone and other terpenoid-quinone biosynthesis	3	1	0.318	1.146	1.000	0.386	0.000
48.	Porphyrin and chlorophyll metabolism	27	2	0.339	1.082	1.000	0.402	0.000
49.	Glyoxylate and dicarboxylate metabolism	18	5	0.367	1.002	1.000	0.427	0.444
50.	Selenoamino acid metabolism	17	1	0.388	0.946	1.000	0.443	0.000
51.	Primary bile acid biosynthesis	36	1	0.426	0.853	1.000	0.458	0.254
52.	Steroid biosynthesis	33	1	0.426	0.853	1.000	0.458	0.054
53.	Steroid hormone biosynthesis	56	1	0.426	0.853	1.000	0.458	0.002
54.	alpha-Linolenic acid metabolism	9	1	0.505	0.684	1.000	0.519	1.000
55.	Butanoate metabolism	22	5	0.506	0.681	1.000	0.519	0.029
56.	Inositol phosphate metabolism	27	1	0.519	0.656	1.000	0.519	0.088
57.	Ascorbate and aldarate metabolism	6	1	0.519	0.656	1.000	0.519	0.000
58.	Arachidonic acid metabolism	31	1	0.125	2.078	1.000	0.255	0.329

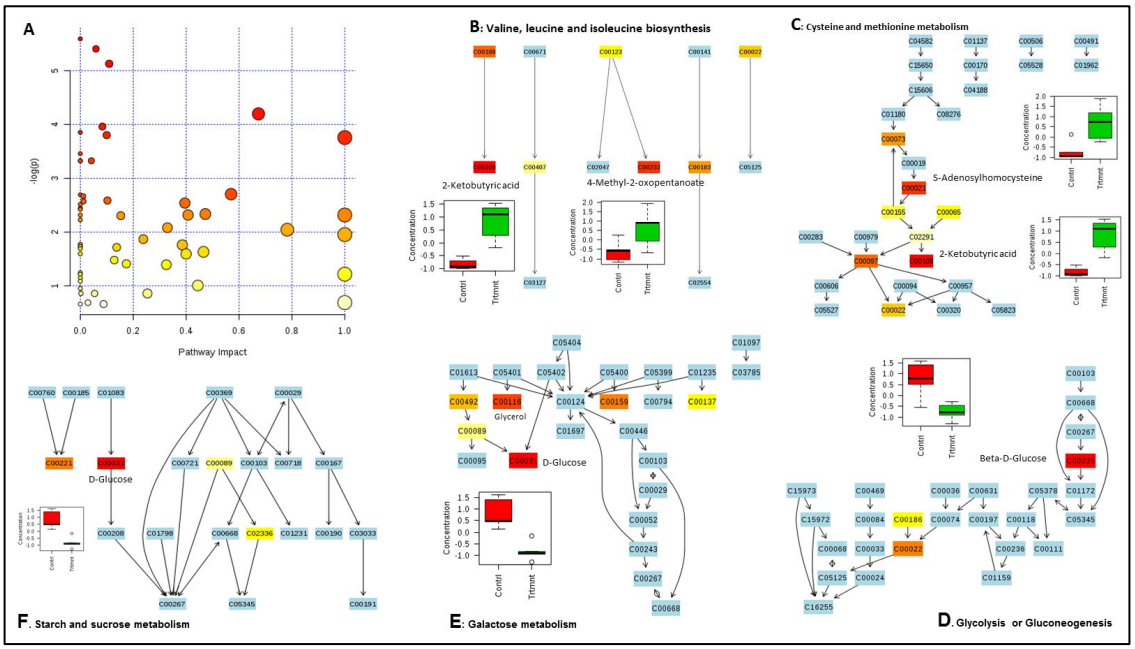


Figure 6.7S Secondary bioinformatics of the profiled metabolites. (A) Topology-based pathway analysis indicating detected networks in fish serum following poly (I: C) *in vivo* challenge. The most affected metabolites are indicated in colour with yellow as least relevant and red most relevant, grouped by their pathway impact and statistical relevance. Five pathways (B, C, D, E and F) of the six containing some of the highest relevant metabolite coverage are indicated above. Boxes in yellow to red represent metabolites within the KEGG database ID codes that were detected and annotated in this analysis. The box colours signify significance, as light yellow ($p > 0.05$), and orange to red ($p < 0.05$) from unpaired t-statistic of the control versus treatment group. A box plot indicates each significant metabolite in a pathway. Light blue boxes indicate metabolites not detected, but were used as background information for pathway analysis to calculate the proportion of identified compounds within each pathway, to determine the position (relative-betweeness centrality) and importance of each metabolite.

7 Chapter 7. Case Study 4: Polyinosinic: Polycytidylic Acid *in vivo* Enhances Chinook salmon (*Oncorhynchus tshawytscha*) Immunity and Alters the Fish Metabolome.

Lulijwa, R., Alfaro, A. C., Merien, F., Burdass, M., Meyer, J., Venter, L., & Young, T. Polyinosinic: polycytidylic acid (poly I:C) *in vivo* enhances Chinook salmon (*Oncorhynchus tshawytscha*) immunity and alters the fish metabolome. *Aquaculture International*, *28*(6), 2437-2463.

7.1 Prelude to case study 4

Building on the results and observations obtained from the short-term poly (I:C) *in vivo* exposure in chapter 6, this study characterised the immuno-metabolic response mechanisms over a five-day post intraperitoneal (i.p) administration. An integrated approach involving assessment of effects on cellular haematology, peripheral blood mononuclear cells (PBMCs) functional parameters, gas chromatography mass spectrometry (GC-MS) serum and liver metabolomics, spleen and head kidney targeted RT-PCR immune cytokine expression were assessed. Findings from this study were discussed in the manuscript and demonstrate poly (I:C) induced innate immune responses through multiple mechanisms and at different levels.

7.2 Introduction

The New Zealand aquaculture sector was valued at over US$ 820 million in 2017, of which Chinook salmon (*Oncorhynchus tshawytscha*) contributed 24% and 13% by value and production volume respectively (FAO, 2019). New Zealand is the leading producer and supplier of farmed *O. tshawytscha* in the world. As the country's aquaculture industry is founded on sustainable practices, *O. tshawytscha* production is considered to be "virus free" (Diggles, 2016) and the current strategy targets US$ 3 billion by 2035 in annual sales (MPI, 2019). However, it is well established that disease outbreaks caused by viruses, bacteria, and parasites, routinely result in significant losses in aquaculture facilities [reviewed in Fazio (2019)]. Viral pathogens are inevitable and present a daunting challenge to aquaculture developments due to the high particle density ($10^7 mL^{-1}$ of seawater), fast replication and persistence in the aquatic environment (Oidtmann et al., 2018; Semple et al., 2018). Due to continued industry growth and a changing aquatic environment (e.g., ocean acidification, rising sea temperatures, spreading of marine diseases), a better understanding of the species' antiviral mechanisms is essential.

Fish possess pattern recognition receptors (PRRs), which recognise and initiate appropriate responses to conserved pathogen-associated molecular patterns (PAMPs) (Castro & Tafalla, 2015; Elward & Gasque, 2003). A common viral PAMP is double stranded ribonucleic acid (dsRNA) (DeWitte-Orr et al., 2010). Vertebrates also possess a multitude of nucleic acid binding PRRs, including surface toll-like receptors (TLRs), class A scavenger receptors (SR-As), and cytosolic PRRs (e.g., melanoma differentiation-associated protein 5 (MDA5), retinoic acid-inducible gene 1 [RIG-I] receptors [RLRs] to recognise dsRNA) (Semple et al., 2018). Following recognition and specific binding by PRR, dsRNA molecules such as polyinosinic: polycytidylic acid (poly [I:C]) initiate signal production of type I interferons (I IFNs) and interferon-stimulated genes (ISGs) to limit viral replication (Monjo et al., 2017). Majority of ISGs enhance host cellular antiviral defence (Zhang & Gui, 2012). The antiviral ISGs are mostly stimulated by type I [interferon alpha (*ifn-α*) and interferon beta (*ifn-β*)], type III interferon lambda (*ifnλ*) and partially by type II interferon gamma (*ifnγ*) which collaboratively target viral replication (Schoggins & Rice, 2011).

Type I IFNs are induced by viruses in most cells (Samuel, 2001), while *ifnγ* is produced by immune cells and is more important in later adaptive immune responses (Schroder et al., 2004). During adaptive immune responses, *ifnγ* is produced by $CD4^+$ or T-helper (Th1) lymphocytes and $CD8^+$ or cytotoxic T lymphocytes (Biron & Sen, 2001). During innate immune responses, *ifnγ* is produced by natural killer cells (NK cells) and $CD4^+$ cells in response to interleukin-12 (*il-12*), *il-18*, mitogens or antigens (Samuel, 2001) or *il-12* and *il-18* secreted by mononuclear cells and antigen presenting cells infected with intracellular pathogens (Shtrichman & Samuel, 2001). Poly (I:C) is a synthetic dsRNA analogue, which mimics a viral infection to induce antiviral immune responses. Following stimulation, the innate immune system acts as if challenged with a viral pathogen and initiates mechanisms designed to fight the aetiological agent. Thus, poly (I:C) is

used for immunological studies in aquaculture (Jensen et al., 2002a; Zhou et al., 2014); and orchestrates its antiviral effect via a broadly expressed and well-conserved toll-like receptor 3 (*tlr3*) (Kitao et al., 2009; Tanekhy, 2016; Zhou et al., 2014), RIG-I and MDA5, both termed RIG-I-like receptors (RLRs), and SR-As (Brisse & Ly, 2019; Semple et al., 2018; Zhou et al., 2014).

For instance, poly (I:C) induced the expression of cytokine-producing genes in common carp (*Cyprinus carpio*) (Kitao et al., 2009). Administration induced *ifnγ* in goldfish (*Carassius auratus*) (Grayfer & Belosevic, 2009), type I and *ifnγ* in Atlantic salmon (*Salmo salar*) in synergy with cytosine-phosphate-guanosine-oligodeoxynucleotides (CpG-ODNs) (Strandskog et al., 2011; Thim et al., 2012), interferon regulatory factors (IRF 1-5, 9) and *tlr3* in Nile tilapia (*Oreochromis niloticus*) (Y. Shen et al., 2018). In addition, several studies reported expression of gene transcripts for type 1 IFNs and Mx proteins in viral-infected and poly (I:C) treated fish (Arnemo et al., 2014; Das et al., 2009; Lockhart et al., 2007; McBeath et al., 2007). Poly (I:C) vaccination also induced antiviral activity, in Japanese flounder (*Paralichthys olivaceus*) infected with megalocytivirus (Zhou et al., 2014), and in viral hemorrhagic septicemia (VHS) virus-infected zebrafish (*Danio rerio*) (Kavaliauskis et al., 2015).

Poly (I:C) is widely applied as an immunostimulant in salmonids due to its type 1 IFN induced antiviral activity in treated fish (Eaton, 1990; Jensen et al., 2002a; Lockhart et al., 2004a; Salinas et al., 2004). For example, supplementary poly (I:C) conferred antiviral protection against various viral infections in *S. salar* (Jensen et al., 2002a; Jensen & Robertsen, 2002; Larsen et al., 2004; Nygaard et al., 2000; Robertsen et al., 1997; Strandskog et al., 2011; Thim et al., 2012). Antiviral activity was also reported in poly (I:C) transfected *O. tshawytscha* embryonic cells CHSE-214 (Jensen et al., 2002b; Monjo et al., 2017), and in triploid *O. tshawytscha* spleen (SP) epithelia derived cells CHSS cells (Semple et al., 2018). In addition, variation in optimal expression of Mx protein following poly (I:C) treatment was observed in *S. salar* (Jensen et al., 2002a; Jensen & Robertsen, 2002; Robertsen et al., 1997; Saint-Jean & Pérez-Prieto, 2007; Salinas et al., 2004).

Assessment of fish peripheral blood cellular composition, differential leucocyte counts and cellular functional parameters are routinely used to illustrate immunological responses in aquaculture (Lulijwa et al., 2019c). This makes the assessment of cellular parameters and their responses in poly (I:C) treated fish essential. Leucocytes attempt to eliminate pathogens such as viruses via reactive oxygen species (ROS) production (Uribe et al., 2011). Using leucocytes, pro-inflammatory *ifnγ* was reported to facilitate intracellular pathogen destruction through nitric oxide (NO) production to enhance respiratory burst (Stafford et al., 1999). Interestingly, *ifnγ* induced activities were demonstrated in poly (I:C) treated *C. auratus* primary head kidney (HK) *in vitro* (Grayfer & Belosevic, 2009) and in *P. olivaceus* HK macrophages and peripheral blood leucocytes following *in vivo* poly (I:C) treatment (Zhou et al., 2014).

Comprehensive metabolite profiling is another approach that has recently attracted attention in aquaculture research (Alfaro & Young, 2018). This omic technology can for example be

applied in farmed fish immunology assessment (Browdy et al., 2012). Metabolomics can allow the identification of sample specific biomarkers following exposure to specific stressors (Alfaro & Young, 2018). Metabolomics studies on salmonids previously focussed on nutrition (Berntssen et al., 2016; Jasour et al., 2017; Roques et al., 2018a; Wagner et al., 2014), alterations induced by contaminants (Olsvik et al., 2015; Olsvik et al., 2017a; Olsvik & Søfteland, 2018; Softeland et al., 2016; Softeland et al., 2014), pre-sampling handling and sedation practices (Young et al., 2019), and following poly (I:C) short-term exposure (Lulijwa et al., 2020a).

At the moment, very few studies have investigated the immuno-metabolic effects of *in vivo* poly (I:C) administration in *O. tshawytscha*. Of the few studies, antiviral state has been demonstrated *in vitro*, in poly (I:C) transfected *O. tshawytscha* embryonic cells CHSE-214 (Jensen et al., 2002b; Monjo et al., 2017); triploid *O. tshawytscha* SP epithelia derived cells CHSS (Semple et al., 2018) and *in vivo* in *O. tshawytscha* post-smolts (Lulijwa et al., 2020a). Although Lulijwa et al. (2020a) showed that short-term poly (I:C) exposure altered haematological parameters, cellular functions of peripheral blood mononuclear cells (PBMC), the serum metabolome, and levels of organ immune gene expressions, the long-term effects remain unknown. Thus, the present study profiled the immuno-metabolic responses of *O. tshawytscha* to *in vivo* poly (I:C), over a five-day period post-exposure via integrated assessment of effects on cellular haematology, PBMC functional ROS and latex bead phagocytosis, gas chromatography-mass spectrometry (GC-MS) serum and liver metabolite profiles, and reverse transcription polymerase chain reaction (RT-PCR) targeted cytokine expression in HK and SP samples.

7.3 Materials and methods

7.3.1 Fish samples and husbandry

Two-year-old *O. tshawytscha* post-smolt (weight = 414.56±52.32 g; total length = 31.06±1.12 cm) were obtained from the Nelson Marlborough Institute of Technology (NMIT) aquaculture facility (Glenduan, Nelson, New Zealand). Fish were previously maintained on a saltwater recirculating system (temp = 16.02±0.94°C; DO = 7.34±0.32 mg L^{-1}; pH = 8.18±0.17; NH_4^+ = 0.17±0.12 mg L^{-1}; NO_2^- ≤ 0.01 mg L^{-1}) at the NMIT aquaculture facility at 16°C and fed to satiation once daily with Orient 3.0 mm commercial diet (Skretting) (44% crude protein; 28% lipid). The system consisted of three 1.6 m^3 cylindrical tanks with a central drain, a flow rate of 1.4 l s^{-1} and aerated at 3.0 l s^{-1}.

7.3.2 Five-day poly (I:C) exposure

Prior to intraperitoneal (i.p.) injection, experimental fish were captured by scoop net and individually anaesthetised with AQUI-S (AQUI-S New Zealand Ltd, Wellington, New Zealand) at 25 mg L^{-1} of culture water for 2-3 min. Fish total length (cm) and wet weight (g) were quickly taken and data recorded. Fish wet weight were used to determine the volume of poly (I:C) for injection. Fifteen experimental fish were i.p. injected with 2.5 µL g^{-1} fish, of sterile filtered (40 µm) phosphate buffered saline (PBS) containing 2 mg mL^{-1} of poly (I:C) (P1530-100MG, Sigma-Aldrich, Auckland, New Zealand). The injection dosage was given at half the dose previously

used by several authors for *S. salar* (Jensen et al., 2002b; Robertsen et al., 2003). Three unstimulated baseline profiles (control fish) were taken and euthanised by Ikijime without sedation to reduce changes in serum metabolome (Young et al., 2019) at day zero. All study fish were free from skin lesions, skeletal deformities and had intact skin.

Three fish were taken daily from the tank by scoop net, and euthanised by Ikijime without sedation. Approximately 4 mL of blood were collected from each fish via caudal vein puncture using non-heparinised 3.0 mL syringes attached to 20-gauge Terumo needles (SH177, Amtech Medical Limited, Auckland, New Zealand). One millilitre of blood was quickly taken for serum metabolomics, and 10 µL used to prepare slide smears. The remaining 3 mL of blood was preserved in lithium heparin tubes (BD 365966, Phoenix Pharm Distributors, Auckland, New Zealand) and used for haematocrit value determination and PBMC processing. Fish were quickly dissected, and approximately 500 mg of liver, HK and SP sampled into separate 2 mL cryovials (BS20NA-PS, BioStor^{TM}, Interlab, Auckland, New Zealand), snap frozen in liquid nitrogen and stored at −80°C, until metabolite (serum and liver) and immune cytokine transcript expression analysis (HK and SP).

7.3.3 Isolation of fish PBMCs

Fish PBMCs were isolated from 284 µL of blood following a protocol previously published (Lulijwa et al., 2019a) using Histopaque sterile filtered density gradient medium (1.077 g mL^{-1}) (10771-6X100ML, Sigma-Aldrich, Auckland, New Zealand). The 284 µL of heparinised blood was quickly diluted 1:1 with sterile PBS pH 7.4 in duplicate. PBMCs were obtained from blood samples by centrifuging at 971 g for 20 min over a layer of 682 µL Histopaque. Cells at the interface were aspirated with a pipette, pooled and washed twice in 500 µL of sterile PBS by centrifuging at 674 g for 7 min. The resultant cell pellet was re-suspended to a final cell concentration of 1×10^5 cells mL^{-1} in 3 mL PBS supplemented with 2% foetal bovine serum (FBS) (MG-FBS0820-500ML, Mediray, Auckland, New Zealand). This working PBMC suspension was used to monitor for cellular ROS production and phagocytosis.

7.3.4 Differential cell counts

Exactly 10 µL of fresh unheparinised blood was used to prepare slide smears. Slides were left to air dry overnight, fixed with methanol and stained with Giemsa (51811826, Sigma-Aldrich, Auckland, New Zealand). Stained slides were observed under a light microscope at 400× magnification for differential cell counts. Two hundred cells were counted in duplicate and the cellular composition recorded for erythrocytes, thrombocytes, monocytes, lymphocytes and neutrophils. For leucocyte differentials, two hundred cells were counted in duplicate and their composition for lymphocytes, monocytes, thrombocytes, and neutrophils recorded.

7.3.5 Haematocrit value

Lithium heparin preserved blood was drawn into un-heparinised capillary tubes and centrifuged in a Haematokrit 210 Hettich Zentrifugen at 6081 g for 10 min (Kortet et al., 2003) to obtain the packed cell volume.

7.3.6 Fish serum processing

Peripheral blood serum was obtained from 1 mL of blood sample for metabolomics analysis. Blood was centrifuged at 16,250 g for 8 min. Serum supernatant (600 µL) was recovered, transferred into 2 mL cryovials, snap frozen in liquid nitrogen and stored at –80°C until further analysis.

7.3.7 PBMC cell count and viability assessment

A sub-sample of 40 µL of the working cell suspension at 1×10^5 cells mL^{-1} was taken and transferred into 1.5 mL microcentrifuge tube, thereafter, 360 µL of the Muse™ Count and Viability reagent (MPMCH100102, Abacus Dx, Auckland, New Zealand) was added. The mixture was incubated for 5 min at room temperature. The viability profile of the isolated mononuclear cells was determined with a Muse® Count & Viability assay protocol on the Muse® Cell Analyzer in duplicates (Lulijwa et al., 2019a).

7.3.8 ROS production assay

Isolated PBMC intracellular ROS production was measured using the Muse® Oxidative Stress Kit protocol, on the Muse® Cell Analyzer (Abacus Dx, Auckland, New Zealand). A PBMC cell suspension of 20 µL was incubated with 180 µL of Muse® Oxidative Stress working solution following the manufacturer protocols. The sample was mixed thoroughly and run on the Muse® Cell Analyzer to determine ROS production. For the positive control, ROS production was primed by pelleting 100 µL of PBMCs (1×10^5 cells mL^{-1}) suspension. The pellet was resuspended in 100 µL of bacterial lipopolysaccharide (LPS) from *Escherichia coli* serotype O111:B4 (L4391, Sigma-Aldrich, Auckland, New Zealand) solution (10 µg mL^{-1}) in L-15 Medium (Leibovitz, L1518, Sigma-Aldrich, Auckland, New Zealand) (Jørgensen & Robertsen, 1995; Solem et al., 1995) supplemented with 10% FBS and incubated at room temperature for 20 min. Production of ROS was accomplished using the Muse® Oxidative Stress Kit (MPMCH100111, Abacus Dx, Auckland, New Zealand) according to previous work (Lulijwa et al., 2019b).

7.3.9 Phagocytic activity determination

This was accomplished following a modified protocol by Sakai et al. (1995) and previous work (Lulijwa et al., 2019b). Exactly 10 µL of PBMC suspension (1×10^5 cells mL^{-1}) were pipetted into sterile glass microscope slides and incubated at room temperature for 1 h. Non-adherent cells were washed off using PBS with 2% FBS. Then, 10 µL of fluorescent latex beads (1×10^5 mL^{-1}) amine-modified polystyrene suspension (1.0-1.3 µm, L9654, Sigma-Aldrich, Auckland, New Zealand) was added and incubated for 2 h at room temperature. Non-phagocytosed latex beads were washed off using PBS with 2% FBS. The slides were subsequently fixed with absolute methanol and later stained with Giemsa at AUT. Around 100 cells were counted microscopically at 400× magnification and the % phagocytic activity (PA) determined according to the equation:

$$Phagocytosis = (Number\ of\ phagocytic\ PBMCs)/(Number\ of\ total\ PBMCs)\ x\ 100$$

The phagocytic index was determined by the equation:

$$Phagocytic\ Index = (Number\ of\ latex\ beads\ per\ cell\ )/(Number\ of\ phagocytic\ cells)$$

7.3.10 Metabolite extraction, derivatisation, analysis and spectral processing

Fish serum and liver metabolites were extracted and derivatised according to standard protocols (Villas-Boas et al., 2011; Young et al., 2019). Briefly, metabolites were co-extracted with 20 µL L-alanine-2,3,3,3-d4 (d_4 alanine 10 mM for methyl chloroformate [MCF] derivatisation) and ribitol (10 mM for trimethylsilyl [TMS] derivatisation) as internal standards in a cold methanol: water (MeOH:H_2O) solution in series of 50% and 80% methanol. One sample set was derivatised by MCF and a second set by TMS. Derivatised samples were transferred to GC vials for analysis on GC7890-MS5970 system (Agilent Technologies, CA, USA) equipped with a ZB-1701 GC capillary column (Phenomenex) and a quadrupole analyser with an electron impact ion source operated at 70 eV. Metabolite spectra data were deconvulated using a version 2.66 Automated Mass Spectral Deconvolution and Identification System (AMDIS) software integrated with the MassOmics R-based script to interrogate an in-house library of derivatised compounds (Aggio et al., 2011). The internal standards and liver weight were used to normalise for peak intensity and compensate for technical variation prior to statistical analysis.

7.3.11 Molecular assessment of immune genes

7.3.11.1 Organ mRNA expression analysis

For total RNA isolation, 10 mg of RNAlater-ICE (AM7030, Life Technologies, Auckland, New Zealand) stabilised sample was immersed in 350 µL of tissue lysis buffer (3604721001, MagNA Pure LC RNA Isolation Tissue Lysis Buffer, Roche Diagnostics, Auckland, New Zealand). The lysates were homogenised in a microcentrifuge tube with a pipette tip to disaggregate tissues for 5–10 times and vortexed. The lysates were incubated at room temperature for 30 min, and centrifuged at maximum speed (21130 g) for 2 min in an Eppendorf centrifuge 5424R, and the supernatant (200 µL) was carefully collected with a precision pipette into a microcentrifuge tube for RNA extraction. Total RNA was extracted from 200 µL of lysate using the Roche MagNA Pure LC2 instrument with the MagNA Pure LC RNA isolation kit–High Performance (03542394001, Roche Life Sciences, Auckland, New Zealand).

After purification, 100 µL of eluted RNA was collected. Relative quantification of cytokine transcript expression by one-step RT-PCR was performed on the LightCycler 480 instrument II (Roche Diagnostics, Auckland, New Zealand) using the LightCycler® EvoScript RNA SYBR® Green I Master Kit (07800134001, Roche Life Sciences, Auckland, New Zealand), primers (Integrated DNA Technologies, Inc., Singapore) at a working concentration of 0.9 µmol l^{-1} and RNA in a final volume of 20 µL. The reactions were performed using the following thermocycling conditions; 60°C for 15 min (reverse transcription/cDNA synthesis), 95°C for 10 min (transcriptase inactivation and initial denaturation step) and 40 cycles of amplification (95°C for 15 s for denaturation and 60°C for 1 min for annealing and extension).

Upon completion, dissociation/melting curve analyses were performed to reveal and exclude nonspecific amplification or primer-dimer issues (all melting analyses in this study presented single reproducible peaks for each target cytokine transcript suggesting amplification

of a single product). The housekeeping gene used for mRNA expression analysis was β-actin (*actb*) due to its stability within fish tissues. All primer sequences were acquired from previously published sources and can be referred to in (Table 7.1). The relative cytokine transcript expression levels were calculated using the comparative Ct (△△Ct) equation (Schmittgen & Livak, 2008). Organ relative cytokine transcript expression was calculated as $2^{-\triangle\triangle Ct}$ and expressed as a fold-change. The mRNA transcripts for all target cytokine transcripts were normalised to the reference gene (*actb*) within the same sample, condition and time point, and presented as fold-change with respect to the control group.

Table 7.1 Primers used to amplify *Oncorhynchus tshawytscha* genes.

Genes	Sequence (5'–3')	Product size (bp)	Gene function	Reference, gene bank accession number
actb	(F)- GTCACCAACTGGGACGACAT (R)- GTACATGGCAGGGGTGTTGA	175	ATP binding, determines cell shape and controls motility	*O. tshawytscha* FJ890357.1 (Monjo et al., 2017)
ifn1	(F)- AAACTGTTTGATGGGAATATGAAA (R)- CGTTTCAGTCTCCTCTCAGGTT	140	Activate intracellular antimicrobial programmes and elicit development of innate and adaptive immunity	Rainbow trout (*Oncorhynchus mykiss*) AJ580911 (Monjo et al., 2017)
Mx1	(F)- CGGAGTTCGTCTCAACGTCT (R)- CCCTTCCACGGTACGTCTTC	140	Interferon-induced dynamin-like GTPase with antiviral activity against a wide range of RNA viruses and some DNA viruses	*O. mykiss* U30253.1 (Monjo et al., 2017)
ifnγ	(F)- CAACATAGACAAACTGAAAGTCCA (R)- ACATCCAGAACCACACTCATCA	129	Triggers cellular response to viral and microbial infections	*O. tshawytscha* GT897806 (Bjork et al., 2014)
il-1β	(F)- ACCGAGTTCAAGGACAAGGA (R)- CATTCATCAGGACCCAGCAC	181	Potent pro-inflammatory cytokine	*O. tshawytscha* DQ778946 (Bjork et al., 2014)
il-6	(F)- CAGTTTGTGGAGGAGTTTCAGA (R)- TGTTGTAGTTTGAGGTGGAGCA	118	Potent pleiotropic cytokine	*O. mykiss* NM_001124657 (Bjork et al., 2014)
tnf-α	(F)- ACCAAGAGCCAAGAGTTTGAAC (R)- CCACACAGCCTCCATAGCCA	154	Multifunctional pro-inflammatory cytokine	*O. tshawytscha* DQ778945 (Bjork et al., 2014)
il-10	(F)- CTACGAGGCTAATGACGAGC (R)- GATGCTGTCCATAGCGTGAC	97	Plays anti-inflammatory roles, helping to limit excessive tissue damage caused by inflammation	*O. mykiss* AB118099 (Bjork et al., 2014)

F: forward primer; R: reverse primer; bp: base pair; *actb*: β-Actin; *ifn1*: type 1 interferon 1; Mx1: Mx1 protein; *ifnγ*: interferon gamma; *il-1β*: interleukin 1beta; *il-6*: interleukin 6; *tnf-α*: tumor necrosis factor alpha; *il-10*: interleukin 10.

7.3.12 Data processing and statistical analysis

Data from haematological parameters and PBMC cellular functional properties were processed to assess the effect of *in vivo* poly (I:C) i.p. injection on fish immune system by one-way time series analysis of variance (ANOVA) using Minitab 17 statistical software at $p = 0.05$ and Dunnett's multiple comparison test. Relative gene expression was calculated using the "delta-delta method" (Schmittgen & Livak, 2008) as explained above and statistical significance determined by one-way time series ANOVA using Minitab 17 statistical software at $p = 0.05$.

For metabolomics, all data were processed using MetaboAnalyst 4.0 a web based comprehensive tool for metabolomics data analysis (Chong et al., 2018). Data were normalised by auto-scaling and one-way time series ANOVA [$p < 0.05$ and false discovery rate (FDR ≤ 0.1)] used to identify differences in metabolite profiles between control fish (day 0) and poly (I:C) i.p. injected fish (days 1-5). Significantly altered metabolites were tested for differences (Dunnett's

test) between control and poly (I:C) treated fish using Minitab 17 at $p < 0.05$. A respective heat map assessment of the ANOVA test was generated. Unsupervised multivariate data analysis was explored using principal component analysis (PCA) to find possibilities of inherent clustering, and a trajectory plot generated using the grand means and standard errors of daily data using R for windows version 3.6.1 (Li, 2017).

7.4 Results

7.4.1 Peripheral blood haematological parameters

Prolonged poly (I:C) i.p. injection induced significant ($p < 0.05$) effects on *O. tshawytscha* haematological parameters except for thrombocyte and lymphocyte counts. Significantly ($p < 0.05$) lower erythrocyte counts were observed on day three and four post-stimulation. In addition, higher monocyte and neutrophil counts were observed at day three and four respectively (Figure 7.1), while no effect was observed on fish haematocrit value, despite alterations from the control (Table 7.2). All assessed peripheral blood parameters exhibited a recovery trend by day five.

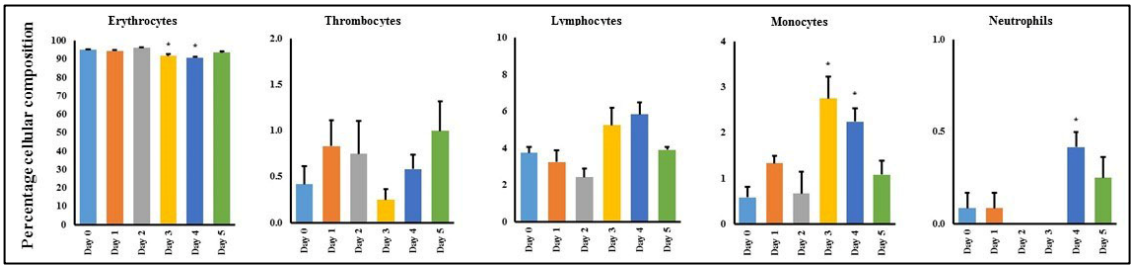


Figure 7.1 Fish peripheral blood differential cell counts following *in vivo* i.p. injection with 2.5 µL g^{-1} fish of poly (I:C). Data come from mean±SEM of duplicate counts of 200 cells on giemsa stained slides of three control (day 0) and three treatment fish each day (1-5) post-poly (I:C) administration. Bars with asterisk are significantly different from the control (day 0), one-way time series ANOVA at $p = 0.05$, with Dunnett's multiple comparison test.

7.4.2 Differential leucocyte and thrombocyte cell counts

Poly (I:C) significantly ($p < 0.05$) induced alterations in differential leucocyte and thrombocyte counts (Figure 7.2). Compared with the control, higher ($p < 0.05$) thrombocyte counts were observed at day two, while lymphocytes were depressed on days two, three and five post-challenge. Poly (I:C) induced significant ($p < 0.05$) elevation in monocyte counts on day one and day three, while neutrophils increased from day three to five in treated fish (Figure 7.2).

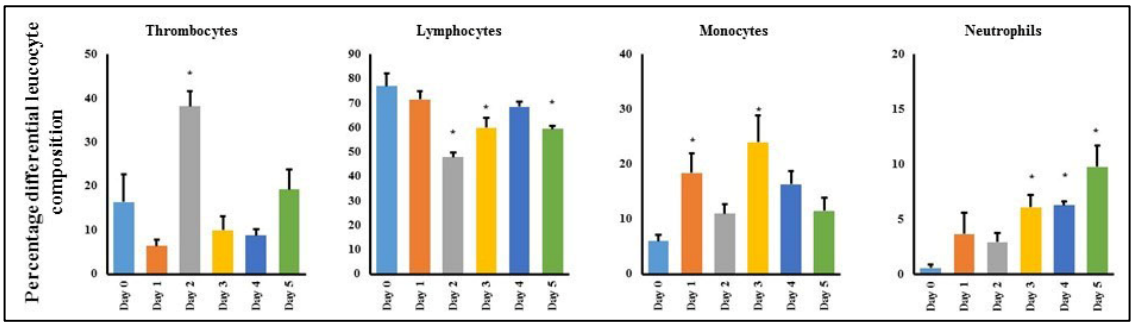


Figure 7.2 Fish leucocyte and thrombocyte differential counts following *in vivo* i.p. injection with 2.5 µL g^{-1} fish of poly (I:C). Data come from mean±SEM of duplicate counts of 200 cells on giemsa stained slides of three control (day 0) and three treatment fish each day (1-5) post-poly (I:C) administration. Bars with asterisk are significantly different from the control (day 0), one-way time series ANOVA at $p = 0.05$, with Dunnett's multiple comparison test.

7.4.3 Fish PBMC cellular functional characterisation

Poly (I:C) administration induced significant ($p < 0.05$) PBMC ROS production, in treated fish at day one to day four, post-challenge. On the other hand, the treatment did not significantly affect fish PBMC viability, phagocytosis and phagocytic index profile over the entire exposure period, despite the observed general increase in these parameters post-poly (I:C) treatment (Table 7.2).

Table 7.2 Cellular functional characteristics and haematocrit value of *Oncorhynchus tshawytscha* PBMCs following *in vivo* i.p. injection with 2.5 µL g^{-1} fish of poly (I:C). Data come from mean±SEM duplicate readings of three control (day 0) and three treatment fish each day (1-5) post-poly (I:C) administration for haematocrit value (%), viability (%) and ROS (%); from counts of 100 cells for phagocytosis (%) and from phagocytic cells for phagocytic index. Values in a column with asterisk are significantly different from the control (day 0), one-way time series ANOVA at $p = 0.05$ with Dunnett's multiple comparison test.

Time	Viability	ROS production	Phagocytosis	Phagocytic Index	Haematocrit value
Day 0	46.31±8.61	10.92±1.28	25.00±2.00	2.39±0.36	39.63±0.61
Day 1	54.60±6.67	38.95±8.09*	28.00±1.53	2.12±0.50	28.30±2.54
Day 2	62.33±9.26	38.10±7.05*	30.33±3.85	2.55±0.50	29.83±1.55
Day 3	42.00±5.63	46.80±2.28*	28.33±7.33	2.76±0.25	34.20±4.33
Day 4	60.88±8.89	59.68±8.05*	30.33±2.73	2.41±0.10	29.48±0.34
Day 5	66.42±9.54	16.88±2.17	26.67±2.73	1.84±0.25	39.62±4.45
F-value	1.12	10.50	0.29	0.82	3.31
P-value	0.373	<0.001	0.909	0.557	0.042

7.4.4 Fish serum and liver metabolomics

A total of 201 and 187 metabolic features were found in liver (Table 7.5S) and serum (Table 7.6S) respectively after GC-MS analysis. Among them, 45 unique features are currently "unknown" since their retention times and mass spectral signatures could not be matched to known compounds in our database. Unsupervised multivariate data analysis was applied using principal component analysis (PCA) to investigate metabolite variations induced by poly (I:C) i.p. injection over a period of five days. An overview of liver PCA trajectory plot shows major metabolite alterations one day post-stimulation (Figure 7.3a and b). From day two to three post-challenge, metabolites closely fluctuated in magnitude to levels observed at day 0. A second spike in metabolites appears on day four, followed by a trend towards baseline levels by day five (Figure

7.3a). The first two principal components (PC1 and PC2) explain 37.2% and 18.1% of total variance in the data, respectively.

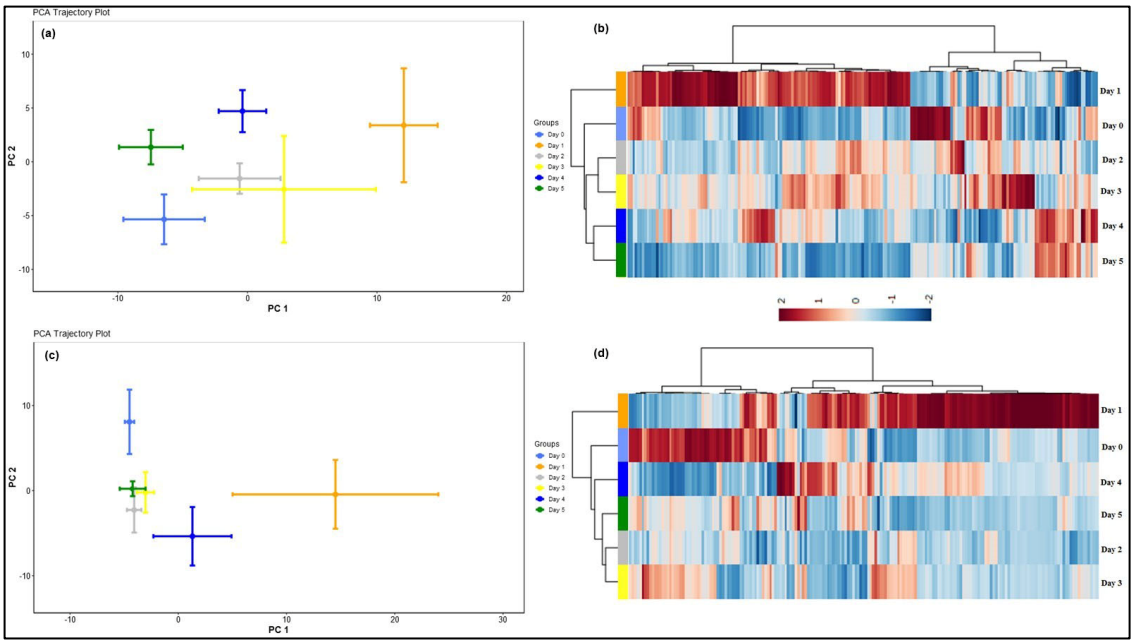


Figure 7.3 Principal component analysis score trajectory plot and heat map for a and b liver and c and d serum metabolic features following *in vivo* i.p. injection of *Oncorhynchus tshawytscha* with 2.5 µL g^{-1} fish of poly (I:C). Data come from means of three control (day 0) and three treatment fish each day (1–5) post-poly (I:C) administration.

Similarly, the PCA trajectory plot of serum features, reflects major alterations at day one post-challenge (Figure 7.3c and d). Serum metabolic features closely fluctuated from day two to three, followed by a second major alteration at day four and a trend towards baseline levels by day five. The first two principal components (PC1 and PC2) explain 46.1% and 19.9% of total variance in the data, respectively (Figure 7.3c) (for detailed individual metabolite data, see Figure 7.9S and Figure 7.10S for liver and serum respectively).

Table 7.3 List of significantly altered liver metabolic features identified by one-way time series ANOVA ($p < 0.05$ and FDR ≤ 0.1) analysis following *in vivo* i.p. injection administration with 2.5 µL g^{-1} fish of poly (I:C). Data come from mean±SEM of three control (day 0) and three treatment fish each day (1-5) post-poly (I:C) administration. (↑: upregulated; ↔: no change and ↓: downregulated metabolites). Features in a row with asterisk are significantly different from the control, one-way time series ANOVA at $p < 0.05$, with Dunnett's multiple comparison test.

Number	Metabolites	F.value	p.value	FDR	Day 1	Day 2	Day 3	Day 4	Day 5
1	D-talo-hexose	18.167	<0.001	0.006	↓*	↓*	↓*	↓*	↓*
2	Alpha-L-galactopyranose, 6-deoxy-1,2,3,4-tetrakis-O-trimethylsilyl-	15.666	<0.001	0.007	↓*	↓*	↓*	↓*	↓*
3	Creatinine	12.076	<0.001	0.010	↑*	↑	↑*	↑	↓
4	Alpha.-D-galactoside, methyl tetrakis-O-trimethylsilyl-	11.732	<0.001	0.010	↓*	↓*	↓*	↓*	↓*
5	Proline	11.496	<0.001	0.010	↑*	↔	↑	↔	↓
6	D-glucose	10.784	<0.001	0.012	↓*	↓*	↓*	↓*	↓*
7	Phosphorylethanolamine	10.315	0.001	0.013	↑	↑	↑	↑*	↑*
8	Dimethyl 2-aminomalonate hydrochloride	8.108	0.002	0.034	↑*	↑*	↑*	↑	↑
9	2-aminobutanoic acid	7.424	0.002	0.043	↑	↑*	↑*	↑	↑
10	Unknown 126	7.313	0.002	0.043	↑*	↑	↑	↑*	↑
11	Unknown 112	6.909	0.003	0.050	↑*	↑	↑*	↑	↓
12	Glycine	6.711	0.003	0.052	↑*	↑	↑*	↑	↑
13	Glutaric acid	6.476	0.004	0.054	↑	↑	↑	↑*	↑
14	Unknown 102100 580	6.347	0.004	0.054	↑	↓	↓	↓	↓
15	4-hydroxyphenylacetic acid	6.327	0.004	0.054	↑*	↑	↑	↑	↓
16	Isoleucine	6.133	0.005	0.058	↑*	↑	↑	↑	↓
17	Unknown 128	6.017	0.005	0.059	↑*	↓	↑	↓	↓
18	Leucine	5.856	0.006	0.060	↑*	↑	↑	↑	↓

19	Valine	5.834	0.006	0.060	↑*	↑	↑	↑	↓
20	Nicotinic acid	5.547	0.007	0.066	↓*	↓*	↓	↓*	↓*
21	Urea	5.175	0.009	0.078	↑*	↑	↑	↑	↑
22	Unknown 102100 326	5.020	0.010	0.084	↑	↓	↑	↓	↓
23	2-ketoglutaric acid	4.788	0.012	0.096	↓	↓	↓	↑	↑

Using one-way time series ANOVA ($p < 0.05$, FDR ≤ 0.1), poly (I:C) injection significantly altered 23 liver (Table 7.3) and 13 serum features post-stimulation (Table 7.4). In liver, six metabolites (1, 2, 4, 6, 20 and 23) were downregulated, while 12 metabolites (3, 5, 7, 8, 9, 12, 13, 15, 16, 18, 19 and 21) were upregulated. In addition, five of the upregulated features, one day post-challenge were unknown (10, 11, 14, 17 and 22). From day two to five, five metabolites (1, 2, 4, 6 and 20) remained significantly lower in comparison to the control. Only levels for metabolite (8) remained significantly elevated from day one to three post-challenge. In addition, levels for metabolite (9) increased from day two to three; while features (3, 11, and 12) increased at day three. Four days post-challenge, significant upregulation was only observed for features (10, 13, and 23); while levels for metabolite (7) significantly increased from day four to day five (Table 7.3 and Figure 7.4).

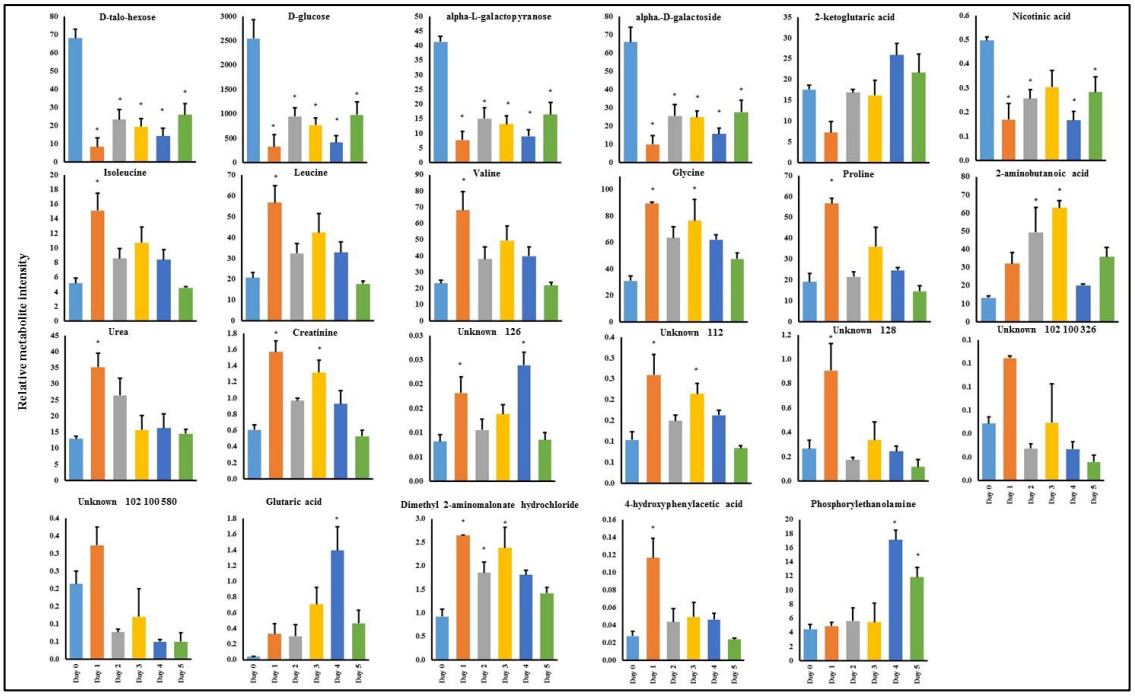


Figure 7.4 List of significantly altered liver metabolic features identified by one-way time series ANOVA ($p < 0.05$ and FDR ≤ 0.1) analysis following *in vivo* i.p. injection administration with 2.5 µL g^{-1} fish of poly (I:C). Data come from mean±SEM of three control (day 0) and three treatment fish (day 1-5) post-poly (I:C) *in vivo* administration. Data bars with asterisk are significantly different from the control (day 0), one-way time series ANOVA at $p = 0.05$, with Dunnett's multiple comparison test.

Table 7.4 List of significantly altered serum metabolic features identified by time series ANOVA ($p < 0.05$ and FDR ≤ 0.1) analysis following *in vivo* i.p. injection administration with 2.5 µL g^{-1} fish of poly (I:C). Data come from mean±SEM of three control (day 0) and three treatment fish each day (day 1-5) post-poly (I:C) administration. (↑: upregulated; ↔: no change and ↓: downregulated metabolites). Metabolites in a row with asterisk are significantly different from the control, one-way time series ANOVA at $p < 0.05$, with Dunnett's multiple comparison test.

Number	Altered metabolites	F.value	p.value	FDR	Day 1	Day 2	Day 3	Day 4	Day 5
1	Unknown 201	15.583	<0.001	0.007	↑*	↑	↓	↔	↓
2	Succinic acid	15.428	<0.001	0.007	↑*	↓*	↓*	↓*	↓*
3	Ethanolamine	9.104	0.001	0.056	↓	↓*	↓*	↓*	↓*
4	Beta-D-glucopyranose	7.356	0.002	0.105	↓*	↓*	↓*	↓*	↓
5	Unknown 112	6.438	0.004	0.105	↑*	↑	↑	↑	↑
6	Unknown 059	6.424	0.004	0.105	↓*	↓*	↓	↓	↓
7	D-glucose	6.412	0.004	0.105	↓*	↓*	↓	↓*	↓
8	Isoleucine	5.920	0.006	0.105	↑*	↑	↑	↑	↔
9	Creatinine	5.839	0.006	0.105	↑*	↑	↑	↑	↑
10	D-Mannose	5.709	0.006	0.105	↓*	↓*	↓*	↓*	↓
11	Unknown 045	5.604	0.007	0.105	↑	↓	↓	↓	↓
12	Unknown 113	5.537	0.007	0.105	↑*	↓	↔	↔	↓
13	Unknown 198	5.512	0.007	0.105	↑*	↓	↑	↑	↓

In fish serum, poly (I:C) *in vivo* resulted in increased levels of eight features (Table 7.4 and Figure 7.5), one day post-challenge. Three of the elevated metabolites were identifiable (2, 8 and 9) and five features were unknown (1, 5, 11, 12 and 13). In addition, four features, three identifiable (4, 7, and 10) and one unknown (6) decreased, one day after treatment (Table 7.4 and Figure 7.5). From day two to day four, levels for downregulated metabolites (4, 7 and 10), remained significantly lower. Furthermore, metabolites (2 and 3) decreased from day two to day five (Table 7.4 and Figure 7.5).

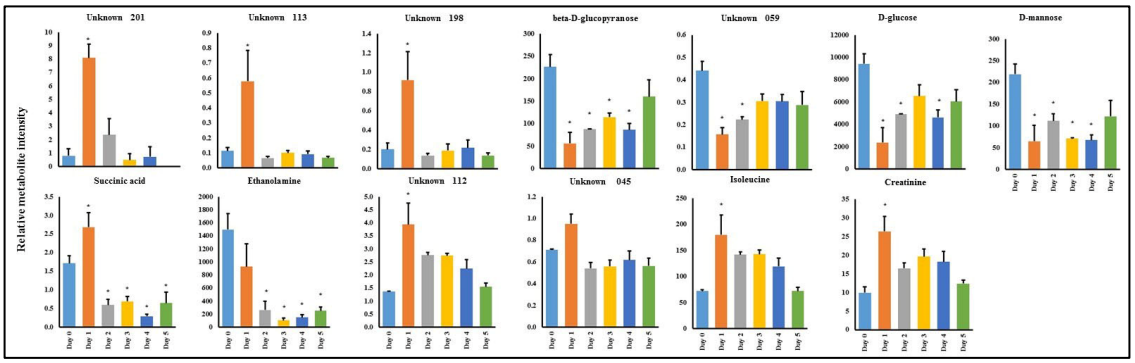


Figure 7.5 List of significantly altered serum metabolic features identified by one-way time series ANOVA ($p < 0.05$ and FDR ≤ 0.2) analysis following *in vivo* i.p. injection administration with 2.5 µL g^{-1} fish of poly (I:C). Data come from mean±SEM of three control (Day 0) and three treatment fish (day 1-5) post-poly (I:C) *in vivo* administration. Data bars with asterisk are significantly different from the control (day 0), one-way time series ANOVA at $p = 0.05$, with Dunnett's multiple comparison test.

7.4.5 Fish head kidney and spleen mRNA immune gene expression

Relative expression levels of HK cytokines in *O. tshawytscha* injected with poly (I:C) are illustrated (Figure 7.6). Poly (I:C) upregulated all target cytokine transcripts in fish HK. However, only cytokine transcripts for *il-10*, Mx1 protein, *ifnγ*, and *il-1β* significantly ($p < 0.05$) peaked on day one, two and four post-challenge respectively. Despite upregulation in treated fish, no significant ($p > 0.05$) changes were noted from the control group in *tnf-α*, *ifn1* and *il-6* (Figure 7.6).

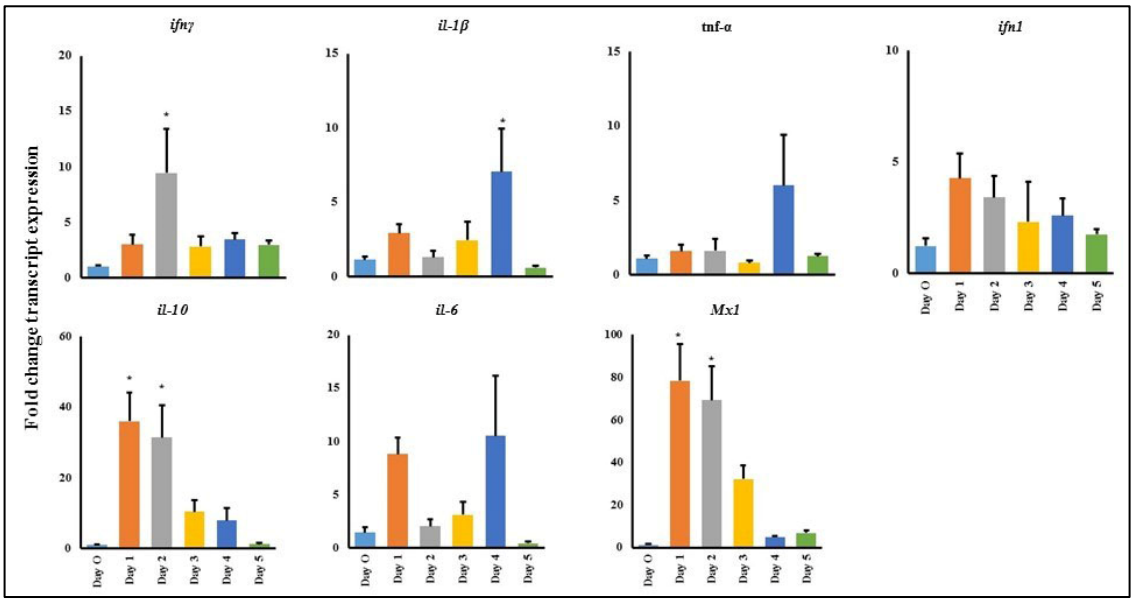


Figure 7.6 Expression mRNA HK cytokines by RT-PCR following *in vivo* i.p. injection of *Oncorhynchus tshawytscha* with 2.5 µL g^{-1} fish of poly (I:C). Data come from mean±SEM of three control (day 0) and three treatment fish (day 1-5) post-poly (I:C) *in vivo* administration. Data bars with asterisk are significantly different from the control (day 0), one-way time series ANOVA at $p = 0.05$, with Dunnett's multiple comparison test.

In fish SP samples, poly (I:C) altered all target cytokine transcript levels during one to five days post-challenge (Figure 7.7). However, only cytokine transcript levels for *il-10*, *il-6* and antiviral Mx1 protein significantly ($p < 0.05$) peaked on days one and two post-challenge, respectively, and appeared to return to baseline levels by day five. However, no significant ($p > 0.05$) alterations were observed in *ifnγ*, *il-1β*, *tnf-α*, and *ifn1* in treated fish at the different time points (Figure 7.7).

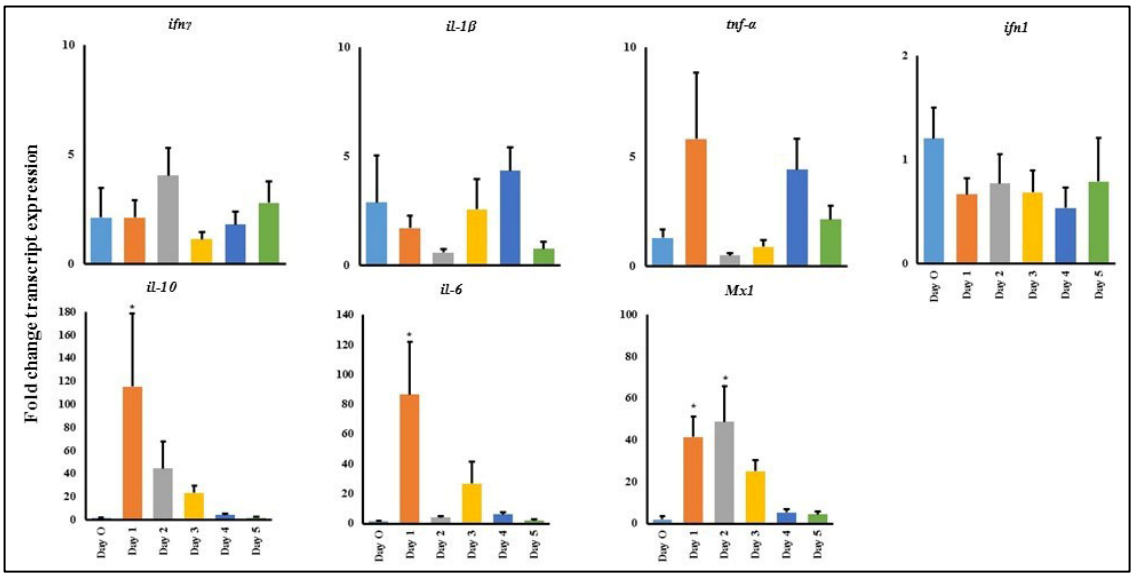


Figure 7.7 Expression mRNA SP cytokines by RT-PCR following *in vivo* i.p. injection of *Oncorhynchus tshawytscha* with 2.5 µL g^{-1} fish of poly (I:C). Data come from mean±SEM of three control (day 0) and three treatment fish (day 1-5) post-poly (I:C) *in vivo* administration. Data bars with asterisk are significantly different from the control (day 0), one-way time series ANOVA at $p = 0.05$, with Dunnett's multiple comparison test.

7.5 Discussion

This study investigated the immunomodulatory effects of *in vivo* poly (I:C) administration over a five-day post-exposure period on *O. tshawytscha*. An integrated approach was employed involving classical peripheral blood haematology, PBMC latex bead phagocytosis and phagocytic index, flow cytometry cellular viability and functional ROS production. Data were also collected using GC-MS-based metabolomics profiling of serum and liver samples, and targeted RT-PCR analysis of immune cytokine expression in HK and SP samples.

Poly (I:C) significantly depressed *O. tshawytscha* erythrocyte counts at days three and four post-injection, which recovered to baseline levels by day five. The reduction in erythrocyte counts may be linked to immunological involvement as previously reported, with poly (I:C) and infectious salmon anaemia virus (ISAV) in teleosts (Lulijwa et al., 2020a; Morera et al., 2011; Y. Shen et al., 2018; Wessel et al., 2015; Workenhe et al., 2008). Evidences of viral PAMPs binding receptors *tlr3*, and RIG-I, have been reported in fish erythrocytes (Morera et al., 2011; Rodriguez et al., 2005; Wessel et al., 2015), and respiratory globins strongly linked with immune function in vertebrates and invertebrates (Iwanaga, 2002; Jiang et al., 2007; Liepke et al., 2003). At the same time point (day three and four), when erythrocyte counts were depressed, significantly higher monocyte and neutrophil counts were recorded, which is suggestive of poly (I:C) induced innate immune stimulation. The quantification of neutrophils in circulating peripheral blood is a strong indicator of enhanced immunocompetence (Neumann et al., 2001). In a previous study, we did not find quantifiable neutrophils in peripheral blood of apparently healthy *O. tshawytscha* smolts (Lulijwa et al., 2019a). Furthermore, poly (I:C) enhanced thrombocyte counts two days post-injection could be indicative of enhanced clotting capacity (Espelid et al., 1996). Elevation in differential monocyte counts on days one and three, in combination with higher neutrophil counts at day three to five post-injection, also suggest enhanced capacity to mount an immune response. It is noted that while other leucocyte populations appeared to return to baseline levels by day five, neutrophil counts remained elevated. Overall, trends in erythrocyte, thrombocyte, lymphocyte, monocyte and neutrophil counts suggest that poly (I:C) enhanced innate immune parameters and cellular migration [similar with bacterial challenges (Costas et al., 2013)] and/or proliferation, corresponding with observed alterations at a metabolic level. Proliferating cells exhibit enhanced nutrient demand to supply energy, to make and translocate molecules across cellular and tissue membranes [reviewed in Fritsch and Weichhart (2016)].

Analysis of cellular functional parameters revealed enhanced ROS production in PBMCs of treated fish, with a biphasic response at days one and four post-challenge. The initial peak in ROS production is due to direct response to poly (I:C) in combination with IFN induction. The second ROS peak may be associated with elevated levels of liver glutaric acid simultaneously observed at that time point (Jacobsen & Hannibal, 2019). According to Shi et al. (2018), mitochondrial ROS production has been implicated in *il-1β* stimulation in higher vertebrates, and

this cytokine increased in treated fish HK and SP samples. In support of the observed results for ROS production, *ifnγ* transcript increased in poly (I:C) treated fish HK and SP. In previous studies, *ifnγ* has been reported to facilitate respiratory burst following poly (I:C) treatment *in vitro* (Grayfer & Belosevic, 2009) and *in vivo* (Zhou et al., 2014; Zhu et al., 2019). Specifically, poly (I:C) administration at a dose of 10 μg g^{-1} fish body weight significantly induced respiratory burst activity at day one post-injection in *P. olivaceus* via *ifnγ* stimulation (Zhou et al., 2014). Also, earlier *in vitro* work with *C. auratus* enhanced macrophage and neutrophil respiratory burst activity and intracellular NO production (Grayfer & Belosevic, 2009). Although poly (I:C) did not significantly alter fish PBMC viability, phagocytosis and phagocytic index, a general increase in these parameters was recorded in this study. Elsewhere, *in vitro* poly (I:C) administration in *C. auratus* increased phagocytic activity (Grayfer & Belosevic, 2009). Thus, poly (I:C) induced temporal immune response via ROS production, which returned to baseline by day five.

While several studies have demonstrated the immunomodulatory effects of poly (I:C) administration at cellular levels in *O. tshawytscha in vitro* (Jensen et al., 2002b; Monjo et al., 2017; Semple et al., 2018), little is known about immuno-metabolic responses to poly (I:C) in this species *in vivo* (Lulijwa et al., 2020a). GC-MS-based metabolomics profiling of liver and serum demonstrated alterations in 23 and 13 metabolites, respectively, over the five days post-exposure. Perturbed metabolic signatures are involved in pathways associated with amino acid (AA) metabolism and protein biosynthesis, glycolysis, the Citric acid cycle, ROS regulation and lipid metabolism.

AAs serve as protein building blocks and as catabolic substrates to provide energy for several biochemical processes (Ballantyne, 2001). Alterations observed in poly (I:C) exposed fish, supports AA use for energy production and also suggests changes to several other pathways. In fish liver, the branched chain amino acids (BCAA) isoleucine, leucine, and valine, along with AA catabolic products urea and creatinine, accumulated one day post-challenge. In fish serum, results only reflected isoleucine and creatinine increases at day one. Except for liver creatinine at day three, these increased metabolites remained elevated, but their levels were similar to baseline from day two to four and appeared to normalise by day five. BCAAs are essential for vertebrate lymphocytes, eosinophils and neutrophils as substrates for energy, protein, RNA and DNA biosynthesis; with lymphocytes exhibiting enhanced proliferation and demand for these AAs following stimulation (Calder, 2006). We identified increased levels of free BCAAs in fish liver, which are associated with protein energy metabolism (Roques et al., 2018c; Wagner et al., 2014). Liver 2-aminobutanoic acid increased from day two and peaked at day three. This is a non-proteinogenic AA produced during transamination of 2-oxobutyric acid (a metabolite involved in isoleucine biosynthesis) (Wishart et al., 2018), which accumulated due to metabolism of BCAAs. In addition, accumulation of liver creatinine and urea indicates muscle phosphocreatine and AA metabolism to supply energy (Salazar, 2014) and of poly (I:C) induced alterations in liver and kidney functions (Ajeniyi & Solomon, 2014). Similarly, observed accumulation of plasma

creatinine further suggests poly (I:C) induced muscle catabolism to keep with enhanced energy demand. These results are in line with enhanced cellular profile of thrombocytes, lymphocytes, monocytes and neutrophils, which could have increased demands for nutrients to supply energy [reviewed in Fritsch and Weichhart (2016)]. By day five, BCAA activity, creatinine and urea levels showed a return to baseline levels, a trend that was also observed in leucocyte counts.

Poly (I:C) also caused an increase in liver glycine, and proline, one day post *in vivo* stimulation. Glycine directly scavenges radicals (Fang et al., 2002) and can be used to produce glutathione, a strong antioxidant (Hall, 1998). Glycine mediates its immunomodulatory effects through its glycine receptor (glycine-gated chloride channels), with which, it controls intracellular Ca^{2+} concentration, and suppresses activation of transcription factors, with consequent regulation of inflammatory cytokine and ROS production (Wang et al., 2013; Zhong et al., 2003). Accumulation of proline may be indicative of poly (I:C) induced collagen degradation, to facilitate proline-based electron production to generate adenosine triphosphate (ATP) for survival under stressful conditions (Phang et al., 2010).

Carbohydrates, AAs and fatty acids (FA) are used by cells to generate ATP through the central respiratory machineries of glycolysis and the krebs cycle or citric acid cycle (Vander Heiden et al., 2009). The glycolytic pathway in particular is the primary route for glucose catabolism to supply ATP needed for multitudes of biochemical processes (Enes et al., 2009). In fish liver, poly (I:C) exposure dramatically decreased levels for D-talo-hexose, D-glucose, alpha-L-galactopyranose, and alpha-D-galactoside from days one to five post-challenge. Similar observations were noted in fish serum, with the carbohydrates beta-D-glucopyranose, D-glucose and D-mannose decreasing from days one to four post-challenge. These sugars are core metabolites within glycolysis/gluconeogenesis pathway topology, and are involved in galactose, starch and sucrose metabolism previously identified as being modulated by poly (I:C) exposure in *O. tshawytscha* (Lulijwa et al., 2020a).

These findings suggest that poly (I:C) enhanced fish energy requirements, as reported previously in proliferating cells (Fritsch & Weichhart, 2016). Proliferating cells demand for energy, needed to produce and transport cytokines during infection (D. Wu et al., 2016) and by several enzymes involved in other biochemical processes such as AA and protein biosynthesis. In agreement with these results, viral infected Chinese shrimp (*Fenneropenaeus chinensis*), and poly (I:C) challenged giant freshwater prawn (*Macrobrachium rosenbergii*) exhibited enhanced energy metabolism (Li et al., 2014; Saray et al., 2018). Thus far, elevated energy demands suggest that poly (I:C) potentially induced "aerobic glycolysis" (Warburg effect) to simulate conditions similar to those observed during intracellular viral replication (Eisenreich et al., 2019) (see postulated pathways in Figure 7.8). Observations at metabolic level appear to align with those at cytokine transcript and cellular levels, since *ifnγ* driven cellular differentiation to enhance immunity (Langevin et al., 2019) is energy demanding (Fritsch & Weichhart, 2016). Aerobic glycolysis has been reported before, as poly (I:C) and IFN stimulated macrophages exhibited a

shift from oxidative phosphorylation [reviewed in Kelly and O'Neill (2015)]. Similarly, thrombocytes, monocyte and neutrophil counts increased in poly (I:C) treated fish, which is suggestive of enhanced fish immunity, and the elevated energy demands observed here. However, enhanced energy demands persisted through the post-exposure period.

In line with the observed metabolic reprograming (aerobic glycolysis), following poly (I:C) treatment, breaks in the Citric acid cycle, manifested as accumulation of intermediate metabolites such as succinic acid, previously reported in mammalian macrophages (Jha et al., 2015) were observed. In fish liver, poly (I:C) induced a dramatic reduction in levels of 2-ketoglutaric acid one day post-stimulation. Levels were re-established by day two, and elevated at day four, suggesting disturbance to citric acid cycle functioning. The dramatic decrease in 2-ketoglutaric acid potentially suggests first break in citric acid cycle conversion of isocitric acid into 2-ketoglutaric acid by the enzyme isocitrate dehydrogenase (IDH), which resulted in the production of nucleotides for cytokine mRNAs and phospholipids needed by proliferating leucocytes (O'Neill & Pearce, 2016) observed in this study. In fish serum, elevated succinic acid levels one day post-poly (I:C) challenge also indicates citric acid cycle disturbance, and reduced energy production; since this metabolite yields less nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) (Chen et al., 2019) in several metabolic processes (Kobayashi & Shimizu, 1999; Yang & Sauve, 2016). Initial accumulation of succinic acid is indicative of a second break in the citric acid cycle (Gaber et al., 2017), which results from elevated glycolytic activity due to *ifnγ* upregulation, where succinic acid in turn promotes mitochondrial ROS activities [reviewed in Fritsch and Weichhart (2016)]. Accumulation of succinic acid was previously reported in viral challenged oysters (Young et al., 2017) bacterial exposed mussels (Nguyen, Alfaro, Young, et al., 2018) and in pro-inflammatory macrophages (O'Neill & Pearce, 2016). Succinic acid levels decreased below baseline levels from day two to day five, probably to limit cellular damage.

In teleosts, several studies have demonstrated *ifnγ* induced ROS production following poly (I:C) administration *in vitro* (Grayfer & Belosevic, 2009) and *in vivo* (Zhou et al., 2014; Zhu et al., 2019). In this perspective, 2-ketoglutaric acid is reportedly important in alleviating oxidative stress (Liu et al., 2018). Thus, changes observed in 2-ketoglutaric acid over the five-day post-exposure period is associated with enhanced ROS production observed at the cellular level. In this regard, 2-ketoglutaric acid directly reacts with hydrogen peroxide, generating succinic acid (Liu et al., 2018). Consequently, succinic acid increased in fish serum, at day one post-stimulation and reportedly promotes mitochondrial ROS activities [reviewed in Fritsch and Weichhart (2016)]. Furthermore, 2-ketoglutaric acid also scavenge nitrogen, to support the production of glutamate and glutamine (N. Wu et al., 2016). In addition, liver glutaric acid, a 2-ketoglutaric acid precursor, increased at day four post-poly (I:C) exposure. Build-up in glutaric acid enhances ROS production (Jacobsen & Hannibal, 2019), which corresponds with a peak in ROS levels at day four post-poly (I:C) stimulation. As a result, the ROS peak at day four, likely led to accumulation

of 2-ketoglutaric acid, probably due to ROS related inhibition of alpha-ketoglutarate dehydrogenase (α-KGDH), an enzyme that converts 2-ketoglutaric acid to succinyl-CoA [reviewed in Tretter and Adam-Vizi (2005)]. ROS induced inhibition of α-KGDH reduces NADH production and supply in the electron transport chain (ETC) [reviewed in Tretter and Adam-Vizi (2005)]. In response to observed changes in ROS, free radical scavengers glycine and proline increased in fish liver one day post *in vivo* stimulation (Fang et al., 2002; Hall, 1998; Phang et al., 2010).

In line with elevated demand for energy demonstrated at AA and sugar levels, in combination with disturbances to citric acid cycle functioning; liver nicotinic acid levels decreased from day one to five. This result suggests increased demand in the formation of nicotinamide, required in generation of coenzymes nicotinamide adenine (NAD^+), and nicotinamide adenine dinucleotide phosphate ($NADP^+$), required in several biochemical processes. This could be linked to the increased demand for electron transporters in glycolysis, the Citric acid cycle, and during oxidative phosphorylation, among other processes (Yang & Sauve, 2016), following poly (I:C) induced interferon stimulation (Fritsch & Weichhart, 2016). Observed reduction in 2-ketoglutaric acid, nicotinic acid and accumulation of succinic acid one day post-poly (I:C) stimulation suggest induction of "aerobic glycolysis" observed as sustained energy demand throughout the five days post-exposure.

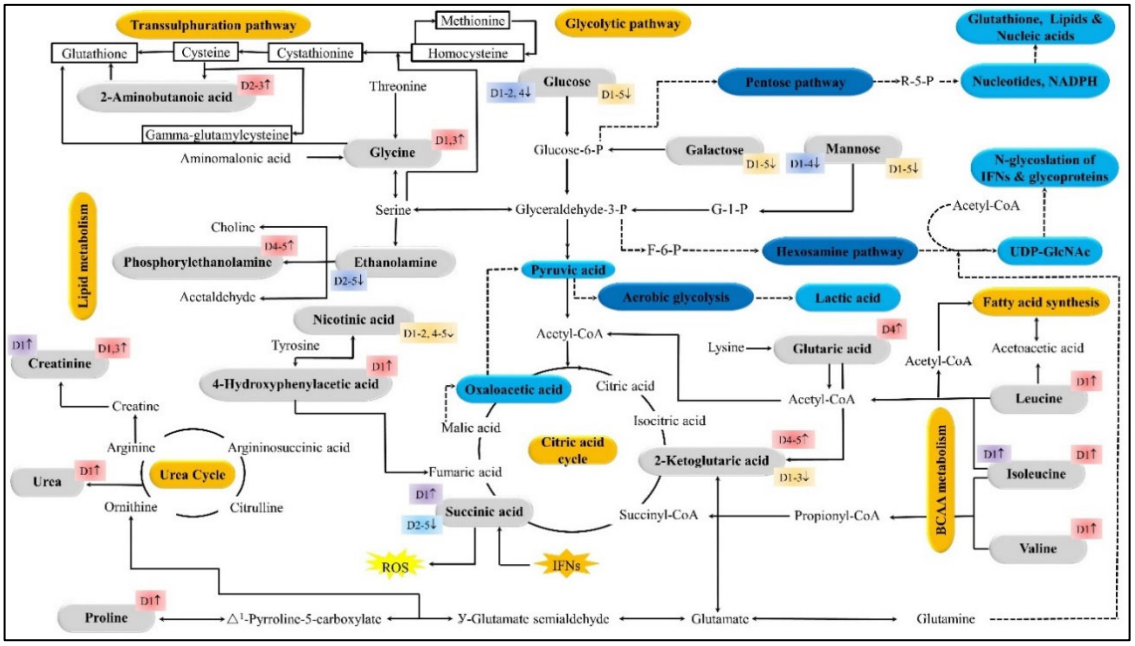


Figure 7.8 Schematic of liver and serum altered metabolite profile following prolonged *in vivo* i.p. injection exposure of *Oncorhynchus tshawytscha* with 2.5 µL g^{-1} fish of poly (I:C). Upregulated (↑) metabolites are indicated in red (liver), purple (serum), while downregulated (↓) metabolites are shown in yellow (liver) and blue (serum). Pathways that were directly affected by poly (I:C) are shown in orange, and those hypothesised to be affected are indicated in deep blue (with respective metabolites in light blue). For abbreviations, R-5-P: ribose-5-phosphate; F-6-P: fructose-6-phosphate; UDP-GlcNAc: uridine diphosphate-N-acetylglucosamine; IFNs: interferons; ROS: reactive oxygen species and D: indicates day 1-5.

Proliferating cells synthesise new phospholipids from diacylglycerols and polar groups like choline or ethanolamine (van Meer et al., 2008). It is possible that "aerobic glycolysis" favoured the pentose pathway at the glucose-6-phosphate shunt to enhance lipid metabolites for incorporation into cellular structures. Results demonstrate that poly (I:C) induced increases in liver phosphorylethanolamine levels from day four to five post-challenge. This metabolite is a precursor for phospholipid synthesis (Wishart et al., 2018). In agreement, levels for phosphorylethanolamine head group ethanolamine (Patel & Witt, 2017) consistently reduced in fish serum from day two to five in treated fish, suggesting incorporation into phospholipid membrane biosynthesis. These notions suggest that poly (I:C) induced viral mimics, involving phospholipid membrane remodelling to favour viral replication, similar to conditions observed in virus infected fish (Schiøtz et al., 2009). The above alterations in liver and serum phospholipid metabolism further strengthen the notion that poly (I:C) induced "aerobic glycolysis", as previously reported in higher vertebrate dendritic cells (Everts et al., 2014). Increased lipid metabolism is necessary to support development of cellular membranes and organelles, including biosynthesis and translocation of molecules (Everts et al., 2014).

Accumulation was also observed for organic acid dimethyl 2-aminomalonate hydrochloride from day one to three with a profile similar to that of altered AAs. Furthermore, there were increased levels for 4-hydroxyphenylacetic acid, one day post-challenge. However, the mechanism underlying upregulation of these metabolites is still poorly understood. In addition, five unknown compounds were found in the liver and serum of poly (I:C) treated fish with a similar profile to that observed in the AAs.

From these metabolic results, it is evident that GC-MS can be employed to identify challenge-specific biomarkers. By comparing the metabolome of unchallenged and challenged animals, we can identify key biomarkers that distinguish groups and develop metabolic strategies aimed at enhancing fish performance against a specific challenge. Metabolome-reprograming is a recently coined procedure that involves exogenous administration of the key metabolites to normalise the metabolome of challenged or diseased animals, resulting into biological phenotype improvement (Peng et al., 2015b). For instance, L-leucine, glucose, and malate have been used to reprogram ineffective metabolomes in teleosts against bacterial pathogens (Du et al., 2017; Peng et al., 2015c; Yang et al., 2020). Findings of this study may act as a primer for targeted metabolome-reprograming as a strategy to remedy viral infections in aquaculture. In our previous study (Lulijwa et al., 2020a), we illustrated poly (I:C) induced short-term effects in fish HK, SP, erythrocytes and serum, and the present study provides detailed prolonged metabolic effects in the liver and serum.

RT-PCR revealed that poly (I:C) induced significant antiviral *ifnγ* transcripts expression in poly (I:C) treated fish HK, while *ifn1* was not significantly upregulated in HK and SP samples. In addition, there was significant upregulation of antiviral Mx1 protein transcript expression in HK and SP at day one and two post-challenge, with higher expression levels in HK than in SP.

This organ differential cytokine transcript expression is explained by the fact that IFN induction initially occurs in HK and SP, followed by a systemic spread (Jørgensen et al., 2003). In addition, HK compared with SP is the production site for immune cells and a centre for antigen processing (Rauta et al., 2012). Head kidney is also richer than SP in NK cells and T-helper lymphocytes, which produce *ifnγ* and *ifn1* during innate immune response (McNab et al., 2015; Shtrichman & Samuel, 2001) and antiviral activities (Zou et al., 2005). Meanwhile, *ifnγ* is more important in later adaptive immunity (Schroder et al., 2004); and is produced by T-helper lymphocytes and $CD8^+$ or cytotoxic T lymphocytes (Biron & Sen, 2001).

The observed significant upregulation of Mx1 and lower *ifn1* transcript expression was also observed in a previous poly (I:C) exposure study (Lulijwa et al., 2020a). This state is due to earlier *ifn1* induction post-poly (I:C) treatment in teleosts. Early poly (I:C) induced *ifn1* expression, was reported in *O. tshawytscha* CHSS cells at, 6 h (Semple et al., 2018), 14 h in *S. salar* HK tissue, 12 h in HK leucocytes declining 24 and 48 h afterwards (Robertsen et al., 2003). Thus, results suggest that observed Mx1 protein levels probably stemmed from partial synergistic *ifnγ* and earlier *ifn1* activities, as previously reported in S. *salar in vitro* (Sun et al., 2011). Similarly, other studies have reported that poly (I:C) treated fish express gene transcripts for type 1 IFN and Mx protein (Das et al., 2009; Eaton, 1990; Jensen & Robertsen, 2002; Lockhart et al., 2004a; Nygaard et al., 2000). Also, poly (I:C) induced *ifnγ* in *O. mykiss* (Zou et al., 2005), *ifnγ*, type I IFN and Mx transcripts in *S. salar in vitro* and in vivo (Arnemo et al., 2014; Strandskog et al., 2011; Thim et al., 2012). Type 1 IFNs play an important role in vertebrate viral defence (Espert et al., 2003); including in fish (Caipang et al., 2003; Ellis, 2001). These results suggest that the antiviral state was induced by poly (I:C) administration in *O. tshawytscha*.

Expression levels for anti-inflammatory *il-10*, Mx1, *ifnγ* and *il-1β* significantly peaked at day one, two and four, respectively, post-challenge in HK. In SP tissue, *il-10* and *il-6* significantly peaked at day one, while Mx1 protein significantly peaked at day two. In both samples, expression levels appeared to return to baseline levels by day five. The observed trends for Mx1 expression in HK and SP are consistent with results from *S. salar* where poly (I:C) induced high Mx protein expression from day one to three (Robertsen et al., 1997), followed by reduction to undetectable levels by day nine (Salinas et al., 2004). Elevated levels of Mx protein have also been reported at 14 days post-stimulation (Jensen, et al., 2002b), while a biphasic response with high Mx expression on day two to six, low levels from day eight to 12 and undetectable expression over the third week were observed in *S. salar* (Lockhart et al., 2004a).

Furthermore, strong up-regulation of anti-inflammatory mRNA *il-10* in the treated fish HK and SP is in line to suppress the effect of pro-inflammatory cytokines at transcriptional level to avoid cellular damage (Aste-Amezaga et al., 1998). This is evident in the current study as high expression levels for *il-10* compared with *il-1β* in both samples at day one to three, accompanied with an apparent increase in *il-1β* at day three to five, when *il-10* levels appeared to return to baseline levels. A similar trend is observed for *il-6* and *tnf-α* in both organs although the trend

appeared more pronounced in HK than in SP. Administered *in vivo*, poly (I:C) induced *il-10* and *il-1β* in Atlantic cod (*Gadus morhua*) (Seppola et al., 2008). In addition, upregulated *il-1β* transcripts in HK is related to enhanced mitochondrial ROS production at that time point, as reported in higher vertebrates (Shi et al., 2018). In general, poly (I:C) *in vivo* induced antiviral *ifnγ* and Mx1 transcripts, and anti-inflammatory *il-10* in fish organs signifying its importance as an immunostimulant in enhancing *O. tshawytscha* adaptive immunity *in vivo*. Early production of pro-inflammatory cytokines in fish HK is commensurate with timely results for BCAAs metabolism and protein biosynthesis, "aerobic glycolysis", altered Citric acid cycle metabolism, enhanced ROS production and phospholipid metabolism, as previously reported in activated macrophages (O'Neill & Pearce, 2016). It should however be noted that *O. tshawytscha* genome duplication occurred in an ancestral chinook over 90 million years ago (Christensen et al., 2018). Thus, majority of *O. tshawytscha* genes have paralogous sequences, which have not yet been sequenced, and this could have affected the gene results obtained in this study. Interestingly, gene sequence work for this species is in progress in a different laboratory, and will greatly improve accuracy of results in future studies.

7.5.1 Conclusions and recommendations

Conclusively, the current study provides insights and enhance our understanding of poly (I:C) *in vivo* immuno-metabolic modulation in *O. tshawytscha*. Poly (I:C) significantly enhanced fish peripheral blood innate immune cellular migration of thrombocytes, monocyte and neutrophils post-challenge. In addition, cellular PBMC ROS production was enhanced from day one to four, post-poly (I:C) stimulation. Enhanced cellular migration and/or proliferation and consequent ROS production at day one reflects poly (I:C) induced immune stimulation, which corresponds to a dramatic decrease for liver and serum metabolites involved in glycolysis, accumulation of metabolites involved in BCAA metabolism and protein biosynthesis, Citric acid cycle regulation. Meanwhile, liver metabolites involved in ROS regulation were perturbed and those involved in phospholipid metabolism were differentially altered. Poly (I:C) also upregulated antiviral *ifnγ* and Mx1 protein and the corresponding regulatory *il-10* transcript in HK and SP samples, which normalised by day five. These results revealed that poly (I:C) *in vivo* induced immune responses at cellular, metabolic, and molecular levels in teleost *O. tshawytscha*. Findings provide potential for design of amelioration strategies against future viral pathogens via metabolome-reprograming. Further studies are recommended to gain a deeper understanding of poly (I: C) induced effects on the identified and postulated biochemical pathways.

7.6 Appendices

Table 7.5S List of liver raw features identified by gas chromatography-mass spectrometry (GC-MS) analysis following *in vivo* i.p. injection administration with 2.5 µL g^{-1} fish of poly (I:C). Data come from three control (Day 0: D0) and three treatment fish (F1-F3) each day (D1-D5) post-poly (I:C) administration.

Number	Metabolite	AMDIS %	Ref.Ion	Ret.Time	F1D0	F2D0	F3D0	F1D1	F2D1	F3D1	F1D2	F2D2	F3D2	F1D3	F2D3	F3D3	F1D4	F2D4	F3D4	F1D5	F2D5	F3D5
1.	1-Monopalmitin	85	371	31.773	0.001	0.002	0.001		0.001	0.004		0.003			0.003	0.002	0.002	0.001	0.002	0.001	0.002	0.003
2.	Mannitol	100	319	20 - 20.12	0.001	0.001	0.001	0.001				0.000	0.001		0.001	0.000	0.000		0.000	0.001	0.000	
3.	Pantothenic acid	73	73	23.431	0.005	0.005	0.006	0.003	0.006	0.003	0.002	0.011	0.005	0.009	0.004	0.003	0.003	0.006	0.002	0.003	0.003	0.005
4.	Doconexent	82	73	31.66	0.003	0.004	0.002	0.003	0.003	0.004	0.002	0.005	0.004	0.015	0.005	0.003	0.003	0.005	0.006	0.004	0.004	0.006
5.	Sorbose	82	73	19.2 - 19.25	0.005	0.007	0.006	0.003	0.003	0.005	0.005	0.005	0.004	0.012	0.005	0.004	0.002	0.006	0.003	0.004	0.006	0.007
6.	4-Hydroxybutanoic acid	82	147	10.9 - 10.95	0.006	0.004	0.001	0.004	0.008	0.002	0.004	0.009	0.004	0.015	0.004	0.002	0.007	0.010	0.003	0.005	0.004	0.014
7.	D-Glucitol	83	319	23.632	0.004	0.010	0.012	0.003	0.004	0.003	0.002	0.005	0.007		0.003	0.004	0.002	0.005	0.004	0.002	0.005	0.005
8.	Oleic Acid	95	117	25.05- 25.15	0.005	0.005	0.006	0.008	0.004	0.006	0.005	0.031		0.010	0.005	0.011	0.027	0.015	0.030	0.021	0.030	0.014
9.	2-methyl-3-oxo-3-[(2-oxo-2-trimethylsilyloxyethyl)-trimethylsilylamino]propanoic acid	85	147	9.59	0.008	0.003	0.002	0.017	0.019	0.005	0.013	0.003	0.005	0.026	0.008	0.006	0.012	0.017	0.004	0.005	0.003	0.014
10.	Scyllo-Inositol	80	73	22.05 - 22.11	0.005	0.006	0.006	0.003	0.006	0.003	0.003	0.004	0.007	0.007	0.003	0.004	0.004	0.005	0.011	0.004	0.003	0.005
11.	D-Xylose	83	73	16 - 16.1	0.007	0.006	0.003	0.005	0.003	0.004	0.004	0.003	0.009	0.020	0.005	0.003	0.004	0.005	0.003	0.004	0.003	0.009
12.	Galactitol	66	73	20.061	0.005	0.011	0.002	0.003	0.010	0.003	0.003	0.020	0.010	0.017	0.005	0.004	0.006	0.009	0.002	0.004	0.003	0.009
13.	Phosphorylethanolamine	86	299	19.366	0.005	0.003	0.005	0.006	0.005	0.004	0.008	0.006	0.002		0.008	0.008	0.019	0.018	0.014	0.014	0.010	0.011
14.	11-Octadecenoic acid	85	339	25.114	0.004	0.007	0.007	0.006	0.004	0.005	0.004	0.033	0.002		0.007	0.011	0.030	0.019	0.030	0.023	0.032	0.016
15.	Uracil	86	241	12.606	0.005	0.004	0.005	0.008	0.008	0.004	0.007	0.008	0.005		0.004	0.011	0.015	0.008	0.010	0.010	0.005	0.015
16.	D-Galactose	90	73	19.59 - 19.7	0.010	0.019	0.013	0.003	0.002	0.005	0.004	0.006	0.008	0.012	0.006	0.004	0.002	0.006	0.005	0.005	0.006	0.010
17.	Cermizine C TFA salt	81	152	6.868	0.009	0.009	0.005	0.003	0.006	0.011	0.004	0.010	0.009	0.012	0.004	0.005	0.009	0.015	0.007	0.010	0.011	0.009
18.	D-Maltose	88	361	33.959	0.006	0.018	0.027	0.005	0.004	0.005	0.009	0.010	0.001	0.008	0.003	0.023	0.004	0.007	0.008	0.004	0.011	0.006
19.	Octadeca-9,12-diynoic acid	74	75	28.159	0.005	0.006	0.003	0.005	0.005	0.006	0.004	0.003	0.007	0.032	0.005	0.003	0.005	0.009	0.006	0.005	0.005	0.008
20.	D-Gluconic acid	83	73	20.93 - 21.05	0.015	0.023	0.023	0.010	0.008	0.016	0.012	0.027	0.020	0.039	0.013	0.015	0.010	0.029	0.025	0.018	0.027	0.029
21.	D-Fructose	89	73	19.1 - 19.2	0.007	0.014	0.014	0.002	0.002	0.004	0.004	0.009	0.012	0.025	0.006	0.005	0.002	0.007	0.006	0.007	0.006	0.009
22.	3-Aminoisobutyric acid	82	174	13.874	0.004	0.006	0.004	0.011	0.005	0.013	0.018	0.007	0.006	0.031	0.013	0.012	0.006	0.021	0.001	0.011	0.004	0.012
23.	Inosine	84	73	33.905	0.010	0.017	0.026	0.006	0.006	0.005	0.010	0.012	0.007	0.025	0.006	0.019	0.015	0.012	0.032	0.012	0.022	0.019
24.	Acetylcarnitine	83	73	20.93 - 21.05	0.009	0.010	0.011	0.003	0.004	0.007	0.011	0.013	0.010	0.023	0.010	0.008	0.018	0.014	0.018	0.017	0.008	0.020
25.	2-Ketoglutaric acid	75	73	20.923	0.018	0.016	0.019	0.005	0.004	0.013	0.016	0.017	0.018	0.023	0.014	0.011	0.025	0.021	0.031	0.025	0.013	0.027
26.	1,6-Methanocycloprop[a]indene, 1,1a,6,6a-tetrahydro-6a-methoxy-7,7-dimethyl-	76	185	10.69	0.004	0.009	0.003	0.004	0.003	0.005	0.007	0.008	0.005	0.018	0.005	0.003	0.004	0.014	0.010	0.016	0.012	0.015
27.	.alpha-L-Galactopyranose, 6-deoxy-1,2,3,4-tetrakis-O-(trimethylsilyl)-	85	73	21.6 - 21.65	0.037	0.043	0.044	0.004	0.005	0.014	0.007	0.018	0.019	0.019	0.011	0.010	0.006	0.007	0.014	0.008	0.019	0.022
28.	6-Benzo[c] thiochromenethione	76	228	12.526	0.010	0.009	0.006	0.007	0.011	0.013	0.009	0.020	0.010	0.021	0.009	0.007	0.011	0.026	0.019	0.021	0.022	0.016
29.	Arabinose	88	73	16.16 - 16.2	0.010	0.016	0.007	0.009	0.014	0.012	0.009	0.020	0.014	0.045	0.012	0.011	0.010	0.022	0.009	0.011	0.012	0.018
30.	1-(3-Methylbutyl)-2,3,4,6-tetramethylbenzene	86	147	8.732	0.014	0.012	0.004	0.016	0.019	0.010	0.009	0.023	0.010	0.027	0.009	0.008	0.013	0.035	0.013	0.021	0.013	0.025
31.	Urea	88	147	13.225	0.012	0.012	0.015	0.043	0.033	0.029	0.036	0.025	0.018	0.018	0.007	0.022	0.016	0.009	0.024	0.012	0.016	0.016
32.	4-Aminobutanoic acid	86	174	14.862	0.014	0.010	0.008	0.016	0.013	0.016	0.038	0.012	0.011	0.024	0.050	0.018	0.003	0.026	0.001	0.021	0.005	0.017
33.	D-Talo-hexose	94	204	21.631	0.060	0.076	0.068	0.004	0.003	0.018	0.013	0.029	0.029	0.028	0.015	0.015	0.007	0.013	0.022	0.014	0.032	0.033
34.	.alpha.-D-Galactoside, methyl tetrakis-O-(trimethylsilyl)-	69	73	22.48	0.051	0.066	0.080	0.004	0.006	0.020	0.016	0.037	0.023	0.030	0.018	0.027	0.010	0.016	0.021	0.015	0.032	0.036
35.	Myo-Inositol	77	73	23.191	0.030	0.044	0.048	0.033	0.081	0.019	0.038	0.035	0.039	0.072	0.052	0.054	0.113	0.048	0.326	0.048	0.049	0.072
36.	alpha-D-Mannose	86	319	19.4 - 19.58	0.032	0.068	0.097	0.008	0.007	0.015	0.027	0.060	0.044	0.032	0.013	0.037	0.010	0.016	0.045	0.016	0.040	0.039
37.	dimethyl-trimethylsilyl-undec-10-enoxysilane	69	147	9.819	0.017	0.025	0.011	0.027	0.037	0.025	0.021	0.054	0.016	0.027	0.019	0.019	0.038	0.041	0.034	0.036	0.043	0.042
38.	6-aminohexanoic acid	73	174	10.721	0.053	0.043	0.040	0.082	0.064	0.020	0.047	0.026	0.050	0.042	0.033	0.039	0.043	0.077	0.011	0.053	0.038	0.065
39.	2-Aminobutanoic acid	90	130	9.679	0.011	0.015	0.013	0.023	0.029	0.044	0.077	0.035	0.036	0.070	0.056	0.062	0.019	0.022	0.019	0.026	0.037	0.044
40.	D-Ribose	78	73	16.3 - 16.35	0.032	0.027	0.061	0.030	0.020	0.098	0.128	0.146	0.038	0.089	0.033	0.032	0.046	0.056	0.022	0.019	0.030	0.035
41.	Phosphoric acid	94	241	11.237	0.118	0.139	0.267	0.077	0.024	0.040	0.132	0.115	0.110	0.094	0.050	0.105	0.059	0.058	0.133	0.092	0.080	0.106
42.	L-5-Oxoproline	96	156	16.137	0.132	0.230	0.159	0.084	0.136	0.117	0.077	1.104	0.086	0.084	0.092	0.277	0.459	0.212	0.353	0.167	0.325	0.160
43.	Trimethylsilyl 2-[(trimethylsilyl)amino]-6-[(trimethylsilyl)oxy]hexanoate	82	73	16.36 - 16.4	0.238	0.097	0.575	0.064	0.006	0.315	0.243	0.189	0.139	0.067	0.185	0.332	0.059	0.016	0.336	0.325	0.435	0.115
44.	Glycerol	99	147	11.035	0.131	0.120	0.096	0.021				0.074	0.137	0.170		0.048	0.063	0.114	0.043	0.091	0.094	0.165
45.	Silanol, trimethyl-, phosphate (3:1)	86	73	12.319	0.341	0.300	0.422	0.277	0.151	0.233	0.387	0.523	0.319	0.342	0.290	0.396	0.547	0.371	0.499	0.494	0.529	0.447
46.	D-Glucose	99	319	19.7 - 19.85	2.161	3.325	2.152	0.077	0.054	0.835	0.587	1.165	1.081	0.506	0.771	1.025	0.316	0.232	0.693	0.454	1.246	1.241
47.	Phosphate	100	299	12.323	1.371	1.332	1.651	1.281	0.614	0.991	1.560	2.010	1.266	1.132	1.188	1.752	2.377	1.613	1.738	1.989	2.252	1.728
48.	Alanine	95	102	11.32	0.134	0.240	0.177	0.257	0.263	0.231	0.152	0.107	0.169	0.288	0.097	0.093	0.088	0.119	0.145	0.057	0.053	0.135
49.	Glutamic acid	100	174	18.4 - 18.6	0.072	0.111	0.087	0.067	0.059	0.108	0.086	0.083	0.090	0.121	0.078	0.070	0.059	0.059	0.084	0.062	0.068	0.082
50.	Palmitic acid	99	74	22.021	0.044	0.084	0.064	0.113	0.090	0.097	0.092	0.071	0.067	0.118	0.054	0.060	0.080	0.079	0.066	0.049	0.056	0.073
51.	Docosahexaenoic acid	99	79	30.164	0.039	0.068	0.048	0.080	0.067	0.073	0.068	0.051	0.061	0.083	0.046	0.042	0.055	0.056	0.053	0.039	0.043	0.053
52.	Aspartic acid	99	160	16.568	0.040	0.060	0.046	0.045	0.040	0.079	0.056	0.042	0.040	0.116	0.049	0.045	0.040	0.039	0.067	0.032	0.032	0.050
53.	Glycine	100	88	11.668	0.024	0.036	0.033	0.087	0.091	0.090	0.079	0.061	0.051	0.106	0.073	0.050	0.057	0.069	0.061	0.041	0.046	0.056
54.	Stearic acid	98	74	24.533	0.021	0.046	0.031	0.063	0.050	0.050	0.055	0.041	0.036	0.070	0.039	0.035	0.046	0.046	0.036	0.030	0.027	0.040
55.	Lactic acid	100	103	9.442	0.036	0.055	0.050	0.071	0.065	0.046	0.033	0.030	0.039	0.057	0.032	0.027	0.040	0.031	0.032	0.017	0.013	0.038
56.	Proline	98	128	15.27	0.012	0.026	0.020	0.054	0.062	0.054	0.017	0.026	0.022	0.055	0.026	0.027	0.027	0.025	0.022	0.011	0.013	0.020
57.	Valine	100	130	13 - 13.05	0.019	0.026	0.024	0.076	0.083	0.045	0.053	0.034	0.028	0.067	0.042	0.039	0.051	0.034	0.035	0.023	0.018	0.024
58.	Leucine	100	144	14.25 - 14.31	0.017	0.026	0.019	0.061	0.068	0.041	0.042	0.028	0.027	0.061	0.034	0.032	0.043	0.027	0.029	0.018	0.016	0.020
59.	Threonine	98	115	15.8 - 15.9	0.015	0.021	0.016	0.037	0.021	0.028	0.022	0.013	0.027	0.022	0.017	0.026	0.015	0.014	0.010	0.007	0.012	0.018
60.	Eicosapentaenoic acid	93	79	27.277	0.012	0.021	0.018	0.026	0.021	0.024	0.022	0.016	0.016	0.030	0.013	0.010	0.023	0.020	0.020	0.013	0.015	0.022
61.	Succinic acid	95	115	9.273	0.032	0.035	0.025	0.003	0.001	0.026	0.008	0.018	0.017	0.017	0.016	0.009	0.005	0.005	0.008	0.008	0.013	0.018
62.	Cystathionine	97	160	35.65 - 35.95	0.001	0.004	0.001	0.016	0.006	0.032	0.026	0.007	0.017	0.025	0.004	0.005	0.005	0.011	0.003	0.001	0.001	0.004
63.	Linoleic acid	94	67	24.39 - 24.45	0.011	0.015	0.013	0.023	0.018	0.019	0.019	0.018	0.015	0.028	0.014	0.011	0.020	0.023	0.016	0.015	0.014	0.020
64.	Lysine	92	142	26.721	0.012	0.022	0.013	0.046	0.051	0.019	0.023	0.020	0.015	0.025	0.024	0.026	0.030	0.024	0.020	0.016	0.014	0.023
65.	Arachidonic acid	99	79	26.969	0.006	0.013	0.009	0.018	0.014	0.014	0.013	0.011	0.010	0.020	0.009	0.009	0.014	0.012	0.013	0.007	0.008	0.012

Number	Metabolite	AMDIS %	Ref.Ion	Ret.Time	F1D0	F2D0	F3D0	F1D1	F2D1	F3D1	F1D2	F2D2	F3D2	F1D3	F2D3	F3D3	F1D4	F2D4	F3D4	F1D5	F2D5	F3D5
66.	Trans-Vaccenic acid	100	55	24.3 - 24.325	0.008	0.014	0.010	0.013	0.012	0.013	0.011	0.010	0.013	0.014	0.011	0.011	0.010	0.011	0.011	0.009	0.008	0.010
67.	Myristic acid	99	74	18.054	0.008	0.011	0.010	0.016	0.012	0.013	0.017	0.009	0.009	0.016	0.008	0.006	0.011	0.016	0.009	0.009	0.007	0.014
68.	Unknown 149(100) 167(32.8) 57(17.4)	100	149	32.538	0.005	0.009	0.006	0.009	0.009	0.009	0.008	0.006	0.008	0.011	0.005	0.007	0.006	0.006	0.007	0.005	0.005	0.006
69.	Malic acid	98	75	15.362	0.015	0.020	0.017	0.009	0.005	0.020	0.009	0.013	0.016	0.016	0.014	0.010	0.006	0.007	0.009	0.007	0.013	0.015
70.	Glutathione	95	142	19.236	0.010	0.015	0.011	0.006	0.004	0.014	0.008	0.012	0.012	0.014	0.012	0.008	0.006	0.006	0.011	0.008	0.007	0.011
71.	Pyroglutamic acid	83	84	16.652	0.016	0.014	0.014	0.007	0.006	0.025	0.011	0.015	0.018	0.025	0.016	0.009	0.008	0.008	0.013	0.010	0.009	0.011
72.	Docosapentaenoic acid	94	79	30.24 - 30.3	0.005	0.009	0.006	0.010	0.008	0.012	0.009	0.008	0.008	0.013	0.007	0.004	0.008	0.009	0.007	0.005	0.004	0.009
73.	(Z)-Docos-13-enamide	75	59	41.045	0.007	0.012	0.007	0.013	0.014	0.013	0.012	0.010	0.012	0.015	0.009	0.010	0.013	0.011	0.012	0.009	0.011	0.011
74.	Beta-Alanine	99	88	12.9 - 12.98	0.003	0.005	0.004	0.011	0.010	0.025	0.014	0.011	0.005	0.055	0.015	0.010	0.012	0.022	0.007	0.004	0.004	0.006
75.	Citramalic acid	100	128	10.914	0.003	0.006	0.004	0.006	0.006	0.006	0.005	0.003	0.005	0.007	0.003	0.004	0.003	0.003	0.004	0.002	0.003	0.004
76.	2-Aminobutyric acid	97	116	12.426	0.005	0.010	0.005	0.012	0.018	0.024	0.029	0.019	0.013	0.047	0.028	0.027	0.013	0.014	0.010	0.009	0.015	0.021
77.	Fumaric acid	93	113	9.415	0.007	0.010	0.007	0.005	0.003	0.008	0.005	0.005	0.008	0.007	0.007	0.003	0.003	0.003	0.004	0.004	0.005	0.006
78.	Isoleucine	100	115	14.31 - 14.37	0.004	0.006	0.005	0.017	0.018	0.010	0.011	0.008	0.007	0.015	0.009	0.008	0.011	0.007	0.007	0.004	0.004	0.005
79.	Unknown 160(100) 59(43.4) 116(22.7)	94	160	14.915	0.003	0.006	0.004	0.006	0.006	0.006	0.003	0.002	0.003	0.007	0.002	0.002	0.001	0.002	0.003	0.001	0.001	0.002
80.	Tyrosine	100	236	29.048	0.003	0.006	0.004	0.015	0.023	0.007	0.011	0.004	0.004	0.014	0.006	0.004	0.010	0.012	0.006	0.003	0.003	0.004
81.	Serine	98	100	17.653	0.002	0.005	0.004	0.009	0.007	0.008	0.005	0.004	0.004	0.013	0.006	0.007	0.005	0.006	0.005	0.003	0.005	0.006
82.	Ornithine	96	128	25.114	0.003	0.005	0.004	0.023	0.025	0.003	0.004	0.002	0.002	0.006	0.002	0.002	0.003	0.004	0.003	0.001	0.002	0.002
83.	Phenylalanine	100	162	20.401	0.002	0.004	0.003	0.009	0.018	0.005	0.005	0.003	0.003	0.006	0.004	0.003	0.006	0.005	0.005	0.003	0.002	0.004
84.	Unknown 113(100) 85(48.1) 59(21.2)	100	113	8.853	0.004	0.006	0.004	0.003	0.002	0.005	0.002	0.003	0.004	0.004	0.004	0.001	0.002	0.001	0.002	0.002	0.003	0.004
85.	Unknown 082(100) 142(56.8) 110(42.6)	94	82	15.15 - 15.2	0.002	0.004	0.004	0.002	0.001	0.003	0.003	0.004	0.003	0.005	0.003	0.002	0.002	0.002	0.004	0.002	0.002	0.003
86.	Palmitelaidic acid	99	55	21.686	0.003	0.004	0.003	0.005	0.005	0.005	0.005	0.004	0.004	0.007	0.003	0.003	0.004	0.006	0.004	0.003	0.003	0.005
87.	Heptadecanoic acid	96	74	23.37	0.002	0.004	0.002	0.005	0.004	0.004	0.004	0.003	0.003	0.006	0.003	0.002	0.004	0.004	0.003	0.002	0.002	0.003
88.	DL-3-Aminoisobutyric acid	96	88	13.311	0.000	0.001	0.001	0.002	0.001	0.002	0.003	0.001	0.001	0.005	0.003	0.002	0.002	0.002	0.001	0.001	0.002	0.002
89.	Cholesterol	81	386	44.185	0.002	0.004	0.003	0.009	0.009	0.004	0.007	0.004	0.002	0.009	0.004	0.005	0.007	0.004	0.004	0.002	0.003	0.004
90.	Alpha-Linolenic acid	78	79	24.45 - 24.525	0.002	0.003	0.003	0.004	0.003	0.005	0.003	0.004	0.003	0.005	0.002	0.001	0.003	0.004	0.003	0.003	0.004	0.004
91.	Tryptophan	97	130	34.038	0.001	0.002	0.001	0.005	0.011	0.003	0.004	0.002	0.003	0.004	0.003	0.002	0.004	0.004	0.003	0.001	0.001	0.002
92.	Gamma-Linolenic acid	90	79	24.57 - 24.64	0.002	0.002	0.002	0.003	0.002	0.005	0.003	0.004	0.003	0.004	0.003	0.002	0.003	0.006	0.003	0.004	0.002	0.005
93.	Unknown 115(100) 118(95.4) 86(69.3)	98	115	15.92 - 16	0.001	0.002	0.002	0.005	0.003	0.003	0.002	0.001	0.002	0.004	0.002	0.003	0.002	0.002	0.002	0.001	0.002	0.003
94.	Histidine	85	139	27.649	0.002	0.003	0.002	0.001	0.001	0.002	0.001	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001
95.	Citric acid	97	143	16.603	0.003	0.003	0.003	0.016	0.016	0.003	0.004	0.004	0.003	0.004	0.004	0.004	0.019	0.013	0.005	0.006	0.003	0.005
96.	Asparagine	95	127	16.795	0.002	0.003	0.005	0.004	0.003	0.012	0.008	0.007	0.006	0.012	0.006	0.007	0.005	0.004	0.007	0.003	0.005	0.004
97.	Unknown 070(100) 42(28.5) 69(8.0)	90	70	7.137	0.001	0.002	0.001	0.002	0.002	0.001	0.001	0.001	0.001	0.002	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001
98.	Unknown 114(100) 59(61.6) 146(33.7)	94	114	17.271	0.001	0.002	0.001	0.003	0.002	0.003	0.002	0.001	0.002	0.002	0.002	0.003	0.001	0.001	0.001	0.001	0.001	0.002
99.	Unknown 201(100) 117(89.6) 119(86.2)	99	201	8.483	0.001	0.002	0.001	0.002	0.001	0.001	0.002	0.001	0.002	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001
100.	Pentadecanoic acid	88	74	19.708	0.001	0.002	0.001	0.003	0.002	0.002	0.003	0.002	0.001	0.003	0.001	0.001	0.002	0.002	0.002	0.001	0.001	0.002
101.	2-Aminoadipic acid	96	114	20.455	0.001	0.003	0.000	0.007	0.005	0.007	0.009	0.002	0.002	0.007	0.004	0.003	0.002	0.004	0.001	0.002	0.001	0.003
102.	Putrescine	98	88	22.22	0.002	0.003	0.001	0.004	0.004	0.004	0.006	0.005	0.002	0.003	0.006	0.005	0.005	0.005	0.005	0.004	0.002	0.008
103.	Dimethyl 2-aminomalonate hydrochloride	90	146	14.914	0.001	0.001	0.001	0.003	0.003	0.003	0.002	0.002	0.001	0.003	0.002	0.002	0.002	0.002	0.002	0.001	0.001	0.002
104.	Vitamin E	82	430	44.377	0.000	0.001	0.000	0.001	0.000	0.001	0.001	0.000	0.001	0.001	0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.000
105.	Unknown 074(100) 46(21.3) 59(0.97)	95	74	7.093	0.000	0.000	0.000	0.000	0.000			0.000	0.000	0.000		0.000		0.000	0.000	0.000		0.000
106.	Unknown 156(100) 96(36.6) 128(31.0)	65	156	20.46	0.000	0.002	0.000	0.005	0.004	0.005	0.006	0.002	0.001	0.005	0.003	0.002	0.001	0.003	0.001	0.001	0.001	0.002
107.	4-Aminobutyric acid	97	102	15.05 - 15.12	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.001	0.003	0.004	0.001	0.001	0.001	0.001	0.001	0.001	0.001
108.	Unknown 081(100) 136(51.6) 152(42.5)	78	81	25.938	0.001	0.001	0.001	0.000	0.000	0.001	0.000	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.001
109.	Methionine	99	147	18.543	0.001	0.001	0.001	0.006	0.006	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.001
110.	Dihomo-gamma-Linolenic acid	85	79	27.15 - 27.2	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
111.	Oxalic acid	82	59	6.236	0.001	0.001	0.001	0.000	0.000	0.001	0.000	0.000	0.001	0.001	0.001	0.000	0.001	0.000	0.001	0.001	0.000	0.001
112.	Cysteine	96	192	20.548	0.000	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001
113.	Citraconic acid	67	127	10.789	0.000	0.000	0.000	0.001	0.000	0.002	0.001	0.000	0.001	0.002	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
114.	(E)-heptadec-10-enoic acid	73	55	22.91 - 22.97	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.000	0.001	0.001	0.001	0.000	0.000	0.001
115.	Cis-4-Hydroxyproline	83	144	20.324	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
116.	Adrenic acid	79	79	29.95 - 30.04	0.000	0.001	0.000	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.000	0.000	0.000
117.	Conjugated linoleic acid	72	67	24.22 - 24.3	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001
118.	Dodecane	87	57	9.858	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
119.	Creatinine	98	202	16.95 - 17	0.001	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001
120.	9E-heptadecenoic acid	94	55	23.223	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.000	0.001
121.	(11Z,14Z,17Z)-Eicosa-11,14,17-trienoic acid	72	79	27.484	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.000	0.001
122.	11,14-Eicosadienoic acid	71	67	27.34 - 27.37	0.000	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.000	0.001
123.	Unknown 128(100) 42(10.5) 129(6.8)	85	128	21.257	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
124.	Unknown 059(100) 73(69.3) 77(45.8)	72	59	19.02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.001
125.	Unknown 086(100) 59(56.6) 128(39.3)	96	86	13.445	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
126.	4-Methyl-2-oxopentanoic acid	86	85	8.1 - 8.155	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
127.	Unknown 232(100) 128(72.0) 156(51.0)	95	232	22.631	0.001	0.001	0.001	0.000	0.000	0.001	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
128.	2-Hydroxybutyric acid	98	117	10.65	0.000	0.000	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.001
129.	Glutamine	82	84	25.16 - 25.25	0.001	0.001	0.001	0.000	0.001	0.001	0.000	0.001	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.001
130.	Tridecane	71	57	12.847	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
131.	Unknown 045(100) 59(89.0) 74(67.8)	95	45	9.157	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.001
132.	2-Oxoglutaric acid	73	115	15.464	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
133.	Nicotinic acid	95	137	11.046	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
134.	Dodecanoic acid	74	74	15.49	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
135.	Unknown 091(100) 106(54.7) 105(21.8)	93	91	5.9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
136.	N-PENTADECANE	75	57	15.537	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
137.	Nonadecanoic acid	66	74	25.864	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
138.	Caproic acid	76	74	6.506	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Number	Metabolite	AMDIS %	Ref.Ion	Ret.Time	F1D0	F2D0	F3D0	F1D1	F2D1	F3D1	F1D2	F2D2	F3D2	F1D3	F2D3	F3D3	F1D4	F2D4	F3D4	F1D5	F2D5	F3D5
139.	Unknown 088(100) 44(30.3) 59(19.6)	85	88	23.616	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
140.	Caprylic acid	78	74	9.7 - 9.75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
141.	NADPH	84	138	11.08	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
142.	Unknown 142(100) 153(51.8) 59(22.0)	91	142	22.393	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
143.	Arachidic acid	74	74	27.515	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
144.	Unknown 142(100) 59(19.9) 141(17.0)	86	142	29.512	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
145.	Guanine	90	307	27.934	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
146.	Unknown 059(100) 148(44.7) 43(33.8)	74	59	23.209	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
147.	Unknown 074(100) 87(68.8) 55(33.1)	70	74	19.024	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
148.	Unknown 102(100) 58(0.86) 59(0.75)	67	102	23.302	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
149.	Unknown 249(100) 57(31.8) 264(22.9)	92	249	17.801	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
150.	Unknown 125(100) 184(90.5) 96(54.3)	93	125	22.747	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
151.	1-Aminocyclopropanecarboxylic acid	74	141	13.676	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
152.	Benzoic acid	83	105	9.896	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
153.	Unknown 056(100) 59(24.8) 115(10.8)	80	56	7.195	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
154.	Dehydroascorbic acid	72	59	15.785 - 15.82	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
155.	Unknown 112(100) 43(60.3) 115(49.2)	90	112	10.717	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
156.	Unknown 114(100) 59(15.3) 82(14.4)	94	114	12.367	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
157.	n-Heptadecane	100	57	16.786	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
158.	Glyoxylic acid	86	75	11.4 - 11.45	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
159.	Cis-Aconitic acid	95	153	15.752	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
160.	alpha-Hydroxyglutarate	70	85	14.55	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
161.	Unknown 135(100) 180(28.0) 77(19.9)	84	135	16.124	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
162.	Salicylic acid	96	135	17.078	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
163.	Unknown 082(100) 153(90.1) 56(65.2)	84	82	18.127	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
164.	Unknown 071(100) 57(94.4) 85(72.9)	74	71	14.111	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
165.	Pyruvic acid	77	89	7.568	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
166.	n-Tricosane	100	57	25.78	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
167.	Nervonic acid	71	55	33.926	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
168.	Unknown 128(100) 139(21.1) 42(19.1)	84	128	19.879	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
169.	Unknown 115(100) 100(70.4) 56(60.1)	79	115	8.223	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
170.	Beta-Citryl-L-glutamic acid	76	143	32.181	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
171.	Unknown 115(100) 59(65.5) 189(50.1)	84	115	19.76	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
172.	Decanoic acid	72	74	12.756	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
173.	Unknown 131(100) 71(89.6) 103(88.8)	71	131	17.25 - 17.32	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
174.	Adipic acid	78	114	12.621	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
175.	2-Oxobutyric acid	75	57	6.086	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
176.	Unknown 102(100) 103(26.6) 44(19.0)	77	102	21.265	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
177.	Unknown 114(100) 147(31.9) 115(27.2)	76	114	23.156	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
178.	Unknown 043(100) 59(16.5) 74(15.0)	65	43	7.161	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
179.	Unknown 071(100) 57(92.3) 43(75.3)	80	71	24.09 - 24.15	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
180.	Unknown 059(100) 81(40.5) 91(34.4)	69	59	37.649	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
181.	Homocysteine	70	114	23.59	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
182.	Unknown 088(100) 57(53.2) 59(45.8)	90	88	7.436	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
183.	Unknown 119(100) 147(96.0) 101(53.9)	67	119	17.64	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
184.	2-Oxovaleric acid	70	71	6.544	0.000	0.000	0.000	0.000	0.000	0.000	0.000			0.000	0.000	0.000						
185.	S-Adenosylhomocysteine	81	147	17.3 - 17.35	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
186.	Glutaric acid	91	100	9.674	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.001	0.001	0.000	0.001	0.002	0.001	0.001	0.000	0.001
187.	2-methyl-octadecanoic acid	78	88	25.357	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
188.	Glyceric acid	66	119	13.977	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
189.	Isocitric acid	78	129	21.444	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
190.	Malonic acid	93	101	7.65	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
191.	Lignoceric acid	68	74	34.256	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
192.	Nicotinamide	86	57	7.45			0.000	0.000	0.000	0.000	0.000		0.000	0.000		0.000	0.000	0.000	0.000			
193.	N-Acetylglutamic acid	68	116	19.519	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
194.	Nonacosane	94	57	36.482	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
195.	Itaconic acid	90	127	10.402	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
196.	Azelaic acid	76	185	16.72 - 16.77	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
197.	3-Methyl-2-oxopentanoic acid	81	57	8.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
198.	2-Hydroxyisobutyric acid	72	73	9.366	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
199.	4-Hydroxyphenylacetic acid	79	121	19.409	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
200.	Trans-4-Hydroxyproline	67	216	17.673	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
201.	Unknown 198(100) 138(61.7) 166(40.8)	84	198	21.159	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
202.	Unknown 126(100) 127(46.7) 59(33.2)	94	126	10.213	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
203.	Ferulic acid	69	222	26.77	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
204.	Unknown 101(100) 119(80.5+A87:A212) 74(35.3)	69	101	8.747	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000			0.000	0.000			

Table 7.6S List of serum raw features identified by gas chromatography-mass spectrometry (GC-MS) analysis following *in vivo* i.p. injection administration with 2.5 µL g^{-1} fish of poly (I:C). Data come from three control (Day 0: D0) and three treatment fish (F1-F3) each day (D1-D5) post poly (I:C) administration.

Number	Metabolite	AMDIS %	Ref.Ion	Ret.Time	F1D0	F2D0	F3D0	F1D1	F2D1	F3D1	F1D2	F2D2	F3D2	F1D3	F2D3	F3D3	F1D4	F2D4	F3D4	F1D5	F2D5	F3D5
1.	D-Glucose	99	319	19.7 - 19.825	8.115	11.112	9.065	1.124	0.969	5.041	4.865	4.913	4.947	8.437	4.894	6.265	5.892	4.254	3.751	4.634	5.417	8.117
2.	2-Amino-3-phenylpropanamide	66	120	8.336	6.447	6.184	6.591	4.859	1.718	1.067	4.104	8.048	7.600	7.473	5.135	6.239	5.848	9.252	4.781	6.553	7.648	8.621
3.	Phosphate	100	299	12.327	4.237	4.726	2.992	5.465	5.576	5.850	2.828	1.904	4.890	0.492	3.151	2.636	3.673	8.141	2.653	3.600	3.323	4.857
4.	Ethanolamine	82	174	12.657	1.888	1.043	1.557	0.894	1.548	0.344	0.530	0.104	0.162	0.049	0.099	0.172	0.071	0.221	0.163	0.207	0.367	0.184
5.	Urea	93	147	13.227	0.093	0.269	0.107	0.684	1.001	2.077	0.957	0.394	0.639	1.764	0.263	0.350	0.269	0.753	0.244	0.152	0.222	0.213
6.	Myo-Inositol	77	73	23.191	0.392	0.255	0.310	0.158	0.752	0.142	0.312	0.290	0.559	0.196	0.509	0.374	0.805	0.214	2.152	0.272	0.287	0.422
7.	Propanoic acid	94	147	9.586	0.979	0.819	0.891	0.935	0.656	0.495	0.542	0.919	0.869	0.724	0.504	0.650	0.546	0.900	0.643	0.782	1.196	0.839
8.	2-(2-hydroxyethyl)-1-isoquinolinone	72	189	13.233	0.042	0.140	0.033	0.380	0.568	1.272	0.542	0.233	0.398	0.969	0.130	0.187	0.147	0.460	0.153	0.087	0.060	0.115
9.	Glycerol	97	147	11.034	0.478	0.385	0.545	1.007	0.396	0.372	0.336	0.293	0.439	0.381	0.299	0.356	0.380	0.720	0.228	0.424	0.412	0.400
10.	Beta-D-Glucopyranose	94	204	21.627	0.251	0.172	0.257	0.036	0.028	0.105	0.073	0.084	0.107	0.131	0.099	0.113	0.086	0.063	0.111	0.086	0.189	0.206
11.	Methyl 6-deoxy-beta-L-Galactopyranoside	68	204	22.484	0.231	0.433	0.272	0.054	0.062	0.236	0.184	0.166	0.122	0.308	0.153	0.206	0.237	0.158	0.163	0.162	0.252	0.246
12.	Putrescine	88	174	16.783	0.056	0.077	0.083	0.090	0.080	0.014	0.026	0.039	0.046	0.092	0.060	0.029	0.020	0.073	0.020	0.049	0.016	0.098
13.	D-Mannose	85	319	19.433 - 19.55	0.180	0.262	0.214	0.026	0.030	0.138	0.080	0.134	0.121	0.073	0.068	0.073	0.057	0.055	0.091	0.071	0.193	0.103
14.	Pyrimidin-2-yl trifluoromethanesulfonate	66	228	12.527	0.115	0.144	0.173	0.068	0.157	0.059	0.085	0.104	0.139	0.077	0.061	0.092	0.072	0.119	0.076	0.124	0.107	0.151
15.	Tert-Butylpentamethyldisiloxane	68	147	11.614	0.166	0.108	0.155	0.241	0.158	0.078	0.145	0.266	0.239	0.189	0.216	0.251	0.161	0.224	0.120	0.140	0.161	0.138
16.	2-Aminobutanoic acid	87	130	9.68	0.189	0.155	0.139	0.246	0.262	0.074	0.238	0.223	0.186	0.179	0.390	0.400	0.251	0.664	0.136	0.267	0.299	0.401
17.	2,2,8-Trimethyl-4,5-dihydro-2H-cyclopenta[de]chromene	76	185	10.69	0.132	0.101	0.099	0.064	0.080	0.030	0.056	0.060	0.090	0.037	0.039	0.056	0.036	0.098	0.044	0.061	0.070	0.098
18.	Isopropanolamine	76	73	10.721	0.123	0.052	0.112	0.069	0.057	0.017	0.029	0.082	0.045	0.022	0.023	0.046	0.024	0.045	0.036	0.050	0.080	0.071
19.	2-Butyne-1,4-diol	67	147	9.987	0.186	0.103	0.120	0.060	0.074	0.012	0.021	0.154	0.096	0.005	0.038	0.034	0.059	0.025	0.023	0.032	0.109	0.100
20.	D-Galactose	87	73	19.55 - 19.7	0.052	0.059	0.101	0.040	0.042	0.017	0.018	0.031	0.032	0.022	0.017	0.028	0.026	0.032	0.066	0.024	0.057	0.039
21.	4-Hydroxybutanoic acid	82	147	10.9 - 10.96	0.098	0.073	0.086	0.057	0.056	0.022	0.027	0.066	0.074	0.010	0.030	0.033	0.028	0.049	0.027	0.040	0.123	0.057
22.	D-Ribose	80	73	16.3 - 16.364	0.053	0.023	0.053	0.079	0.066	0.016	0.014	0.022	0.025	0.013	0.018	0.017	0.017	0.024	0.054	0.020	0.045	0.050
23.	Scyllo-Inositol	80	73	22.05 - 22.1	0.059	0.031	0.043	0.022	0.058	0.010	0.022	0.029	0.055	0.023	0.028	0.028	0.044	0.019	0.139	0.018	0.047	0.030
24.	Raffinose	88	73	31.867	0.086	0.019	0.058	0.014	0.029	0.001	0.009	0.038	0.043	0.011	0.016	0.018	0.011	0.028	0.219	0.018	0.092	0.052
25.	L-5-Oxoproline	87	156	16.148	0.051	0.099	0.068	0.398	1.704	0.182	0.097	0.079	0.074	0.088	0.104	0.094	0.096	0.264	0.695	0.096	0.075	0.071
26.	Sucrose	73	361	31.8 - 32		0.018		0.011	0.024		0.002	0.052	0.057	0.011	0.018	0.021	0.010	0.039	0.249	0.012	0.037	0.049
27.	Arabinose	97	73	16.144 - 16.2	0.073	0.059	0.079	0.138	0.314	0.042	0.046	0.039	0.032	0.037	0.046	0.041	0.044	0.104	0.231	0.036	0.067	0.058
28.	D-Frutose	100	73	19.1 - 19.2	0.039	0.025	0.050	0.024	0.029	0.005	0.013	0.028	0.023	0.006	0.010	0.018	0.011	0.015	0.055	0.011	0.018	0.015
29.	3-Aminoisobutyric acid	74	174	13.85 - 13.9	0.051	0.021	0.026	0.041	0.046	0.014	0.021	0.045	0.033	0.029	0.049	0.055	0.030	0.053	0.022	0.042	0.061	0.067
30.	1,2,7,8-Tetrahydro-thiino[2,3-f]-(1)-benzopyran-2,8-dione	74	174	14.866	0.079	0.046	0.086	0.015	0.026	0.086	0.006	0.030	0.029	0.032	0.035	0.029	0.014	0.020	0.037	0.026	0.044	0.072
31.	1H-Indole-1-acetic acid	88	73	25.111	0.082	0.033	0.040	0.024	0.102	0.018	0.015	0.036	0.030	0.012	0.028	0.037	0.018	0.014	0.207	0.024	0.088	0.024
32.	D-Xylose	69	73	16 - 16.1	0.035	0.025	0.055	0.027	0.144	0.023	0.011	0.018	0.014	0.008	0.011	0.012	0.011	0.016	0.138	0.009	0.034	0.016
33.	beta-D-Arabinopyranose	69	217	14.2 - 14.25		0.010		0.015	0.011	0.009	0.007	0.019	0.017	0.011	0.016	0.020	0.014	0.024	0.020	0.010	0.016	0.017
34.	Alanine	95	102	11.32	0.505	0.596	0.490	1.605	2.461	0.488	0.294	0.264	0.463	0.421	0.374	0.378	0.385	0.844	0.580	0.214	0.140	0.470
35.	Glutamic acid	100	174	18.4 - 18.6	0.016	0.016	0.021	0.143	0.181	0.015	0.014	0.017	0.014	0.020	0.018	0.016	0.018	0.038	0.011	0.019	0.010	0.017
36.	Palmitic acid	99	74	22.021	0.820	0.459	0.707	0.245	0.519	0.337	0.273	0.560	0.528	0.390	0.581	0.597	0.258	0.076	0.476	0.531	0.496	0.377
37.	Docosahexaenoic acid	99	79	30.164	0.545	0.339	0.468	0.147	0.340	0.210	0.174	0.361	0.366	0.236	0.411	0.417	0.178	0.034	0.366	0.376	0.367	0.240
38.	Aspartic acid	99	160	16.568	0.006	0.006	0.005	0.068	0.087	0.010	0.006	0.005	0.005	0.010	0.008	0.006	0.005	0.014	0.003	0.006	0.003	0.005
39.	Glycine	100	88	11.668	0.130	0.130	0.122	0.468	0.631	0.098	0.105	0.092	0.138	0.083	0.186	0.226	0.104	0.263	0.128	0.141	0.112	0.192
40.	Stearic acid	98	74	24.533	0.282	0.142	0.211	0.081	0.173	0.088	0.077	0.161	0.147	0.105	0.188	0.172	0.079	0.026	0.136	0.162	0.124	0.101
41.	Lactic acid	100	103	9.442	0.558	0.448	0.773	0.867	1.118	0.354	0.212	0.281	0.381	0.490	0.372	0.265	0.342	0.476	0.308	0.267	0.129	0.668
42.	Proline	98	128	15.27	0.054	0.076	0.051	0.228	0.530	0.090	0.033	0.055	0.082	0.042	0.073	0.108	0.090	0.152	0.054	0.036	0.040	0.059
43.	Valine	100	130	13 - 13.05	0.403	0.353	0.372	0.831	1.173	0.492	0.609	0.581	0.609	0.502	0.727	0.701	0.550	0.687	0.430	0.447	0.363	0.327
44.	Leucine	100	144	14.25 - 14.31	0.247	0.279	0.237	0.643	0.926	0.417	0.476	0.422	0.486	0.454	0.510	0.499	0.466	0.490	0.292	0.285	0.254	0.217
45.	Threonine	98	115	15.8 - 15.9	0.042	0.039	0.030	0.162	0.144	0.025	0.030	0.019	0.053	0.016	0.038	0.079	0.053	0.090	0.021	0.028	0.020	0.031
46.	Eicosapentaenoic acid	93	79	27.277	0.161	0.083	0.149	0.032	0.078	0.042	0.036	0.097	0.084	0.053	0.099	0.095	0.037	0.006	0.074	0.080	0.090	0.048
47.	Succinic acid	95	115	9.273	0.001	0.002	0.002	0.002	0.003	0.003	0.000	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.001
48.	Cystathionine	97	160	35.65 - 35.95	0.000	0.000	0.000	0.006	0.002	0.001	0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000
49.	Linoleic acid	94	67	24.39 - 24.45	0.087	0.042	0.071	0.024	0.052	0.036	0.025	0.063	0.049	0.039	0.060	0.063	0.022	0.005	0.045	0.055	0.057	0.032
50.	Lysine	92	142	26.721	0.226	0.321	0.203	0.545	0.819	0.127	0.220	0.248	0.332	0.080	0.417	0.421	0.361	0.510	0.237	0.304	0.184	0.303
51.	Arachidonic acid	99	79	26.969	0.088	0.032	0.064	0.016	0.039	0.014	0.014	0.037	0.034	0.016	0.040	0.044	0.014	0.002	0.034	0.035	0.034	0.018
52.	Trans-Vaccenic acid	100	55	24.3 - 24.325	0.060	0.056	0.052	0.058	0.084	0.050	0.045	0.050	0.056	0.053	0.062	0.056	0.047	0.019	0.060	0.053	0.058	0.057
53.	Myristic acid	99	74	18.054	0.085	0.042	0.072	0.020	0.040	0.025	0.025	0.051	0.043	0.035	0.056	0.070	0.021	0.006	0.042	0.051	0.039	0.033
54.	Unknown 149(100) 167(32.8) 57(17.4)	100	149	32.538	0.038	0.030	0.034	0.054	0.086	0.017	0.020	0.019	0.021	0.021	0.027	0.023	0.027	0.048	0.037	0.020	0.036	0.051
55.	Malic acid	98	75	15.362	0.001	0.001	0.001	0.005	0.006	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.001
56.	Glutathione	95	142	19.236	0.002	0.002	0.002	0.013	0.017	0.003	0.002	0.002	0.002	0.002	0.003	0.002	0.002	0.005	0.001	0.003	0.001	0.002
57.	Pyroglutamic acid	83	84	16.652	0.003	0.002	0.003	0.025	0.031	0.002	0.002	0.002	0.003	0.003	0.004	0.004	0.004	0.006	0.002	0.004	0.002	0.003
58.	Docosapentaenoic acid	94	79	30.24 - 30.3	0.054	0.029	0.043	0.009	0.022	0.015	0.012	0.040	0.030	0.020	0.038	0.038	0.011	0.002	0.023	0.029	0.024	0.018
59.	(Z)-Docos-13-enamide	75	59	41.045	0.012	0.018	0.016	0.012	0.025	0.012	0.008	0.014	0.017	0.019	0.023	0.022	0.022	0.022	0.021	0.019	0.027	0.028
60.	Beta-Alanine	99	88	12.9 - 12.98	0.004	0.005	0.005	0.010	0.017	0.008	0.002	0.007	0.003	0.014	0.008	0.005	0.002	0.003	0.001	0.009	0.001	0.002
61.	Citramalic acid	100	128	10.914	0.021	0.017	0.013	0.033	0.053	0.015	0.010	0.010	0.014	0.010	0.018	0.014	0.018	0.027	0.022	0.008	0.024	0.035
62.	2-Aminobutyric acid	97	116	12.426	0.032	0.032	0.022	0.075	0.149	0.034	0.057	0.042	0.041	0.041	0.101	0.100	0.066	0.102	0.038	0.051	0.044	0.059
63.	Fumaric acid	93	113	9.415	0.000	0.000	0.000	0.002	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
64.	Isoleucine	100	115	14.31 - 14.37	0.072	0.076	0.068	0.181	0.245	0.113	0.138	0.135	0.153	0.125	0.152	0.150	0.130	0.139	0.089	0.084	0.072	0.059
65.	Unknown 160(100) 59(43.4) 116(22.7)	94	160	14.915	0.005	0.007	0.005	0.026	0.041	0.005	0.003	0.002	0.004	0.004	0.004	0.003	0.004	0.009	0.006	0.002	0.002	0.005
66.	Tyrosine	100	236	29.048	0.033	0.038	0.022	0.183	0.345	0.049	0.139	0.023	0.045	0.101	0.053	0.038	0.111	0.256	0.046	0.042	0.022	0.025
67.	Serine	98	100	17.653	0.007	0.008	0.008	0.034	0.045	0.006	0.005	0.005	0.007	0.006	0.012	0.014	0.012	0.030	0.011	0.010	0.008	0.014
68.	Ornithine	96	128	25.114	0.007	0.012	0.007	0.273	0.380	0.014	0.027	0.008	0.010	0.020	0.017	0.021	0.022	0.056	0.008	0.007	0.003	0.010
69.	Phenylalanine	100	162	20.401	0.038	0.044	0.032	0.117	0.251	0.051	0.067	0.035	0.057	0.054	0.052	0.052	0.067	0.112	0.058	0.049	0.033	0.041
70.	Unknown 113(100) 85(48.1) 59(21.2)	100	113	8.853	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
71.	Unknown 082(100) 142(56.8) 110(42.6)	94	82	15.15 - 15.2	0.000	0.000	0.000	0.002	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
72.	Palmitelaidic acid	99	55	21.686	0.025	0.012	0.021	0.007	0.014	0.010	0.007	0.019	0.015	0.011	0.018	0.020	0.006	0.002	0.013	0.016	0.015	0.010

Number	Metabolite	AMDIS %	Ref.Ion	Ret.Time	F1D0	F2D0	F3D0	F1D1	F2D1	F3D1	F1D2	F2D2	F3D2	F1D3	F2D3	F3D3	F1D4	F2D4	F3D4	F1D5	F2D5	F3D5
73.	Heptadecanoic acid	96	74	23.37	0.029	0.013	0.022	0.006	0.014	0.008	0.007	0.017	0.015	0.010	0.019	0.018	0.007	0.002	0.013	0.019	0.012	0.009
74.	DL-3-Aminoisobutyric acid	96	88	13.311	0.001	0.001	0.001	0.006	0.007	0.001	0.002	0.002	0.001	0.002	0.003	0.005	0.002	0.003	0.001	0.003	0.002	0.003
75.	Cholesterol	81	386	44.185	0.062	0.027	0.042	0.006	0.020	0.012	0.011	0.039	0.032	0.029	0.035	0.041	0.012	0.001	0.031	0.033	0.021	0.019
76.	Alpha-Linolenic acid	78	79	24.45 - 24.525	0.012	0.005	0.013	0.004	0.007	0.007	0.003	0.012	0.007	0.007	0.008	0.009	0.002	0.001	0.006	0.007	0.012	0.004
77.	Tryptophan	97	130	34.038	0.021	0.020	0.017	0.056	0.165	0.020	0.053	0.027	0.050	0.026	0.040	0.039	0.049	0.083	0.030	0.025	0.017	0.018
78.	Gamma-Linolenic acid	90	79	24.57 - 24.64	0.013	0.005	0.012	0.004	0.006	0.007	0.003	0.013	0.009	0.006	0.011	0.010	0.003	0.002	0.006	0.012	0.008	0.006
79.	Unknown 115(100) 118(95.4) 86(69.3)	98	115	15.92 - 16	0.002	0.002	0.002	0.012	0.014	0.002	0.002	0.001	0.002	0.001	0.003	0.005	0.003	0.008	0.002	0.002	0.002	0.003
80.	Histidine	85	139	27.649	0.007	0.006	0.005	0.007	0.011	0.003	0.002	0.002	0.006	0.002	0.003	0.005	0.006	0.011	0.007	0.004	0.003	0.004
81.	Citric acid	97	143	16.603	0.030	0.068	0.023	0.205	0.243	0.017	0.019	0.018	0.019	0.012	0.028	0.035	0.224	0.286	0.013	0.044	0.013	0.021
82.	Asparagine	95	127	16.795	0.032	0.027	0.026	0.036	0.025	0.018	0.015	0.015	0.041	0.016	0.022	0.041	0.027	0.044	0.031	0.022	0.021	0.020
83.	Unknown 070(100) 42(28.5) 69(8.0)	90	70	7.137	0.002	0.002	0.002	0.009	0.018	0.002	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.004	0.002	0.001	0.000	0.002
84.	Unknown 114(100) 59(61.6) 146(33.7)	94	114	17.271	0.004	0.005	0.004	0.014	0.013	0.003	0.003	0.002	0.005	0.002	0.004	0.007	0.005	0.007	0.002	0.003	0.003	0.004
85.	Unknown 201(100) 117(89.6) 119(86.2)	99	201	8.483		0.002	0.001	0.006	0.009	0.009	0.005	0.001	0.001	0.001				0.002				0.000
86.	Pentadecanoic acid	88	74	19.708	0.021	0.008	0.016	0.004	0.009	0.005	0.005	0.010	0.011	0.007	0.011	0.013	0.004	0.001	0.008	0.011	0.009	0.006
87.	2-Aminoadipic acid	96	114	20.455	0.001	0.001	0.001	0.003	0.006	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.002	0.003	0.002	0.001	0.001	0.001
88.	Dimethyl 2-aminomalonate hydrochloride	90	146	14.914	0.002	0.003	0.003	0.009	0.011	0.002	0.002	0.002	0.003	0.002	0.003	0.004	0.002	0.005	0.003	0.003	0.002	0.004
89.	Vitamin E	82	430	44.377	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
90.	Unknown 074(100) 46(21.3) 59(0.97)	95	74	7.093	0.002	0.003	0.003	0.006	0.008	0.004	0.004	0.005	0.004	0.005	0.003	0.003	0.003	0.004	0.004	0.004	0.002	0.003
91.	Unknown 156(100) 96(36.6) 128(31.0)	65	156	20.46	0.000	0.000	0.000	0.002	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
92.	4-Aminobutyric acid	97	102	15.05 - 15.12	0.002	0.001	0.002	0.001	0.002	0.004	0.000	0.001	0.001	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.001
93.	Unknown 081(100) 136(51.6) 152(42.5)	78	81	25.938	0.001	0.001	0.001	0.001	0.002	0.001	0.000	0.000	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.000	0.001
94.	Methionine	99	147	18.543	0.018	0.017	0.014	0.070	0.095	0.012	0.017	0.009	0.023	0.009	0.014	0.014	0.023	0.043	0.016	0.013	0.013	0.015
95.	Dihomo-gamma-Linolenic acid	85	79	27.15 - 27.2	0.006	0.003	0.004	0.001	0.002	0.001	0.001	0.002	0.003	0.002	0.003	0.003	0.001	0.000	0.003	0.002	0.003	0.001
96.	Oxalic acid	82	59	6.236	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
97.	Cysteine	96	192	20.548	0.001	0.001	0.001	0.003	0.004	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.002	0.003	0.001	0.001	0.001	0.001
98.	Citraconic acid	67	127	10.789	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.001
99.	(E)-heptadec-10-enoic acid	73	55	22.91 - 22.97	0.006	0.002	0.005	0.001	0.004	0.002	0.002	0.003	0.003	0.002	0.004	0.005	0.002	0.000	0.003	0.004	0.003	0.002
100.	Cis-4-Hydroxyproline	83	144	20.324	0.001	0.001	0.001	0.003	0.005	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.001
101.	Adrenic acid	79	79	29.95 - 30.04	0.003	0.002	0.002	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.002	0.002	0.001	0.000	0.001	0.002	0.001	0.001
102.	Conjugated linoleic acid	72	67	24.22 - 24.3	0.003	0.002	0.003	0.001	0.002	0.002	0.001	0.002	0.002	0.001	0.002	0.002	0.001	0.000	0.001	0.002	0.004	0.001
103.	Dodecane	87	57	9.858	0.003	0.002	0.002	0.002	0.003	0.001	0.001	0.001	0.002	0.001	0.002	0.002	0.001	0.002	0.002	0.001	0.002	0.002
104.	Creatinine	98	202	16.95 - 17	0.008	0.013	0.009	0.029	0.032	0.018	0.014	0.017	0.019	0.017	0.024	0.018	0.021	0.021	0.013	0.013	0.014	0.010
105.	9E-heptadecenoic acid	94	55	23.223	0.004	0.002	0.003	0.001	0.002	0.002	0.001	0.002	0.002	0.002	0.003	0.003	0.001	0.000	0.002	0.003	0.003	0.001
106.	(11Z,14Z,17Z)-Eicosa-11,14,17-trienoic acid	72	79	27.484	0.003	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.002	0.002	0.001	0.000	0.001	0.002	0.002	0.001
107.	11,14-Eicosadienoic acid	71	67	27.34 - 27.37	0.005	0.002	0.003	0.001	0.002	0.001	0.001	0.002	0.002	0.001	0.002	0.002	0.001	0.000	0.002	0.002	0.002	0.001
108.	Unknown 128(100) 42(10.5) 129(6.8)	85	128	21.257	0.001	0.001	0.001	0.005	0.013	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.000	0.000	0.000	0.001
109.	Unknown 059(100) 73(69.3) 77(45.8)	72	59	19.02	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
110.	Unknown 086(100) 59(56.6) 128(39.3)	96	86	13.445	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
111.	4-Methyl-2-oxopentanoic acid	86	85	8.1 - 8.155	0.001	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
112.	Unknown 232(100) 128(72.0) 156(51.0)	95	232	22.631	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
113.	2-Hydroxybutyric acid	98	117	10.65	0.003	0.003	0.002	0.014	0.015	0.007	0.015	0.012	0.006	0.016	0.015	0.017	0.015	0.023	0.005	0.006	0.005	0.009
114.	Glutamine	82	84	25.16 - 25.25	0.001	0.001	0.001	0.002	0.004	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.002	0.001	0.002	0.001	0.001
115.	Tridecane	71	57	12.847	0.001	0.001	0.002	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.002	0.001
116.	Unknown 045(100) 59(89.0) 74(67.8)	95	45	9.157	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.000	0.001
117.	Alpha-Ketoglutaric acid	73	115	15.464	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
118.	Nicotinic acid	95	137	11.046	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
119.	Dodecanoic acid	74	74	15.49	0.001	0.001	0.001	0.001	0.002	0.001	0.000	0.001	0.000	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001
120.	Unknown 091(100) 106(54.7) 105(21.8)	93	91	5.9	0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.001	0.001	0.000	0.001	0.001
121.	N-PENTADECANE	75	57	15.537	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
122.	Nonadecanoic acid	66	74	25.864	0.002	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.000
123.	Caproic acid	76	74	6.506	0.002	0.001	0.001	0.002	0.003	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.001	0.001	0.001	0.000	0.001	0.001
124.	Unknown 088(100) 44(30.3) 59(19.6)	85	88	23.616	0.002	0.004	0.003	0.008	0.014	0.001	0.002	0.003	0.004	0.001	0.006	0.005	0.004	0.008	0.003	0.004	0.002	0.004
125.	Caprylic acid	78	74	9.7 - 9.75	0.001	0.000	0.001	0.001	0.002	0.000		0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.001
126.	NADPH	84	138	11.08	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
127.	Unknown 142(100) 153(51.8) 59(22.0)	91	142	22.393	0.003	0.004	0.003	0.010	0.018	0.002	0.003	0.004	0.005	0.001	0.008	0.007	0.005	0.009	0.003	0.004	0.002	0.005
128.	Arachidic acid	74	74	27.515	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001
129.	Unknown 142(100) 59(19.9) 141(17.0)	86	142	29.512	0.003	0.005	0.003	0.010	0.014	0.002	0.003	0.003	0.004	0.001	0.007	0.007	0.005	0.008	0.003	0.005	0.003	0.005
130.	Guanine	90	307	27.934	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.000	0.000	0.001	0.001	0.000	0.000
131.	Unknown 059(100) 148(44.7) 43(33.8)	74	59	23.209	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.001
132.	Unknown 074(100) 87(68.8) 55(33.1)	70	74	19.024	0.002	0.000	0.001	0.000	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.000	0.001	0.001	0.001	0.001
133.	Unknown 102(100) 58(0.86) 59(0.75)	67	102	23.302	0.000	0.000	0.001	0.002	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
134.	Unknown 249(100) 57(31.8) 264(22.9)	92	249	17.801	0.000	0.000	0.000	0.001	0.001	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.001
135.	Unknown 125(100) 184(90.5) 96(54.3)	93	125	22.747	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
136.	1-Aminocyclopropanecarboxylic acid	74	141	13.676	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
137.	Benzoic acid	83	105	9.896	0.001	0.000	0.001	0.001	0.001	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001
138.	Unknown 056(100) 59(24.8) 115(10.8)	80	56	7.195	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
139.	Dehydroascorbic acid	72	59	15.785 - 15.82	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
140.	Unknown 112(100) 43(60.3) 115(49.2)	90	112	10.717	0.001	0.001	0.001	0.004	0.005	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.002	0.003	0.002	0.002	0.001	0.001
141.	Unknown 114(100) 59(15.3) 82(14.4)	94	114	12.367	0.005	0.003	0.003	0.012	0.012	0.001	0.010	0.003	0.007	0.003	0.007	0.008	0.012	0.024	0.012	0.004	0.008	0.008
142.	n-Heptadecane	100	57	16.786	0.000	0.000	0.001	0.001	0.002	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.001	0.001	0.001	0.000	0.000	0.000
143.	Glyoxylic acid	86	75	11.4 - 11.45	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Number	Metabolite	AMDIS %	Ref.Ion	Ret.Time	F1D0	F2D0	F3D0	F1D1	F2D1	F3D1	F1D2	F2D2	F3D2	F1D3	F2D3	F3D3	F1D4	F2D4	F3D4	F1D5	F2D5	F3D5
144.	Cis-Aconitic acid	95	153	15.752	0.001	0.003	0.001	0.008	0.011	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.009	0.011	0.001	0.002	0.001	0.001
145.	alpha-Hydroxyglutarate	70	85	14.55	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
146.	Unknown 135(100) 180(28.0) 77(19.9)	84	135	16.124	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
147.	Salicylic acid	96	135	17.078	0.003	0.002	0.004	0.003	0.004	0.002	0.002	0.004	0.003	0.003	0.004	0.005	0.002	0.003	0.004	0.003	0.003	0.003
148.	Unknown 082(100) 153(90.1) 56(65.2)	84	82	18.127	0.001	0.001	0.001	0.001	0.002	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001
149.	Unknown 071(100) 57(94.4) 85(72.9)	74	71	14.111	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
150.	Pyruvic acid	77	89	7.568	0.001	0.001	0.002	0.007	0.006	0.002	0.001	0.001	0.002	0.003	0.001	0.001	0.002	0.004	0.002	0.001	0.001	0.003
151.	n-Tricosane	100	57	25.78	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
152.	Nervonic acid	71	55	33.926	0.002	0.001	0.002	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.000	0.001	0.002	0.001	0.001
153.	Unknown 128(100) 139(21.1) 42(19.1)	84	128	19.879	0.000	0.000	0.000	0.004	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
154.	Unknown 115(100) 100(70.4) 56(60.1)	79	115	8.223	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
155.	Beta-Citryl-L-glutamic acid	76	143	32.181	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
156.	Unknown 115(100) 59(65.5) 189(50.1)	84	115	19.76	0.001	0.002	0.001	0.006	0.007	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.006	0.007	0.000	0.001	0.000	0.001
157.	Decanoic acid	72	74	12.756	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
158.	Unknown 131(100) 71(89.6) 103(88.8)	71	131	17.25 - 17.32	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
159.	Adipic acid	78	114	12.621	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
160.	2-Oxobutyric acid	75	57	6.086	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
161.	Unknown 102(100) 103(26.6) 44(19.0)	77	102	21.265	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
162.	Unknown 114(100) 147(31.9) 115(27.2)	76	114	23.156	0.000	0.001	0.000	0.001	0.002	0.000	0.001	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.000	0.000	0.000	0.000
163.	Unknown 043(100) 59(16.5) 74(15.0)	65	43	7.161	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
164.	Unknown 071(100) 57(92.3) 43(75.3)	80	71	24.09 - 24.15	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
165.	Unknown 059(100) 81(40.5) 91(34.4)	69	59	37.649	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
166.	Homocysteine	70	114	23.59	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
167.	Unknown 088(100) 57(53.2) 59(45.8)	90	88	7.436	0.001	0.001	0.001	0.007	0.006	0.002	0.002	0.002	0.002	0.004	0.002	0.003	0.002	0.006	0.001	0.001	0.001	0.001
168.	Unknown 119(100) 147(96.0) 101(53.9)	67	119	17.64	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
169.	2-Oxovaleric acid	70	71	6.544	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
170.	S-Adenosylhomocysteine	81	147	17.3 - 17.35	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
171.	Glutaric acid	91	100	9.674	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
172.	2-methyl-octadecanoic acid	78	88	25.357	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
173.	Glyceric acid	66	119	13.977	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
174.	Isocitric acid	78	129	21.444	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000
175.	Malonic acid	93	101	7.65	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
176.	Lignoceric acid	68	74	34.256	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
177.	Nicotinamide	86	57	7.45	0.000	0.001	0.000	0.003	0.002	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.002	0.000	0.000	0.000	0.000
178.	N-Acetylglutamic acid	68	116	19.519	0.000	0.000	0.000	0.006	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
179.	Itaconic acid	90	127	10.402	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.002	0.000	0.000	0.000	0.000
180.	Azelaic acid	76	185	16.72 - 16.77	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
181.	3-Methyl-2-oxopentanoic acid	81	57	8.016	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.002	0.001	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.000
182.	2-Hydroxyisobutyric acid	72	73	9.366	0.001	0.000	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.001	0.001
183.	4-Hydroxyphenylacetic acid	79	121	19.409	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
184.	Trans-4-Hydroxyproline	67	216	17.673	0.001	0.003	0.001	0.011	0.009	0.002	0.002	0.001	0.003	0.001	0.001	0.002	0.002	0.005	0.001	0.001	0.001	0.002
185.	Unknown 198(100) 138(61.7) 166(40.8)	84	198	21.159	0.000	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
186.	Unknown 126(100) 127(46.7) 59(33.2)	94	126	10.213	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
187.	Unknown 101(100) 119(80.5) 74(35.3)	69	101	8.747	0.000	0.000	0.000		0.000	0.000				0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

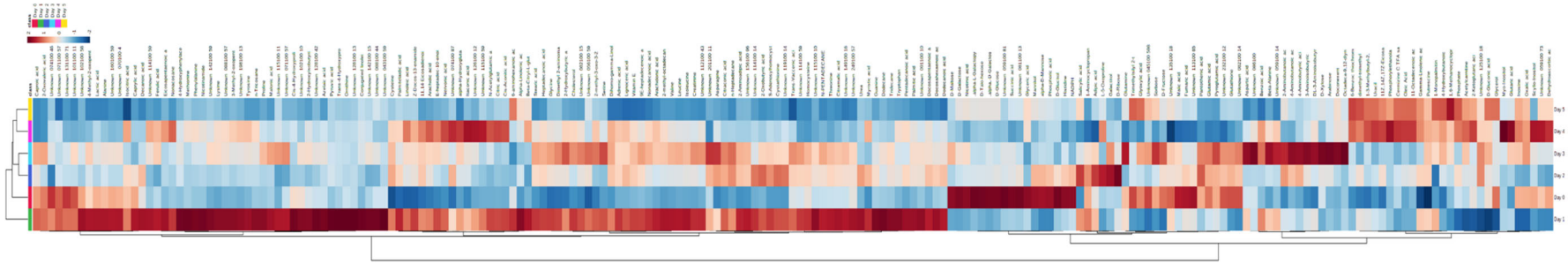


Figure 7.9S Detailed heat map for liver metabolic features following in vivo i.p. injection of *Oncorhynchus tshawytscha* with 2.5 µL g^{-1} fish of poly (I:C). Data come from means of three control (Day 0) and three treatment fish each day (Day 1-Day 5) post-poly (I:C) administration.

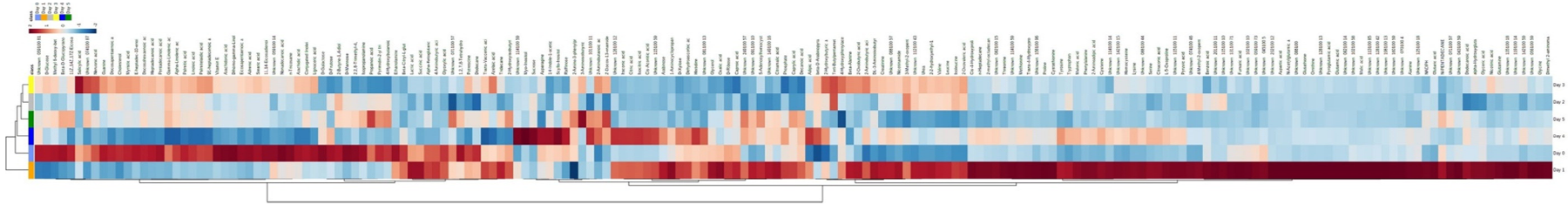


Figure 7.10S Detailed heat map for serum metabolic features following *in vivo* i.p. injection of *Oncorhynchus tshawytscha* with 2.5 µL g^{-1} fish of poly (I:C). Data come from means of three control (Day 0) and three treatment fish each day (Day 1-Day 5) post-poly (I:C) administration.

8 Chapter 8. Case Study 5: Uncoupling Interdependence of Thermal Stress and Growth Performance on Chinook salmon (*Oncorhynchus tshawytscha*) Blood Biochemistry and Immune capacity.

This chapter has been submitted for publication as:

Lulijwa, R., Young., Delorme, N. J., Walker, P.S., Symonds, J. E., & Alfaro, A. C. Uncoupling interdependence of thermal stress and growth performance on Chinook salmon (*Oncorhynchus tshawytscha*) blood biochemistry and immune capacity. Submitted to the journal *Metabolites*.

8.1 Prelude to case study 5

Water temperature profoundly affects fish metabolism and growth, with maximum and minimum tolerance values varying across species. Climate change-driven ocean warming, and abnormally extreme sea surface temperatures (SST) are threatening aquatic life globally, with ecological and production consequences. Although critical, the mechanisms behind thermotolerance and differences in growth performance at individual and population levels remain scanty in the New Zealand farmed Chinook salmon (*Oncorhynchus tshawytscha*). Of recent, heavy summer mortalities have been recorded in some New Zealand farmed *O. tshawytscha* locations. This study was thus designed to investigate blood biomarkers of thermal stress and growth performance in *O. tshawytscha*. An integrated approach involving haematology, erythrocyte osmotic fragility (EOF), the Muse® flow cytometry PBMC viability, reactive oxygen species (ROS) production and colorimetric apoptosis, plus serum biochemistry were employed. Results from this study were discussed in the manuscript and may be useful in selective breeding programs against thermal stress.

8.2 Introduction

Chinook salmon (*Oncorhynchus tshawytscha*) are large anadromous fish native to North America and regions in the western Pacific. Valued for their high-quality fillet, global fishery landings peaked in the late 1980s but have since declined markedly to less than 4000 tons partly due to overfishing. *O. tshawytscha* were introduced to New Zealand more than a century ago, and the country's development of a successful aquaculture industry now provides almost 90% of the world's supply (Tucker, 2014). Salmon are reared only in the South Island of New Zealand where cooler water temperatures are more favourable. However, chronic and acute water temperature variations associated with climate change are starting to pose a significant threat to industry.

Sea surface temperatures (SSTs) around New Zealand have been rising at a rate of approximately 0.2°C per decade over the past forty years, and end-of-century forecasts predict a 1.1–2.5°C increase above the current average (Law et al., 2018; Sutton & Bowen, 2019). Exacerbating the effects of this relatively rapid thermal rise on ecosystems are the increasing incidences of marine heatwaves during summer months (Salinger et al., 2020; Salinger et al., 2019). As examples, New Zealand salmon farmers reported substantial stock losses (ca. 20%) over the 2017/2018 and 2018/2019 austral summers due to unprecedented SSTs reaching 2–5°C above the species preferred upper threshold (17°C) where peak health and growth can be maintained (Broekhuizen et al., 2021; Chiswell & Sutton, 2020; NZKS., 2019; Richter & Kolmes, 2005). Temperature-stressed salmon generally have increased metabolic rates and somatic maintenance costs, grow slower with lower condition factors (*K*), have suppressed appetite, experience reproductive issues, display compromised immunity, and exhibit increased sensitivity to other stressors (Alfonso et al., 2020; Myrick & Cech, 2001; Rebl et al., 2020; Wade et al., 2019; Whitney et al., 2016). However, plasticity in thermal tolerance and growth performance is well documented among fishes (Alfonso et al., 2020) which affords an optimistic outlook to build potential thermal tolerance into New Zealand farmed *O. tshawytscha* stocks.

Some individual fish seem better equipped at dealing with stressors than others, and the appearance of divergent growth phenotypes within cohorts is relatively common (Barton, 2002; Damsgård et al., 2019; Montero et al., 2009; Unrein, 2014). Confronted with rapid global climate change, understanding what makes wild and domesticated fish at individual and population levels more or less likely to perform well under dynamically shifting conditions has major ecological and economic implications. Unravelling biological mechanisms underpinning thermal tolerance in fish is crucial to developing mitigation strategies to safeguard and futureproof the *O. tshawytscha* aquaculture sector.

Accordingly, we performed an elevated temperature challenge on commercial stocks from two of New Zealand's selective breeding programs to establish thermal tolerance trait heritability based on genetic associations with survival and growth performance (unpublished). The current study extends this research by investigating the influence of thermal stress on a targeted suite of 43 blood parameters with clinical significance, and their associations with highly

divergent growth trajectories. We achieve this by selectively sampling individuals which gained or lost weight under both ambient and high temperature conditions. The objectives of this study are to: 1) establish cellular haematology and plasma biochemistry reference data for New Zealand farmed *O. tshawytscha* presenting suboptimal health, 2) utilise quantitative blood profiles to inform mechanism and severity of the thermal stress response, and 3) gain functional insight into poor growth performance.

8.3 Results and Discussion

8.3.1 Survival and growth performance

A three-month thermal challenge at 19–20°C resulted in 20% cumulative mortality and hampered overall growth of surviving fish (Figure 8.1). These results align with performance metrics recorded by farmers during recent marine heatwave events (Chiswell & Sutton, 2020), reinforcing that 19–20°C approximates the chronic thermal maxima for this species. Overall, mean body weight in the challenged group were similar pre- and post-treatment. However, high variation in performance under both thermal regimes were highlighted by positive and negative specific growth rate (SGR). This trend afforded opportunity to focus attention on divergent growth performance phenotypes decoupled from specific thermal stress-induced effects through selective sampling. Representative fish which gained or lost weight under each of the temperature treatments were nominated for targeted assessments of cellular haematology (n=12) and plasma biochemical (n=31) parameters (Table 8.2S). See Supplementary Material (Table 8.1S) for biometric information on selected candidates.

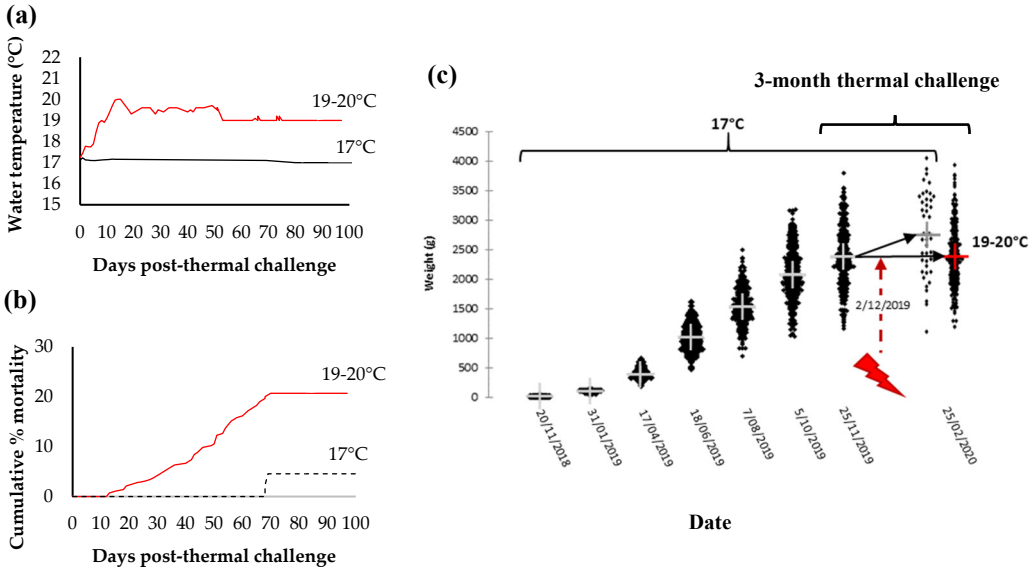


Figure 8.1. Effects of a chronic three-month thermal challenge (17°C versus 19–20°C) on *Oncorhynchus tshawytscha* performance. (a) Water temperature data for the thermal regimes (plotted as daily averages); (b) Cumulative percent mortality during the challenge period; (c) Body weight of fish over the preceding one year of grow-out at 17°C, and in the two temperature treatment groups on completion of the trial.

8.3.2 Thermal stress associations

Regarding cellular haematology, a main effect of temperature was detected on erythrocyte osmotic fragility (EOF) presented as mean corpuscular fragility (MCF) (Figure 8.2b) and higher temperature also activated caspases in peripheral blood mononuclear cells (PBMCs) (Figure 8.2a). The lower MCF values in fish acclimated at the higher temperature indicates an increase in cellular membrane stability of erythrocytes. These results align with the inverse thermal–EOF relationship previously demonstrated in carp (Jóźwiak & PaŁecz, 1988). A mechanistic basis may correspond to differences in cell membrane composition. Erythrocyte membrane strength in fish is strongly influenced by the unsaturation index of the polar lipid component; higher incorporation of omega-3 fatty acids in phospholipids promote changes in the lamellar packing of the membrane and alters viscosity and fragility (Kiron et al., 1994).

Caspase (-3/-8) activities were non-detectable in PBMCs of fish reared under ambient temperature but were detected in their thermally stressed counterparts. Caspases are highly conserved proteases crucial to mediating programmed cell death, or apoptosis (Sakamaki & Satou, 2009). Caspase-8 is an initiator enzyme which regulates immune gene expressions and the activation of downstream caspases, whereas caspase-3 is an executioner enzyme responsible for cleavage of intracellular proteins and fragmentation of DNA. The apoptotic cascade cumulates in self-elimination of surplus or injured cells through chromatin condensation and nuclear fragmentation. Apoptosis is considered a ‘clean’ type of cell death whereby the membrane is left intact, and, unlike cell necrosis, there is no consequential inflammatory response (AnvariFar et al., 2017). In agreement with these results, there was no evidence to suggest that PBMC membrane integrity had been compromised (i.e., non-discernible difference in PBMC viability through dye exclusion principal). Upregulated transcription of various pro-apoptotic genes, including caspases, have previously been established as key thermal stress responses in *O. tshawytscha* (Tomalty et al., 2015); our results compliment these findings at the functional enzyme level.

Acute phase proteins (APPs) such as haptoglobin (HAPT) are potential biomarkers of trauma or infection in fish. Local inflammatory cells secrete cytokines (e.g., interleukin-1 [*il-1*], interleukin-6 [*il-6*], tumor necrosis factor alpha [*tnf-α*]) in response to tissue damage which stimulates hepatocytes to produce APPs and release them into circulation (Roy et al., 2017). In salmonids, gradual thermal rises in water temperature are reported to influence transcription of APP-encoding genes (Rebl et al., 2013; Rebl et al., 2018). Although we detected an increase in plasma haptoglobin levels under elevated thermal conditions (Figure 8.3a), the limited effect on other inflammatory-related markers (i.e., total leucocyte counts and levels of c-reactive protein [CRP], prostaglandin E2 [PGE2], and albumin [ALB]) indicate a relatively mild immune response (Figure 8.2a & Figure 8.3a). In corroboration, immune-based plasma parameters in fish under both thermal regimes were within the normal reference range of healthy O. *tshawytscha* (Casanovas et al., 2021). Furthermore, no evidence of excessive redox imbalance was uncovered

(i.e., via relative production of reactive oxygen species [ROS] in PBMCs, plasma activities of catalase [CAT; ROS-detoxifying enzyme], or enhanced lipid peroxidation [LPx; oxidative damage biomarker]) (Figure 8.2 & Figure 8.3).

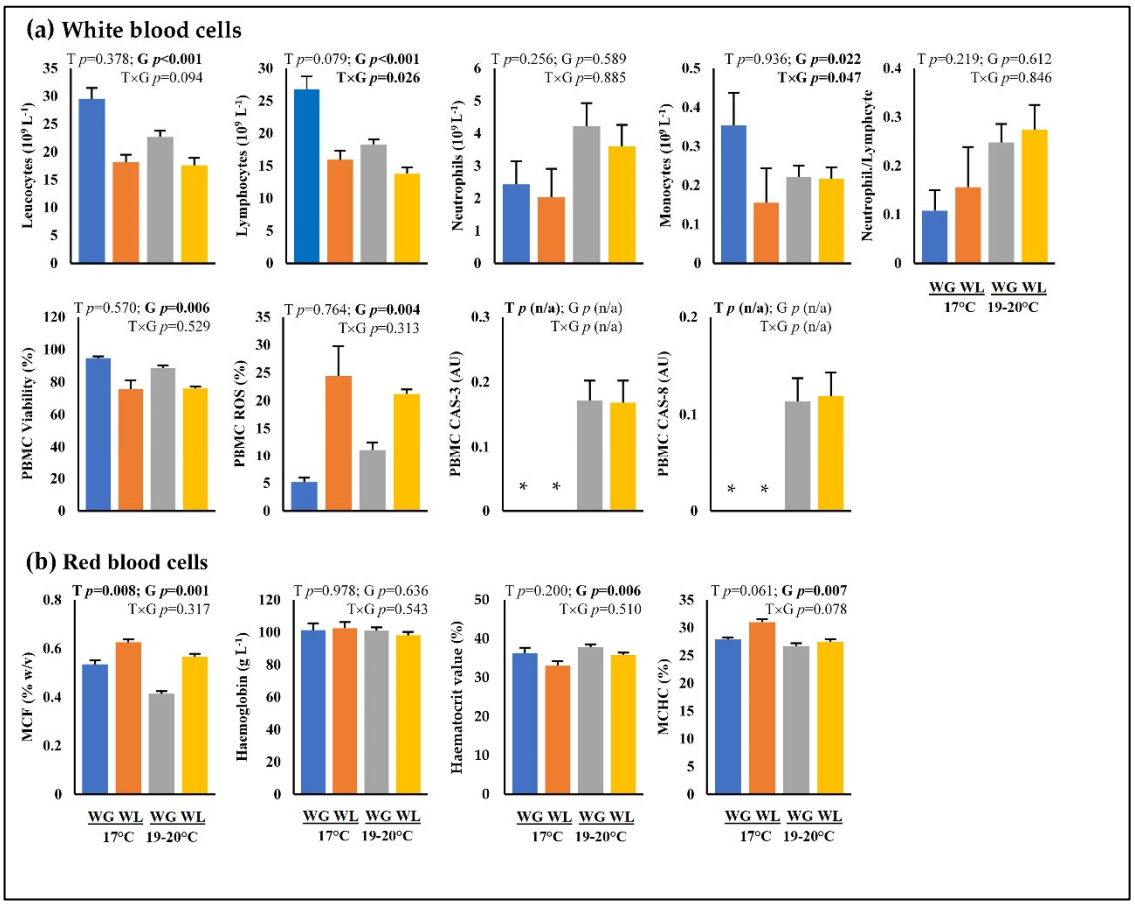


Figure 8.2. Cellular haematology associations with chronic thermal stress (17°C vs 19–20°C) and growth performance (weight gain [WG] vs weight-loss [WL] phenotypes) in *Oncorhynchus tshawytscha*. (a) White blood cell parameters; (b) Red blood cell parameters. Data = group means ± SE; Asterisks signify non-detectable levels. Main effects of temperature (T), growth (G), and their interaction (T×G) are presented in the upper section of each plot (GLMs; α=0.05). PBMC: peripheral blood mononuclear cells, ROS: reactive oxygen species, CAS-3: caspase 3, CAS-8: caspase 8, MCF: mean corpuscular fragility, MCHC%: mean corpuscular haemoglobin concentration.

Levels of four ions (Cl^-, Na^+, K^+, Mg^{2+}) were elevated in *O. tshawytscha* acclimated at 19–20°C, indicating a shift in osmoregulation (Figure 8.3c). Gill chloride cells utilise a Na^+ gradient (established by a gill Na^+/K^+-ATPase in the basolateral membrane) to secrete Cl^- and uptake Na^+ from saltwater to regulate plasma osmolality (Kammerer et al., 2010). Diminished capacity for ion regulation in Atlantic salmon (*Salmo salar*) and sea bream (*Sparus aurata*) during thermal stress is associated with reduced Na^+/K^+-ATPase activity (Vargas-Chacoff et al., 2020; Vargas-Chacoff et al., 2018). Evaluating our results beyond observed relative differences, chronic thermal stress triggered plasma levels of Cl^- and Na^+ to rise outside of the normal reference range (Casanovas et al., 2021), indicating severity of the effect. Further appraisal is needed to ascertain whether these ion levels are being maintained under homeostatic control and within an adaptive regulatory capacity.

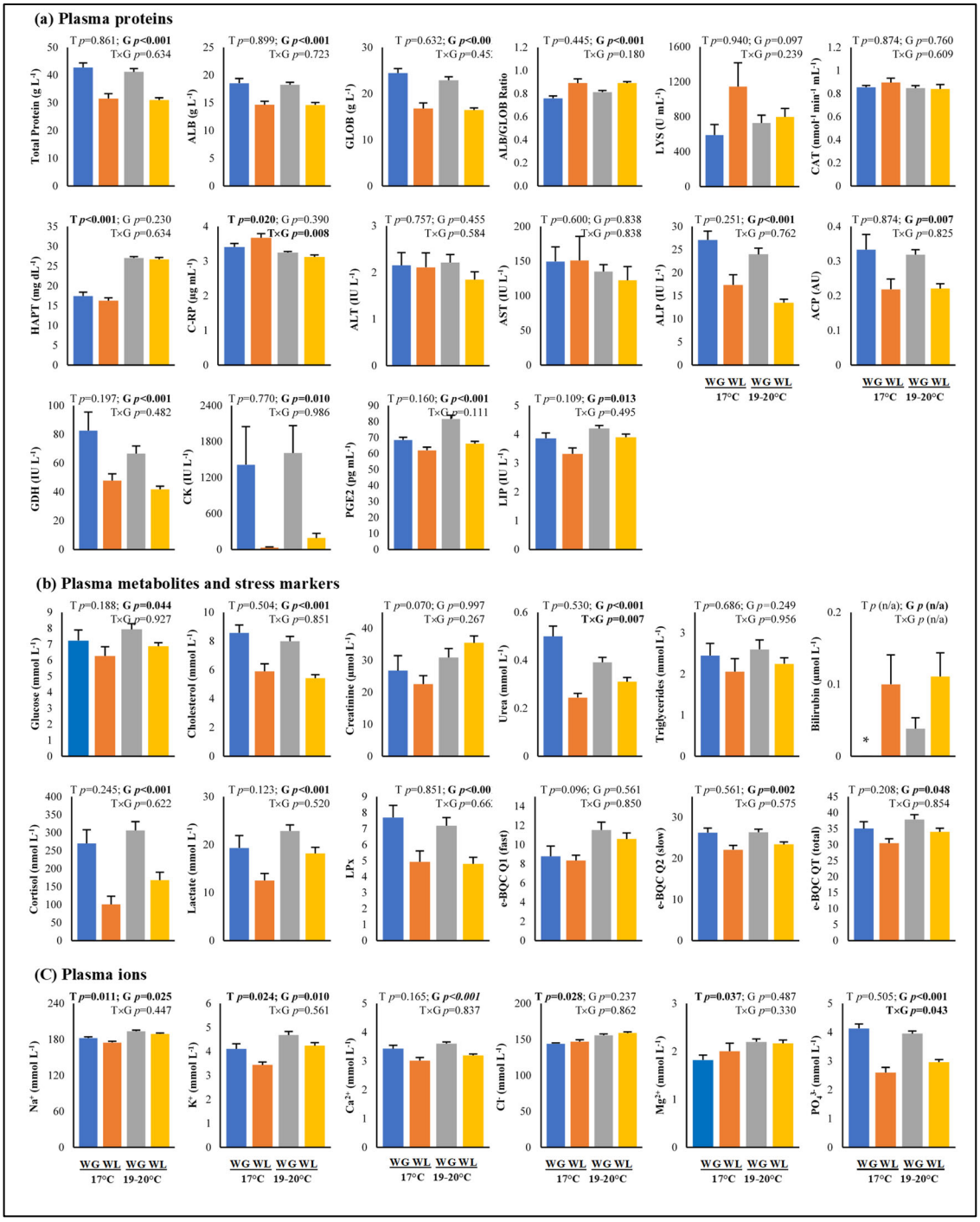


Figure 8.3. Plasma biochemistry associations with chronic thermal stress (17°C vs 19–20°C) and growth performance (weight gain [WG] vs weight-loss [WL] phenotypes) in *Oncorhynchus tshawytscha*. (a) Protein parameters; (b) Metabolite parameters; (c) Ion parameters. Data = group means ± SE; Asterisks signify non-detectable levels. Main effects of temperature (T), growth (G), and their interaction (T×G) are presented in the upper section of each plot (GLMs; α=0.05). ALB: albumin, GLOB: globulin, ALB/GLOB: albumin/globulin ratio, LYS: lysozyme, CAT: catalase, HAPT: haptoglobin, C-RP: c-reactive protein, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, ACP: acid phosphatase, GDH: glutamate dehydrogenase, CK: creatine kinase, PGE2: prostaglandin E2, LIP: lipase, e-BQC Q1: fast antioxidants, e-BQC Q2: slow antioxidants, and e-BQC QT: total antioxidants.

8.3.3 Growth performance associations

Fish with highly divergent growth phenotypes presented markedly different cellular haematology and plasma biochemical profiles irrespective of culture temperature (Figure 8.2 & Figure 8.3). Twenty eight out of the 43 blood parameters assessed were influenced by growth performance. Fish which lost weight during the experiment likely had suppressed appetite and or had reduced feeding activity. This is supported by the positive Pearson correlation (r_s = 0.57; p = 0.001) of the feed intake at sampling versus weight gain/loss during the trial, the collective responses of plasma clinical biomarkers, and use of liver lipid reserves as evidenced by decreased hepatosomatic index (HSI) (Table 8.1S).

Classical signatures of starvation in fish include reduced plasma levels of cholesterol, glucose, and total protein (TP) (Peres et al., 2013), all of which were significantly lower in the weight-loss group (Figure 8.3). Complimenting these findings were low levels of creatine kinase [CK] indicative of reduced ATP turnover and muscle weakening/deterioration (Lowery & Somero, 1990; Racicot et al., 1975; Wade et al., 2019), lower activities of acid phosphatase (ACP) and alkaline phosphatase (ALP) pointing towards suppressed appetite and impaired energy metabolism (Amri et al., 2020; Chandra, 1982; Ray et al., 2017; Tripathi & Verma, 2003), and higher levels of bilirubin symptomatic of liver dysfunction and/or reduced feeding (Barrett, 1971; Meyer et al., 1995; Sakai et al., 1985). Interestingly, visceral fat score and circulating levels of total triglycerides (TAGs) were similar between growth performance groups, whereas HSI was reduced which suggests preferential utilization of liver lipid reserves during reduced calorie intake (Table 8.1S). Correspondingly, lower body girth, stomach width, and gastrointestinal tract signify lower condition factor in the weight-loss group. In addition, there was higher swim bladder fluid build-up (ascites) in the weight-loss group, further suggesting disturbance to buoyancy and additional energy cost to achieve neutral positioning (Loh, 2007; Macaulay et al., 2020).

Cellular haematology biomarker signals associated with reduced feed intake in fish also include symptoms of suppressed immunocompetence in the weight-loss group, as evidenced by lower total leucocyte counts (leucopenia), and reduced viability of PBMCs (Figure 8.2a). Such characteristics increase the risk of infections from pathogens (Rios et al., 2005). Coupled with diminished viability of these white blood cells was an increase in ROS production, revealing that PBMCs were also under enhanced oxidative pressure (potentially as an underpinning mechanism of necrosis). Lower total antioxidant capacity (TAC) (i.e., e-BQC QT values) in plasma compliments these data and provides further evidence of redox imbalance in the weight-loss group (Figure 8.3b). According to the relative e-BQT Q1/Q2 values, primary drivers for this increase in TAC are endogenous metabolites with slow-reacting antioxidant behaviour (e.g., polyphenols). Lipid peroxidation products were diminished in the weight-loss group which may be explained by fish having lower metabolic activities and/or through ketogenesis of protective metabolites (e.g., butyrate derivatives, acetoacetate) under reduced calorie intake (Heming & Paleczny, 1987; Mejía et al., 2006).

Cortisol and lactate are routinely used biomarkers for stress in fish and were detected at relatively high (Casanovas et al., 2021) concentrations in both growth performance groups (Figure 8.3b). We attribute this to crowding and scoop netting of fish prior to handling and euthanisation via anaesthetic overdose. An initial aversion response to the active compound stimulates the hypothalamic–pituitary–interrenal (HPI) axis leading to cortisol accumulations; rising lactate levels follow as a secondary stress response (Readman et al., 2013; Young et al., 2019; Zahl et al., 2010; Zahl et al., 2012). Relative differences between growth performance groups were however still attributable. The lower cortisol levels in the weight-loss group may indicate exhaustion of the HPI axis as a response to one or more previously experienced persistent stressors (e.g., suppressed appetite and or reduced feeding) (Bermejo-Poza et al., 2017; Madaro et al., 2015). The endocrine-based regulation of feeding and weight appears to be tightly coupled with associations between stress factors, corticotropin-releasing factor, and appetite (Conde-Sieira et al., 2018; Volkoff, 2019).

Glutamate dehydrogenase (GDH) reversibly catalyses the oxidative deamination of glutamate to produce α-ketoglutarate (as an energy substrate for the citric acid cycle) and ammonia, playing a key role in nitrogen excretion in fish (Gaspar et al., 2018). The lower GDH activity in the weight-loss group and mean urea levels (Figure 8.3) seems counterintuitive to an expected increase in protein catabolism during reduced feeding. It is therefore possible that the weight-loss group preferentially utilised liver lipid reserves for energy metabolism, as revealed by reduced HSI (Table 8.1S). Our results also contrast findings in slow- versus fast-growth hybrid mandarin fish phenotypes being characterized by higher GDH activity (L. Liu et al., 2017). Further interrogation of this mechanism in *O. tshawytscha* is warranted through experiment to interpret these data.

Albumin and globulin (GLOB) are principal plasma proteins essential for maintaining immunocompetence; their levels indirectly reflect the condition of specific humeral immunity and their ratio is widely used as an index of physiological state (Ahmed & Ali, 2013; Haghighi et al., 2017; Kaleeswaran et al., 2012). Considered non-specific biomarkers, lower levels can indicate a range of issues including liver and kidney dysfunctions, acute haemolytic anemia, infection, and inefficiencies in protein digestion and absorption (Abdel-Tawwab & Hamed, 2018; Ahmed & Ali, 2013; Kumar et al., 2017). ALB/GLOB ratios provide causative insight into lower total protein levels, and increased values may indicate protein energy malnutrition (Rahman & Begum, 2005).

Altered levels of plasma cations (Na^+, K^+ and Ca^{2+}) between the growth performance groups signal differential osmotic balance. Changes in the regulatory system of ionic interchange can alter blood pH and reduce erythrocyte volume, in turn affecting haematocrit (Hct) values (Vosylienė, 1999). In line with this, cellular parameters in fish with negative growth trajectories included lower Hct values possibly due to mild microcytic anemia (Witeska, 2015), increased mean corpuscular haemoglobin concentration (MCHC) suggesting a level of cell shrinkage and

morphological degeneration (Ciepliński et al., 2019), and higher MCF values point to increased membrane fragility to stressors (Kiron et al., 1994; Malekar et al., 2018).

8.3.4 Overview and future directions

In summary, we have established cellular haematology and plasma biochemistry reference data for New Zealand farmed *O. tshawytscha* presenting suboptimal health in association with chronic thermal stress and divergent growth phenotypes. We expected stronger signatures reflecting negative impacts of thermal stress within the blood biomarkers than were detected. Measured effects suggest a low–moderate stress and osmoregulatory disturbance in response to 19–20°C although, various other physiological processes and pathological characteristics need evaluation to ascertain this. Various blood biomarker signatures in fish with negative growth trajectories were clinically relevant to suppressed appetite and or reduced feeding (substantiating their significance), and some provide insights to formulate and explore new hypotheses (e.g., roles of GHD, corticotropin-releasing factor, ketone bodies). Future studies involving elevated thermal stress in *O. tshawytscha* should incorporate temporal assessments of behaviour, metabolic rates, organ histology, compartmentalization/use of energetic reserves, and broader evaluations of endocrine target complements (transcript–to–metabolite). It will also be important to establish whether there exists an interdependency between thermal tolerance and growth performance, and to discern if other pre-existing performance-based metrics are influential to an individuals' fitness in coping with elevated temperatures. Heritable and non-heritable mechanistic bases underpinning variability in thermal tolerance are key areas for research to capitalize on selective breeding potential and to assist advances in husbandry-based practices. Building of robust and resilient salmon will help wild and domesticated fish stocks to withstand global change and extend opportunities for aquaculture development.

8.4 Materials and methods

8.4.1 Experimental design and sampling

Smolts from the breeding programmes of two commercial hatcheries (Sanford Ltd and New Zealand King Salmon) were passive integrated transponder (PIT) tagged, transferred to the Cawthron Finfish Research Centre (FRC) (Glenduan, Nelson, New Zealand), and maintained at 17°C on a saltwater recirculating aquaculture system (RAS) fed to satiation daily with commercial diet (Skretting, Australia). After one year of rearing, 329 fish were randomly distributed among 12 × 500L tanks. Ten tanks were assigned to a high temperature treatment (for evaluation of thermal tolerance trait heritability); the water temperature was gradually increased from 17°C to 20°C at 0.13°C per day for 15 days. The other two tanks were utilised as in situ controls (17°C). Finetuning of the high thermal condition was conducted during the first few weeks of the experiment based on reduced feeding behaviour and incidences of mortality, with a final thermal challenge temperature being reduced to 19°C. Levels of dissolved oxygen within the elevated-temperature treatment tanks were also manipulated (lowered to 6.61±0.29 mg L^{-1}) to mimic conditions of the natural marine environment during warmer summer periods. Fish were fed to

satiation once daily, and mortalities recovered. The entire experiment ran for 102 days. At the conclusion of the experiment, fish were selectively sampled to provide representative numbers of individuals which gained weight and lost weight from every tank across the two thermal treatments. Depending on numbers of fish available, 5–16 fish from each of the 12 tanks were obtained for blood extraction; an unbalanced sampling regime was consequently implemented with biological replication for each of the fixed effects (temperature, growth) ranging from 10–60 fish (Table 8.2S). Fish were crowded and then individually captured from tanks via scoop net, euthanised by anaesthetic overdose (AQUI-S®; 80 ppm; 7 min), and 3–4 mL of peripheral blood was withdrawn from the caudal vein. Sub-aliquots of blood were allocated for analyses of various cellular haematology and plasma biochemistry parameters.

8.4.2 Biometrics

All fish assigned for blood analyses were evaluated for biometric parameters comprising: body weight (BW [g]), fork length (FL [mm]), body girth (mm), Fulton's K (i.e., [BW × 10^5] / FL^3), weight gain as a % of start weight per day, SGR (% BW gain per day during the three-month challenge period), swim bladder fluid (SBF [mL]), stomach width (mm), gastrointenstinal tract weight (g), visceral fat score (VFS [1–4 scale]), belly-flap thickness (in three locations: cranial to the pectoral fin, caudal to the pelvic fin, at the vent), cardiosomatic index (CSI [heart weight / BW]), HSI (liver weight / BW), gonadosomatic index (GSI [gonad weight / BW]), and gut content as % of body weight.

8.5 Cellular haematology parameters

Differential cell counts were evaluated using two whole blood smears per fish. Slides were air dried and transported to an accredited laboratory (Gribbles Veterinary Pathology [GVP], Christchurch, New Zealand) for staining (Leishman) and processing within 72 h of collection. Leucocyte counts (i.e., white blood cells [WBC]) were estimated based on the average counts of 10 fields. Data were presented as a range and absolute values of the differentials were estimated based on the mean of this range. Differential leucocyte counts were manually determined based on a count of 100 cells. Absolute values for lymphocytes, neutrophils and monocytes were determined from the fraction of the 100-cell differential multiplied by the mean of the WBC count range.

Blood MCHC were calculated from haemaglobin (Hb) contents and Hct values (Witeska, 2015). For Hb content, 50–100 µL of whole blood were transferred to a 1.3 mL tube containing lithium heparin, stored at 4°C, and analysed by GVP within 48 h of collection using a HemoCue® Hb201+ system (HemoCue®, Angelholm, Sweden). Hct values were determined on whole blood immediately after withdrawal using heparinised micro hematocrit capillary tubes (Kimble Chase). Tubes were centrifuged in a Haematokrit 210 Hettich Zentrifugen at 9000 g for 7 min.

Blood EOF was measured following (Sarkar et al., 1999). A series of saline solutions were prepared to provide NaCl concentrations of 0.00, 0.10, 0.20, 0.40, 0.60, 0.80, and 0.85% w/v in distilled water. 10 µL of whole blood which had been collected in lithium heparin BD-

Microtainers (BD 365966, Phoenix Pharm Distributors, Auckland, New Zealand) were mixed thoroughly with 2.0 mL of the ascending series of NaCl solutions. Suspensions were incubated at room temperature for 30 min then centrifuged with Eppendorf 5452 Minispin at 900g for 10 min). The optical density of the supernatant was determined spectrophotometrically at 540 nm with distilled water as a blank. The percentage haemolysis is expressed relative to the solution having the highest optical density reading (i.e., maximum haemolysis). Erythrocyte MCF, or the EC_{50} value (effective concentration causing 50% haemolysis), was determined using the equation derived from a four-parameter dose-response curve: $x = c\ ((a-d)/(y-d) - 1)\hat{}(1/b)$; where a = minimum value of the fitted sigmoid curve; b = Hill's slope of the curve; c = point of inflection; d = maximum value of the fitted sigmoid curve; x = MCF (EC_{50}); and y = 50 (% haemolysis).

PBMC isolation and purification was performed using our established protocol (Lulijwa et al., 2019a). Briefly, 284 µL of whole blood (collected in lithium heparin BD-Microtainers) was diluted 1:1 with sterile filtered (40 µm) PBS (SF-PBS) (pH 7.4) and centrifuged (971 g; 20 min) over a layer of 682 µL Histopaque sterile filtered density gradient medium (10771-6X100ML; Sigma-Aldrich, New Zealand) in 1.5 mL Eppendorf tubes. Cells at the interface were aspirated with a pipette and washed twice in 500 µL of SF-PBS (being centrifuged between [674 g; 7 min]). Resulting PBMC pellets were re-suspended to final cell concentrations of 10^5–10^6 cells mL^{-1} in SF-PBS supplemented with 2% fetal bovine serum and immediately processed for PBMC viability and ROS production.

PBMC viability assessments were performed via flow cytometry (MUSE® Flow Cell Analyzer; Merk, Darmstadt, Germany) and a commercial assay kit (MUSE® Cell Count & Viability Kit; Merck) using validated protocols for *O. tshawytscha* leucocytes (Lulijwa et al., 2019a). To first establish correct MUSE® gating parameters, a PBMC sample (10^5–10^6 cells mL^{-1}) from a single fish was used to prepare two matched suspensions of live versus dead cells. PBMC death was quickly induced by adding 10 µL of diluted (1:100) Trigene detergent to one of the suspensions. 20 µL of each suspension was subsequently mixed with 380 µL of assay reagent, vortexed, and incubated at 18°C for 5 min before being analysed via flow cytometry. Cell size gating and viability thresholds were checked based on these data and set for subsequent sample analyses. Purified PBMC samples of experimental fish were similarly analysed, without addition of detergent.

Intracellular ROS production (i.e., specifically superoxide anions) in PBMCs was measured via flow cytometry (MUSE® Cell Analyzer) using a commercial assay kit (Muse® Oxidative Stress Kit [Merk]). Minor modifications to the manufacturers' protocol were implemented to account for the normal physiological temperature range of the fish model: 20 µL of PBMC suspension were incubated for 30 min at 18°C with 180 µL of Muse® Oxidative Stress working solution prior to analysis (Lulijwa et al., 2019b). To first establish correct MUSE® gating parameters, a PBMC sample (10^5–10^6 cells mL^{-1}) from a single fish was used to prepare two

matched calibration suspensions of cells with differing ROS profiles. ROS production was quickly induced by adding 10 µL of 2 mM menadione:EtOH solution to one of the suspensions and incubating at 18°C for 30 min. Calibration suspensions were stained as above with assay kit reagents and their ROS profiles were used to set the gating and threshold parameters for subsequent sample analyses.

Caspase-3 and -8 activities in PBMCs were measured colorimetrically using commercial assay kits (ab39401 and ab39700; Abcam, Cambridge, MA, USA) with minor modifications for frozen samples. Briefly, fish PBMCs (10^5–10^6 cells mL^{-1}) were lyophilised overnight at –75°C. Samples were re-suspended in 300 µL of chilled cell lysis buffer, incubated on ice for 10 min, then centrifuged (10,000 g; 1 min; 2°C). Total protein contents of supernatants were first quantified using a commercial assay kit (ab102536; Abcam, Cambridge, MA, USA) following the manufacturers' instructions, and each sample was adjusted to 50–200 µg protein per 50 µL cell lysis buffer. Duplicate samples and controls were prepared on a 96-well microplate (3364, Corning, Australia) and protein content was measured at 562 nm against a bovine serum albumin standard curve. For caspase enzyme activity, reactions (96-well microplate) comprised 50 µL of sample or blank (2x reaction buffer) in duplicate, 50 µL of caspase reaction mix, and 5 µL of 4 mM DEVD-p-NA substrate (DEVD-p-NA or IETD-pNA [200 µM final concentration]). Plates were incubated at 37°C for 1 h, and absorbances were measured at 405 nm (Thermo Scientific Multiskan Go). Data were normalized to total protein.

8.6 Plasma biochemistry parameters

Fresh peripheral blood samples were centrifuged (16,250 g; 8 min) to obtain plasma, transferred to 2 mL cryovials and immediately snap frozen in liquid nitrogen. Subsamples were sent on dry ice to GVL for targeted and quantitative analyses of selected biochemical and cellular parameters. Biochemical analytes comprised six ions (K^+, Na^+, Mg^{2+}, Ca^{2+}, Cl^-, PO_4^{3-}), seven metabolites (urea, creatinine, lactate, glucose, cortisol, biribulin, cholesterol), TAGs, TP, ALB, GLOB [calculated as TP minus ALB]), HAPT, PGE2, C-RP, and six enzymes (ALP, alanine aminotransferase [ALT], GDH, CK, and lipase [LIP]). Plasma samples were analysed for electrolytes and most clinical chemistries using a 'Cobas c 501' automated chemistry analyser (Roche Diagnostics; Mannheim, Germany). Plasma cortisol levels were determined using a 'Cobas e 411' automated endocrinology analyser (Roche). Each of the assays used a standard kit (Roche) developed for the autoanalyser. The inflammatory markers HAPT, C-RP and PGE2 were analysed in plasma samples using ELISA kits (My BioSource; CA, USA) and read on a Spectramax® ABS plate reader (Molecular Devices; CA, USA).

Lysozyme (LYS) activity was assessed spectrophotometrically (Paredes et al., 2013). Briefly, 200 µL *Micrococcus lisodeikticus* (M3770 [Sigma Aldrich; Auckland, New Zealand]) suspension (0.2 mg mL^{-1} in 0.05 M PBS) were added to wells of a 96-well microplate (3364, Corning, Australia) and mixed with 50 µL of freshly thawed plasma. Absorbance (520 nm) was recorded after 1, 3, 6- and 9-min (with shaking in between) on a UV-vis microplate reader

(Thermo Scientific Multiskan Go). One unit of enzyme activity was defined as the volume of sample required to cause a 0.001/min decrease in absorbance from the slope of the linear portion of the curve.

ACP activity was assessed spectrophotometrically (Dalmo & Seljelid, 1995). Fish plasma was thawed and used without further processing. To 80 µL of sample in a well, 20 µL of 0.2 M, pH 5.0 acetate buffer (S7899 [Sigma Aldrich; Auckland, New Zealand]) and 2 µL of 24 mM, pH 5.0 para-nitrophenyl-phosphate (N7653 [Sigma Aldrich]) were added. The suspension was incubated for 30 min at 12°C. After 30 min, 200 µL of 0.2 M borate buffer (pH 9.8) were added to stop the reaction. Absorbance at 405 nm was recorded using a Thermo Scientific multiscan Go UV-vis microplate reader. Results were recorded in absorbance units.

CAT activity was determined using a colorimetric assay kit (ab83646 according to the manufacturer's instructions (Abcam; Cambridge, MA, USA). In this assay, catalase decomposes H_2O_2 to water and oxygen, and then unconverted H_2O_2 reacts with OxiRed probe to produce a product that can be measured at 570 nm. Briefly, plasma samples in a 96-well plate were treated with H_2O_2, incubated for 30 min at 21°C, then followed by addition of stop solution and development solution. Absorbance was measured on a microplate reader and results expressed as nmol H_2O_2 converted per min per mL of plasma.

Plex was determined using a colorimetric assay kit (KB03002 [Bioquochem; Spain]) according to manufacturer's instructions. This assay measures two major byproducts of lipid peroxidation: malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Reaction between MDA and HNE and the assay reagent A results in a chromophore (diindolylalkane) with maximum absorbance measured at 586 nm. Salmon plasma samples were thawed on ice and 100 µL mixed thoroughly with 325 µL of Reagent A solution and 75 µL of Reagent B in a 1.7 mL microcentrifuge tube. Tubes were incubated in a water bath at 40°C for 40 min, then centrifuged (5,000 g; 5 min; 21°C). 200 µL of supernatant from each sample were aliquoted in duplicate into wells of a 96-well plate together with calibration standards (0–60 µM). Absorbance was measured at 586 nm (EnSpire® microplate reader; PerkinElmer®). MDA and HNE concentrations were calculated using the sample absorbance (blank corrected) and the slope from the linear regression of the standard curves (R^2 = 0.9902–0.9996).

TAC was assayed using an e-BQC portable TCA device (Bioquochem; Spain) with disposable strips which determines antioxidant capacity using electrochemistry; results obtained are in micro-Coulomb (µC). This device has recently been used to determine thermal stress in shellfish (Delorme et al., 2021). A 50 µL sample of chilled plasma was placed onto a disposable strip, obtaining results for fast (Q1) and slow (Q2) antioxidant responses as well as total antioxidant response (QT: sum of Q1 and Q2).

8.7 Statistical analyses

Mixed general linear models (GLMs) and fisher's least significant difference (LSD) were used to assess the effects of thermal stress and growth performance on all cellular haematology

and plasma biochemistry parameters (tank was included as a random factor where possible). Marginal means plots (±SEM) are presented to summarise parameters which were influenced (p<0.05) by the main effects. Individual MCF values were used to assess the effect of rearing temperature and growth performance on EOF and their interaction using a general linear model (GLM) with LSD posthoc test. Analysis of PBMC viability and ROS production was similarly performed but with 'time-to-analysis-after-blood-withdrawal' being included as a potential covariate due to variability in sample processing times (mean ± SD = 105.7 ± 28.2 min). Data were analysed using SPSS v.26 statistical software (IBM Corp.; Armonk, NY, USA).

8.8 Appendices

Table 8.1S Biometric parameters of *Oncorhynchus tshawytscha* following a thermal challenge (17°C versus 19°C) and nominated for subsequent blood analyses. Individual fish were selected based on divergent growth performance characteristics from a larger population (WG = weight gain; WL = weight loss groups). Data represent means ± SEM for each group (n = 9–47).

Biometric parameter	17°C, WG	17°C, WL	19°C, WG	19°C, WL
Body weight (g)	3154.15 ± 147.38	2093.56 ± 124.98	2618.51 ± 95.25	2291.39 ± 68.20
Fork length (mm)	515.62 ± 9.14	478.78 ± 5.76	496.17 ± 5.10	490.41 ± 3.77
Body girth (mm)	400.77 ± 7.03	339.67 ± 9.52	371.62 ± 5.36	350.22 ± 4.31
Condition factor *K*	2.28 ± 0.04	1.89 ± 0.06	2.10 ± 0.03	1.92 ± 0.03
Weight gain as a % of start weight/day	0.27±0.03	-0.13±0.01	0.14±0.01	-0.10±0.01
Specific growth rate (%/day)	0.24 ± 0.02	-0.14 ± 0.01	0.13 ± 0.01	-0.11 ± 0.01
Swim bladder fluid (mL)	0.19 ± 0.13	3.17 ± 2.43	1.11 ± 0.34	5.32 ± 2.37
Stomach width (mm)	15.69 ± 1.18	13.56 ± 2.98	14.13 ± 0.90	12.63 ± 0.77
Gastrointestinal tract weight (g)	253.52 ± 25.22	148.24 ± 13.30	220.28 ± 11.36	183.71 ± 7.44
Visceral fat score	2.31 ± 0.13	2.78 ± 0.15	2.53 ± 0.07	2.59 ± 0.07
Belly-flap thickness (mm)[a]	14.41 ± 0.53	12.98 ± 0.67	13.68 ± 0.36	13.27 ± 0.34
Belly-flap thickness (mm)[b]	10.84 ± 0.51	9.39 ± 0.31	11.69 ± 0.35	10.37 ± 0.32
Belly-flap thickness (mm)[c]	3.55 ± 0.27	3.28 ± 0.26	3.16 ± 0.15	3.00 ± 0.12
Cardiosomatic index (CSI)	1.24 ± 0.06	1.36 ± 0.07	1.41 ± 0.04	1.53 ± 0.05
Hepatosomatic index (HSI)	1.41 ± 0.13	0.62 ± 0.02	1.05 ± 0.06	0.77 ± 0.02
Gonadosomatic index (GSI)	0.18 ± 0.03	0.15 ± 0.02	0.15 ± 0.01	0.17 ± 0.01
Gut content % of body weight	3.20±0.57	2.49±1.37	1.35±0.24	0.36±0.22
Sample size (n)	13	9	47	47

[a] *cranial to the pectoral fin,* [b] *caudal to the pelvic fin and* [c] *at the vent.*

Table 8.2S Sample numbers of selected *Oncorhynchus tshawytscha* from each thermal treatment and growth performance groups allocated for various blood parameter assessments (WG = weight gain; WL = weight loss groups).

Profile Type	Biological Assessment	Tanks[a]	Total Fish	17°C WG Fish	17°C WL Fish	19°C WG Fish	19°C WL Fish
Cellular parameters	Differential cell counts (n=4)	10/2	116	13	9	47	47
	Haemoglobin	10/2	116	13	9	47	47
	% Haematocrit	10/2	116	13	9	47	47
	% Mean corpuscular haemoglobin content	10/2	116	13	9	47	47
	PBMC % Viability	1/1	20	5	5	5	5
	PBMC % ROS	1/1	20	5	5	5	5
	PBMC Caspases 3	1/1	20	5	5	5	5
	PBMC Caspases 8	1/1	20	5	5	5	5
	Erythrocyte osmotic fragility	1/1	20	5	5	5	5
Plasma biochemistry	Ions (n=6)	10/2	116	13	9	47	47
	Metabolites (n=7)	10/2	116	13	9	47	47
	Triglycerides	10/2	116	13	9	47	47
	Total protein	10/2	116	13	9	47	47
	Albumin	10/2	116	13	9	47	47
	Globulin	10/2	116	13	9	47	47
	Albumin:Globulin	10/2	116	13	9	47	47
	Prostaglandin E2	10/2	116	13	9	47	47
	C-reactive protein	10/2	116	13	9	47	47
	Haptoglobin	10/2	116	13	9	47	47
	Alkaline phosphatase	10/2	116	13	9	47	47
	Alanine aminotransferase	10/2	116	13	9	47	47
	Aspartate aminotransferase	10/2	116	13	9	47	47
	Creatine phosphokinase	10/2	116	13	9	47	47
	Glutamate dehydrogenase	10/2	116	13	9	47	47
	Lipase	10/2	116	13	9	47	47
	Lysozyme	2/2	40	11	9	10	10
	Acid phosphatase	2/2	40	11	9	10	10
	Catalase	2/2	40	11	9	10	10
	Antioxidant capacities (e-BQC Q1/Q2/QT)	10/2	113	13	9	46	45
	Lipid peroxides	10/2	114	13	9	46	46

[a] X/Y where X = N 19°C tanks and Y = N 17°C tanks.

9 Chapter 9. Summary and Conclusion

9.1 Thesis background

Globally, aquaculture production has tripled over the last two decades and is projected to be the main source of fish by 2050 (FAO, 2018c, 2020b; Naylor et al., 2021). Chinook salmon (*Oncorhynchus tshawytscha*) remains the only economically farmed finfish in New Zealand (Lulijwa et al., 2020b). In the next thirty years, demand for high-quality animal protein such as *O. tshawytscha* is predicted to accelerate due to population growth (Gordon, 2018). While the New Zealand government strategy is to expand production and annual sales by 2035 to tap into the growing demand (MPI, 2019), climate change effects and disease incidences remain big risks to this plan (**Chapter 1**). Moreover, existing knowledge gaps to *O. tshawytscha* haematology, immunology and metabolomics present an enormous challenge to health management, industry performance and sustainability. These challenges guided the research experiments conducted in this thesis (**Chapter 1**). The thesis was designed to profile the cellular components of fish blood and characterise the immuno-metabolic responses of *O. tshawytscha* to biological and physical stressors. This chapter summarises findings of this thesis, highlights the limitations faced during the study and suggests future research.

9.2 Summary

9.2.1 Salmon metabolomics research

As the salmon aquaculture industry continues to expand, several challenges with broad categorization under ecotoxicology (pollution), thermotolerance, nutrition, postharvest quality, health risks and disease and husbandry practices have emerged (**Chapter 2**). Traditional techniques including biometrics, proximate composition, digestibility assessments, immune assays, and omics approaches including genomics, transcriptomics and proteomics have been used to solve problems in aquaculture. However, these approaches may not depict downstream effects, a position metabolomics can exploit.

The aim of this section was to summarise findings and identify knowledge gaps from the studies that used metabolomics in salmonid aquaculture research. The review compared common metabolomics analytical platforms, identified popularly used salmonid species, discussed sample types, and highlighted statistical methods. It identified the latest research themes and summarised mechanistic responses. The review also identified research gaps under each theme and suggested future application opportunities in salmonid aquaculture research. The section also provided general perspectives for improving metabolomics research in aquaculture. It also suggested inter-laboratory coordination to identify the growing list of unknown features and establishment of the fish metabolome database to aid data interpretation and inference. We expect that showcasing metabolomics-based approaches in salmonid aquaculture research will facilitate uptake and integration in academia and industry programs. For example, metabolomics combined with genomics could enhance the prediction of desirable health and growth traits. This would

contribute towards improving the productivity, efficiency, and sustainability of the salmon aquaculture industry.

9.2.2 Salmon leucocyte immunology

With the increasing demand for fish and fishery products, aquaculture has rapidly expanded to fill the gap due to declining capture fishery. But with the drive to increase production, farmers keep fish at high densities, providing a suitable environment for the proliferation of pathogens. Emergence of aquaculture pathogens has thus driven research into the development of techniques for assessing immunocompetence alongside the traditional growth parameters. Fish immunocompetence is commonly studied, e.g., using lymphoid organs and peripheral blood mononuclear cells (PBMC) functional parameters. Besides the review of metabolomics applications (**Chapter 2**), a second literature survey (**Chapter 3**) aimed to identify methods used to isolate salmonid lymphoid leucocytes and peripheral blood leucocytes (PBL), and to find popularly applied assays to determine immunocompetence in aquaculture research.

The review identified a more affordable and ethically appropriate leucocyte isolation technique to reduce research costs. We found a density gradient-based approach and modified it to develop a micro-volume blood technique to isolate *O. tshawytscha* PBMCs (**Chapter 4**) for subsequent experimental studies (**Chapters 5-8**). It also discusses major assays used in salmonid immunology, from which we chose cellular functional parameters assessed in subsequent case studies (**Chapters 5-8**). The analysis also identified technological gaps to allow integration of traditional techniques with the recent omic and innovative flow cytometry approaches to *in vitro* immunological studies. We hope that isolation techniques and assays presented provide a shopping list for researchers working on other fish species to consult and drive aquatic health research.

9.2.3 PB cellular characterisation and PBMC isolation

The race to provide fish has been accelerated by rapid technological advancements in aquaculture. Globally, aquaculture has become one of the fastest-growing food production sectors (FAO, 2014). New Zealand is a global leader in *O. tshawytscha* farming and supply. Production remains free of major salmonid pathogens found elsewhere. However, recent studies reported pathogen associated summer mortality events since 2012 with heavy losses in the Marlborough Sounds in 2015 (Brosnahan, 2020; Brosnahan et al., 2019c). Haematological assessments form an established tool for health and welfare assessments in aquaculture (Fazio, 2019). However, we knew little regarding New Zealand farmed *O. tshawytscha*. This provided an opportunity to profile the cellular composition of *O. tshawytscha* PB. The shortage of New Zealand farmed *O. tshawytscha* immunological information also led to the development of a micro-blood volume method to isolate PBMCs for immunological studies *in vitro*.

Using PB samples from *O. tshawytscha* yearling smolts, this study demonstrated for the first time that total blood cell counts range from 1.9 - 2.7x10^6 μL^{-1}. The study found five cell types dominated by erythrocytes, lymphocytes, thrombocytes, and monocytes, while neutrophils were

detected in unquantifiable numbers. This contribution described *O. tshawytscha* PB cellular morphology, staining properties and immunological roles. These findings provided insights important in future haematological assessments (Clauss et al., 2008). Although logistical constraints limited the establishment of a reference range for this species in this study, the observed cellular parameters were within range for the same reported in other salmonids.

The review conducted in **Chapter 3**, demonstrated that most of the fish immunological studies are *in vivo* and require euthanisation. In addition, researchers majorly isolated leucocytes from the head kidney (HK), spleen (SP) and thymus, which all necessitate animal euthanisation. We also know that all fish blood cells are nucleated, and require the separation of leucocytes from other cells using expensive density gradient media (**Chapter 3**). This experiment successfully developed an affordable micro-volume method to isolate *O. tshawytscha* PBMCs from PB. From < 300 µL of PB, PBMCs were isolated by continuous density gradient centrifugation. Through this approach, we recovered up to 1.0-1.5 mL of PBMCs at 10^5-10^6 cells mL^{-1}, sufficient to conduct phagocytosis, ROS, and nitric oxide (NO) production assays. The technique can be used non-invasively, offering a sampling advantage over the traditional approaches that involve fish euthanisation. This strategy can also allow on-farm individual sampling for longitudinal studies. Isolated PBMCs also enable the use of novel flow cytometry Muse® Cell Analyser protocols in the field for rapid physiological assessments. Depending on fish size, researchers may integrate the PBMC approach with non-invasive mucus and faecal sampling for metabolomics to give comprehensive physiological assessments. Findings from this chapter may pave the way for future integration of novel flow cytometry, metabolomics, and conventional haematology.

9.2.4 *In vitro* PBMC immunomodulation by Gram-negative bacterial LPS

Disease outbreaks in aquaculture are common and have been reported to cause up to 50% of losses (Assefa & Abunna, 2018; Fazio, 2019). Majority of the disease incidents in aquaculture are caused by 13 genera of Gram-negative (e.g., *Edwardsiella*, *Aeromonas*, *Flavobacterium*, *Francisella*, *Photobacterium*, *Piscirickettsia*, *Pseudomonas*, *Tenacibaculum*, *Vibrio* and *Yersinia*) and Gram-positive bacteria (e.g., *Lactococcus*, *Renibacterium* and *Streptococcus*), especially when water temperatures rise (Pridgeon & Klesius, 2012). Pathogenic Gram-negative bacteria have conserved pathogen associated molecular patterns (PAMPs), such as LPS, that are recognised by the vertebrate immune system via pattern recognition receptors (PRRs) on the cell surfaces, following a breach of physical barriers (Lulijwa et al., 2019a).

Farmed *O. tshawytscha* is significantly important to the New Zealand economy. Recently, climate change-induced rising seawater temperatures exacerbated New Zealand rickettsia-like organism (NZ-RLO2) summer mortality (Brosnahan et al., 2019c). Thus, this case study characterised the cellular functional and molecular immunomodulatory responses of farmed *O. tshawytscha* PBMCs *in vitro* using LPS from *E. coli* serotypes O111: B4 and O55: B5.

Study results demonstrated that *O. tshawytscha* PBMCs recognised bacterial LPS by mounting immune defence via biphasic ROS production, with peaks at 2 h and 12 h post-

stimulation. The fast surge in ROS production by cells reflected innate immune response (Li et al., 2021). This initial ROS peak 2 h post-stimulation suggested direct PBMC interaction with LPS which activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and the inhibition of anti-oxidative enzymes involved in ROS regulation (L. Li et al., 2010). The enzyme NADPH stimulates ROS production by promoting the transfer of electrons to molecular oxygen, producing superoxide anions (Briggs et al., 1975). The second ROS peak resulted from LPS induced pro-inflammatory immune cytokine transcript production. This result was supported at a molecular level, by LPS induced PBMC pro-inflammatory mRNA cytokine transcripts for interferon gamma (*ifnγ*) and tumour necrosis factor alpha (*tnf-α*), the fast responders to pathogenic infections (Bjork et al., 2014). In addition, *O. tshawytscha* PBMCs also exhibited homeostasis via enhanced production of anti-inflammatory interleukin-10 (*il-10*), which evidently regulated LPS induced pro-inflammatory interleukin-6 (*il-6*) and interleukin-1 beta (*il-1β*) release. These findings demonstrated innate cellular ROS production, involvement of pro-inflammatory *ifnγ* and *tnf-α* and regulatory *il-10* during pathogenic Gram-negative bacterial infection in *O. tshawytscha*. Results suggest the activation of inflammation via inflamatory marker genes as previously reported *in vitro* (Holen et al., 2012; Martins et al., 2019; Nguyen et al., 2021; Stenberg et al., 2019).

Despite reports of LPS induced immunomodulation in several salmonids *in vitro* and *in vivo* (Jang et al., 1995; Martins et al., 2019; Nya & Austin, 2010; Paulsen, 2020; Solem et al., 1995), this study offered insights into *in vitro* LPS induced signalling in farmed *O. tshawytscha* PBMCs, important in pathogenic bacterial studies. The experiment postulated that immune related cytokine release could be regulated via Toll-like receptors (TLRs), particularly TLR4, or synergistic TLR1 and TLR2 ligands, probably via the myeloid differentiation primary response gene 88 (MyD88) and Toll-interleukin-1 receptor (TRIF) pathways. We recommend further studies into these observations in *O. tshawytscha*, considering that most teleosts lack TLR4 while impurities rather than LPS itself, have been suggested to stimulate immune reactions (Álvarez de Haro et al., 2021). Finally, since PBS was used as culture media, it could have influenced the results obtained in this study. Therefore, future studies are encouraged to use cell culture media such as Eagle's minimum essential medium (MEM), and Leibovitz's L-15 to avoid stress related biases and unwanted immune reactions.

9.2.5 Short-term in vivo poly (I:C) immuno-metabolic modulation

The expanding growth in international trade of live aquaculture species and their products promotes the emergence of viral diseases (Kibenge, 2019). Although the New Zealand O. *tshawytscha* industry remains relatively secure (Diggles, 2016), viruses are ubiquitous (10^7 mL^{-1} of seawater), replicate fast, and persist in the environment (Oidtmann et al., 2018; Semple et al., 2018). Thus, continued industry growth, a changing climate and emergence of viral diseases necessitates the study of antiviral mechanisms in farmed fish. In addition, scanty literature exists regarding *O. tshawytscha*'s *in vivo* immuno-metabolic response to viral infections or poly (I:C).

Furthermore, most poly (I:C) studies in *O. tshawytscha* have been *in vitro* with poly (I:C) transfected embryonic CHSE-214 and triploid *O. tshawytscha* CHSS cells (Jensen et al., 2002b; Monjo et al., 2017; Semple et al., 2018).

To contribute towards filling this knowledge gap, this study investigated the mechanisms that *O. tshawytscha* uses to respond to *in vivo* poly (I:C) 24 h post-exposure. We used an integrated strategy involving classical haematology, PBMC latex bead phagocytosis, the Muse® flow cytometry assay of viability, ROS, and NO production; liver and serum GC-MS metabolomics; and reverse-transcription polymerase chain reaction (RT-PCR) targeted immune cytokine expression in red blood cells (RBCs), SP and HK samples.

Findings showed that *O. tshawytscha* responds to poly (I:C) in vivo at cellular, metabolic, and molecular levels. Changes in haematological parameters can be excellent indicators of fish health state given availability of reference values (Seibel et al., 2021). In this study, the lack of poly (I:C) effects on haematological and cellular PBMC functional parameters 24 h post-stimulation suggested the need for a longer incubation or requirement for a larger sample size to detect differences. However, the general increase in monocytes, neutrophils, PBMC ROS, NO and phagocytosis parameters suggested enhanced cellular proliferation and PBMC functional activation of inflammation. We observed the most interesting results at metabolic and molecular levels. Poly (I:C) elevated serum metabolites involved in branched chain amino acid (BCAA)/glutathione pathways and phospholipid metabolism, and downregulated those under the glycolytic and energy metabolism 24 h post-stimulation. Therefore, poly (I:C) induced changes suggestive of viral mimics to reprogram the host metabolome for own replication (Eisenreich et al., 2019). At the molecular level, poly (I:C) upregulated antiviral *ifnγ* in HK, anti-inflammatory *il-10* in fish HK and SP and Mx1 protein in HK, SP, and fish RBC, suggestive of a host orchestrated inflammatory and antiviral immune defence against infection (Semple et al., 2018). For the first time, findings enhanced our knowledge of short-term poly (I:C) induced immuno-metabolic modulation in *O. tshawytscha*, important in viral pathogenesis.

9.2.6 Prolonged in vivo poly (I:C) immuno-metabolic modulation

We designed this case study as an extension of the experiment, which looked at the immuno-metabolic effects of *in vivo* poly (I:C) administration in *O. tshawytscha* 24 h post-stimulation (**Chapter 6**). Here the immuno-metabolic responses of *O. tshawytscha* post-smolt to *in vivo* poly (I:C), over a five-day post-exposure period were examined. We adopted a multipronged approach that involved haematology, PBMC functional ROS and latex bead phagocytosis, serum and liver metabolomics and RT-PCR targeted cytokine expression in HK and SP.

Results revealed that poly (I:C) *in vivo* stimulated *O. tshawytscha* immuno-metabolic responses at cellular, metabolic, and molecular levels. At cellular level, depression in erythrocyte counts at day three and day four post-challenge may suggest changes to respiratory capacity or immunological involvement (Lulijwa et al., 2020b; Tang et al., 2019). Increases in counts for monocytes at day one and day three and neutrophils from day three upto day five, post-stimulation

suggested immunocompetence and increased cellular proliferation. At the cellular functional level, poly (I:C) induced temporal ROS production in *O. tshawytscha* PBMCs from day one to day four suggest activation of inflammation in fish PBMCs, an important defence mechanism against infectious pathogens (Lulijwa et al., 2019c).

Metabolically, poly (I:C) elevated liver and serum metabolites involved in BCAA metabolism and protein biosynthesis, and caused perturbations to the citric acid cycle, ROS regulation and liver lipid metabolism. Liver and serum molecules involved in glycolysis were significantly reduced from day one upto day five. Amino acids and sugars are used to generate metabolic energy through the glycolytic pathway and the citric acid cycle. Metabolic results suggested that poly (I:C) heightened energy demand and triggered response mechanisms like those observed in viral-infected teleosts where the virus hijacks the host metabolome to favour own survival (Huang et al., 2020; Tang et al., 2019). At molecular levels, upregulated fish HK *ifnγ* and both organ transcripts for antiviral Mx1 protein and the regulatory *il-10* transcripts, suggested *O. tshawytscha* antiviral defence as previously reported (Martins et al., 2019; Paulsen, 2020; Zhou et al., 2014).

It is important to highlight that the obtained results could have been influenced by external variables, time, daily sampling, handling stress and experimental design shortcomings (e.g., lack of sham PBS controls and negative control) through the study period. However, these could be teased out in future studies to verify some of the hypotheses raised in this study. Nevertheless, these results enhanced our knowledge of poly (I:C) induced immuno-modulation at the cellular, metabolic, and molecular levels several days post-stimulation. Metabolic results may act as a primer for developing amelioration strategies against viral pathogens in aquaculture.

9.2.7 Haemato-biochemical biomarkers associated with thermal stress and growth performance

Over the past four decades, SSTs have been rising at 0.02°C annually (Sutton & Bowen, 2019). Fuelled by global warming, marine heatwaves have hit New Zealand since the 2017/2018 and 2018/2019 period. For instance, a 2-5°C rise in SSTs above the optimum 17°C in 2017/2018 and 2018/2019 contributed to significant summer *O. tshawytscha* mortalities in the Marlborough Sounds (Salinger et al., 2020; Salinger et al., 2019). Temperature affects fish metabolism and growth, and stressed salmon show compromised immunity, growth, metabolism, and appetite (Malekar et al., 2018; Wade et al., 2019). Despite the negative effects of thermal stress, evidence of thermal tolerance and differences in growth performance at individual and population levels are documented in teleosts (Alfonso et al., 2020; Clark et al., 2012). This study was designed to disentangle haematological biomarkers associated with thermal stress (17°C vs 19-20°C) and growth performance (weight gain vs weight loss). An integrated approach that involved cellular haematology, erythrocyte osmotic fragility (EOF), the Muse® flow cytometry PBMC viability, ROS production and caspase activity, including serum biochemistry were employed.

Assessing fish performance, elevated temperature induced a 20% aggregate mortality, in agreement with field observations recorded by farmers during summer marine heatwave events in the Marlborough Sounds. Reid et al. (2019) noted in their review that increasing water temperatures beyond the species upper limit can be lethal. Importantly, negative, and positive specific growth rates (SGR) corresponded with weight loss and weight gain groups irrespective of culture temperature. This is corroborated with lower condition factor (*K*) in the weight loss groups, and evidence of suppressed feed intake. The result confirms different growth performance paths under thermal stress, suggesting the need for future studies to tease out the influence of other intrinsic factors (e.g., genetic variation) that maybe responsible.

Elevated thermal stress, resulted in increased erythrocyte resistance to osmolysis, but activated mild immune responses in form of caspase (-3/-8) activities, and plasma acute phase protein (APP) haptoglobin release, with no evidence of oxidative stress at both thermal regimes studied outside the species range (Casanovas et al., 2021). These results may suggest within optimal range or adaptation to the final temperature challenge (19°C). However, thermal stress induced minor increases to plasma ions levels for Cl^-, Na^+, K^+ and Mg^2 suggesting disturbance to osmoregulatory capacity via reduced Na^+/K^+-ATPase activity (Vargas-Chacoff et al., 2020; Wade et al., 2019). In a similar study, osmoregulatory ion-transporters increased in temperature (21°C and 25°C) stressed *O. tshawytscha* (Tomalty et al., 2015).

Most strikingly, growth performance influenced most of the haematological parameters, irrespective of temperature. Individuals that lost weight were characterised with reduced feed intake and hepatosomatic index (HIS) indicative of liver lipid use for metabolic energy. Indeed, we observed reduced plasma signatures of energetic molecules (cholesterol, glucose, and total protein), including activities for creatine kinase (CK), acid phosphatase (ACP), alkaline phosphatase (ALP), plus elevated bilirubin and albumin/globulin ratio. Findings indicate starvation/malnutrition or reduced feed intake, liver malfunction and poor health in the weight loss group. Results suggest reduced fitness and poor health in the weight loss group, which is associated with low survival and productivity in *O. tshawytscha* (Xu et al., 2020; Yasumiishi et al., 2019). These data are collaborated with reduced lactate and cortisol in the weight loss group, pointing to potential exhaustion from suppressed appetite and reduced feeding (De Boeck et al., 2000).

Collaboratively, reduction in cellular haematology biomarkers of leucocyte count, haematocrit values (HCT) and PBMC viability, suggested compromised immunity and starvation in the weight loss group (Fazio et al., 2017; Rios et al., 2005). Low HCT point to anaemia, erythrocyte size shrinkage, enhanced mean corpuscular haemoglobin concentration (MCHC) and increased oxygen demand (Ciepliński et al., 2019; Rios et al., 2005; Witeska, 2015). Cellular oxidative stress biomarkers included elevated PBMC ROS production and erythrocyte MCF (increased osmolysis) in combination with reduced antioxidant capacity and lipid peroxidation in

the weight loss group (Heming & Paleczny, 1987; Kiron et al., 1994; Malekar et al., 2018; Mejía et al., 2006).

Lastly, significantly reduced levels for plasma osmolytes of Na^{+}, K^{+}, Ca^{+2} and PO_4^{3-} in the weight loss group illustrate disturbance to osmoregulatory capacity. Reduced plasma osmolarity increases the energy budget for osmoregulation (De Boeck et al., 2000), induces erythrocyte dehydration, reduces cell volume, and elevates MCHC as observed in this study (Rios et al., 2005). Findings established cellular haematological and plasma biochemistry parameters associated with thermal stress and growth performance, that may be important in selective breeding programs.

9.3 Limitations

Despite the achievements registered regarding farmed salmonid immunological and metabolomics research, we still know little about the New Zealand farmed *O. tshawytscha*. However, understanding the fish health state requires the development of protocols and tools for health assessment. Despite the successes, we encountered several challenges.

First, there was no *O. tshawytscha* cellular haematology and blood biochemistry literature. Knowledge of species haematology is an important step towards health assessment and requires proper characterisation to identify reliable reference ranges for cellular and plasma or serum biochemistry. Because of this challenge, we referenced cellular values reported in this study (**Chapter 4**) to those obtained from salmonids farmed in the northern hemispheres. More recently, however, reference range haematological data has trickled in thanks to the Ministry of Business, Innovation and Employment (MBIE) funding (Casanovas et al., 2021).

Second, the study aimed to develop *in vitro* assays for *O. tshawytscha* health assessment using PBMCs (**Chapter 4**). While PBMC isolation techniques had previously been developed for Atlantic salmon (*Salmo salar*) and rainbow trout (*Onchorhycus mykiss*), there were no working protocols for use with New Zealand farmed *O. tshawytscha*. Thus, during the study, we trialled several PBMC isolation techniques using methods such as SepMate density gradient centrifugation, with limited success. Similarly, the use of preserved blood couriered from Nelson to Auckland did not work at all. Over time, it became clearer that freshly taken blood was the best sample for isolation of *O. tshawytscha* PBMCs. Subsequently, we developed a micro-volume continuous density gradient centrifugation method and used it to isolate cells to enable health assessments on Gram-negative bacterial LPS (**Chapter 5**), poly (I:C) (**Chapters 6 and 7**) and thermal stress (**Chapter 8**). In addition, the Muse® flow cytometer ROS and NO kits used in these chapters haven't been validated and a recommendation is included below under future studies.

The third challenge involved the lack of a complete *O. tshawytscha* genome sequence. Part of this thesis involved the study of fish response mechanisms to bacterial and viral PAMPs at the molecular level (**Chapters 5, 6 and 7**). However, the lack of genome data limited the choice of primers to those developed for *O. tshawytscha* and *O. mykiss* farmed in the northern hemispheres. To complicate matters, the *O. tshawytscha* and other salmonid genomes were duplicated for a

fourth time over 90 million years ago (Castillo, 2020; Christensen et al., 2018). This means that *O. tshawytscha* has paralogous gene sequences which are not yet known. Other technical challenges relate to the inaccuracy with the reverse primer sequence for the in housekeeping β-actin gene which could have affected the robustness of gene results obtained in **Chapters 5, 6 and 7**. This might affect the present gene results. However, genome sequence is underway for New Zealand farmed *O. tshawytscha*. This will enhance specificity of gene work and improve accuracy in future studies. In addition, the qPCR conditions used in these studies were based on previously published primers, which were not optimised for the LightCycler 480 instrument II used in this work, and this could have limited results reliability. Although the kit used to extract RNA in **Chapters 5, 6 and 7** contained DNase, the molecular workflow did not account for gDNA contamination via inclusion of reverse transcriptase negative, RT (-) controls for all genes, which could also have affected gene findings in these chapters.

Finally, there is a lack of a proper fish metabolome database, which limits the identification of several metabolic features currently reported as unknowns in this study. This thesis found over 200 metabolic features and 45 of them remain unknown (**Chapters 6 and 7**). Statistical analysis found treatments to significantly affect some of the unknown metabolic features (**Chapter 7**). But because these features remain unknown, we omitted them from the discussion and interpretation of findings in this study.

9.4 Future research

From the metabolomics literature review (**Chapter 2**), we identified gaps and suggested future studies into unravelling response mechanisms to current use pesticides, microplastics, sex reversal hormones, thermal tolerance, nutritional manipulations, host-pathogens relationships, and daily farm husbandry practices. We also encourage research into metabolic profiling of farmed fish fillets. Based on the second review (**Chapter 3**), we suggested future effort into the use of the *O. tshawytscha* PBMCs to reduce the requirement for fish euthanasia. Lagging *S. salar* and *O. mykiss*, the New Zealand farmed *O. tshawytscha* is the least studied farmed salmonid. Embracing this strategy will establish an *in vitro* approach to harness several facets of basic research on New Zealand farmed *O. tshawytscha*.

Based on the challenges encountered during case study 1 (**Chapter 4**), we recommend the establishment of a healthy New Zealand farmed *O. tshawytscha* comprehensive cellular haematological and plasma or serum biochemistry reference range database. The database should include apparently healthy fish from alevin, fry, parr, smolt, juvenile to adults in freshwater and marine operations. The effect of sex also needs to be carefully considered at the appropriate stage, including seasonal variation. Availability of this database will enhance the reliability on haematological parameters for health assessments of the New Zealand farmed *O. tshawytscha*.

With the recent emergence of NZ-RLO2 associated summer mortalities in some farmed sites in New Zealand, we suggest the use of the developed *in vitro* PBMC techniques to model the fish response mechanisms to NZ-RLO2 at cellular, molecular, and metabolic levels. Using

PBMCs, we have demonstrated use of the Muse® flow cytometer to investigate cellular response mechanisms via ROS and NO production, which will require future validation. In addition, the Muse® Caspase -3/7, and the Muse® MitoPotential assay of apoptosis will have to be brought onboard. These kits maybe be important in modulating fish responses to physical, biological, and chemical stressors *in vitro* and in vivo. Since the study (**Chapter 5**) did not investigate the signalling pathway for LPS *in vitro* in *O. tshawytscha* PBMCs, we recommend studies into the involved PRRs and signalling pathways. Further studies to increase the repertoire of genes involved in pro- and anti-inflammatory LPS signalling are encouraged. Further studies to increase the repertoire of genes involved in pro- and anti-inflammatory crude and pure LPS signalling in comparison with β-glucan and peptidoglycan are encouraged, using atleast two β-actin housekeeping genes.

We strongly encourage research into the host-pathogen relationships of notifiable bacterial diseases and trials on metabolome reprogramming. The *in vitro* PBMC technique also offers the opportunity to model host response mechanisms to viral pathogens that have devastated global salmon aquaculture. Here, findings in **Chapter 6** and **Chapter 7** could be of value in future studies. For the case-study in Chapter 6 a more robust design would include a negative control, and a sham control with PBS. To reduce variability of haematological assessments, sample size of 10 is recommended. To capture early *ifn1* induction, we recommend sampling points at 6, 12 and 24 h post-challenge. For the study in **Chapter 7**, improvements could involve inclusion of a negative control and a sham PBS control over the experimental period. Sample size could be increased to 5 per group and the period extended to 20 days post-stimulation. Further studies are recommended into postulated poly (I:C) induced effects on the pentose pathway, hexosamine pathway and aerobic glycolysis, common findings in viral infections. The use of poly (I:C) affected metabolites under the glycolysis or gluconeogenesis pathways in metabolome reprogramming is recommended. Individual or family susceptibility to viral mimic would also benefit the New Zealand aquaculture industry. Overall, poly (I:C) studies should consider the collection of samples for poly (I:C) cellular signalling, DNA methylation, genomics, and transcriptomics for wholistic understanding of involved immune-related genes. In Chapters 6 and 7, further studies of immune-related genes will be improved using atleast two β-actin housekeeping genes with the correct reverse primer sequences. The Pfaffl (2001) method instead of the comparative Ct (△△Ct) equation (Schmittgen & Livak, 2008) would be used to compute for relative gene expression, while accounting for the differences in primer efficiency between the housekeeping gene and the target gene. Care must be taken to crosscheck primer s against the specific gene sequences.

In addition, the adoption of New Zealand farmed *O. tshawytscha* PBMCs will enable the design of nutritional, health and toxicological *in vitro* studies, prior to field trials. This approach will help to reduce the cost requirement for maintaining fish for *in vivo* studies. The strategy will

potentially initiate *in vitro* research opportunities with reduced cost and time to allow fast metabolomics and flow cytometry assaying in future *O. tshawytscha* studies.

Although oxygen levels were varied in the thermally stressed fish group to reflect field conditions, we focussed our discussion to the effect of temperature stress and growth performance. Since temperature influences levels of dissolved oxygen, future studies into how thermal stress affects *O. tshawytscha* metabolism, feed intake and use, growth and health are encouraged to better understand effects of climate change. In addition, characterising immunocompetence, metabolism and genetic variation between individuals with low thermal tolerance and survivors will help aquatic scientists understand phenotypic performance differences exhibited by *O. tshawytscha* during warmer temperatures.

9.5 Conclusion

As the *O. tshawytscha* aquaculture industry expands, knowledge of the fish health state will become more valuable to safeguard against potential future pathogens. This research characterised for the first time healthy *O. tshawytscha* cellular haematological parameters important in health studies. The research also developed a non-invasive micro-volume PBMC isolation method. This strategy enables on-farm individual sampling for longitudinal experiments and allows *in vitro* immunological studies. The study also developed an *in vitro* PBMC based technique to model cellular and molecular immune responses and used metabolomics to reveal *in vivo* host responses to poly (I:C). Finally, the research used plasma biochemistry, cellular haematology, and *in vitro* PBMC assays to identify biomarkers associated with thermal stress and growth performance. This research demonstrated an integrated approach for fish health assessments. The thesis contributes to the *O. tshawytscha* aquaculture industry and academia. It also offers insights into cellular haematology, LPS, and poly (I:C) induced immunomodulation. We recommend an integrated approach to further gain a deeper understanding of the species haematology and host-pathogen relationships.

10 References

Abdel-Tawwab, M., & Hamed, H. S. (2018). Effect of bisphenol A toxicity on growth performance, biochemical variables, and oxidative stress biomarkers of Nile tilapia, *Oreochromis niloticus* (L.). *Journal of Applied Ichthyology, 34*(5), 1117-1125.

Abro, R., Moazzami, A. A., Lindberg, J. E., & Lundh, T. (2014). Metabolic insights in Arctic char (*Salvelinus alpinus*) fed with zygomycetes and fish meal diets as assessed in liver using nuclear magnetic resonance (NMR) spectroscopy. *International Aquatic Research, 6*(2), 63.

Abu-Elala, N., Marzouk, M., & Moustafa, M. (2013). Use of different Saccharomyces cerevisiae biotic forms as immune-modulator and growth promoter for Oreochromis niloticus challenged with some fish pathogens. *International Journal of Veterinary Science and Medicine, 1*(1), 21-29.

Ackerman, P. A., Wicks, B. J., Iwama, G. K., & Randall, D. J. (2006). Low levels of environmental ammonia increase susceptibility to disease in Chinook salmon smolts. *Physiological and Biochemical Zoology, 79*(4), 695-707.

Adan, A., Kiraz, Y., & Baran, Y. (2016). Cell proliferation and cytotoxicity assays. *Current pharmaceutical biotechnology, 17*(14), 1213-1221.

Adenan, M. N. H., Moosa, S., Muhammad, S. A., Abrahim, A., Jandric, Z., Islam, M., Rodionova, O., Pomerantsev, A., Perston, B., & Cannavan, A. (2020). Screening Malaysian edible bird's nests for structural adulterants and geographical origin using Mid-Infrared–Attenuated Total Reflectance (MIR-ATR) spectroscopy combined with chemometric analysis by Data-Driven–Soft Independent Modelling of Class Analogy (DD-SIMCA). *Forensic Chemistry, 17*, 100197.

Aggio, R., Villas-Boas, S. G., & Ruggiero, K. (2011). Metab: an R package for high-throughput analysis of metabolomics data generated by GC-MS. *Bioinformatics, 27*(16), 2316-2318.

Ahmed, S. M., & Ali, A. H. (2013). Serum proteins and leucocytes differential count in common carp (*Cyprinus carpio* L.) infested with ectoparasites. *Mesopotamian Journal of Marine Science, 28*(2), 151-162.

Ainsworth, A. J. (1992). Fish granulocytes: Morphology, distribution, and function. *Annual Review of Fish Diseases, 2*, 123-148.

Ajeniyi, S. A., & Solomon, R. J. (2014). Urea and creatinine of *Clarias gariepinus* in three different commercial ponds. *Nature and Science, 12*(10), 124-138.

Alcorn, S. W., Murray, A. L., & Pascho, R. J. (2002). Effects of rearing temperature on immune functions in sockeye salmon (*Oncorhynchus nerka*). *Fish & shellfish immunology, 12*(4), 303-334.

Alcorn, S. W., Pascho, R. J., Murray, A. L., & Shearer, K. D. (2003). Effects of ration level on immune functions in chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture, 217*(1), 529-545.

Alfaro, A. C., Nguyen, T. V., & Mellow, D. (2019). A metabolomics approach to assess the effect of storage conditions on metabolic processes of New Zealand surf clam (*Crassula aequilatera*). *Aquaculture, 498*, 315-321.

References

Alfaro, A. C., & Young, T. (2018). Showcasing metabolomic applications in aquaculture: a review. *Reviews in Aquaculture, 10*(1), 135-152.

Alfonso, S., Gesto, M., & Sadoul, B. (2020). Temperature increase and its effects on fish stress physiology in the context of global warming. *Journal of Fish Biology*.

Álvarez de Haro, N., Van, A. P., Robb, C. T., Rossi, A. G., & Desbois, A. P. (2021). Release of chromatin extracellular traps by phagocytes of Atlantic salmon, *Salmo salar* (Linnaeus, 1758). *Fish & shellfish immunology, 119*, 209-219.

Amri, A., Kessabi, K., Bouraoui, Z., Sakli, S., Gharred, T., Guerbej, H., Messaoudi, I., & Jebali, J. (2020). Effect of melatonin and folic acid supplementation on the growth performance, antioxidant status, and liver histology of the farmed gilthead sea bream (*Sparus aurata* L.) under standard rearing conditions. *Fish Physiology and Biochemistry, 46*(6), 2265-2280.

Andersen, S. M., Assaad, H. I., Lin, G., Wang, J., Aksnes, A., Wu, G., & Espe, M. (2015). Metabolomic analysis of plasma and liver from surplus arginine fed Atlantic salmon. *Frontiers in bioscience (Elite edition), 7*, 67.

Andersen, S. M., Taylor, R., Holen, E., Aksnes, A., & Espe, M. (2014). Arginine supplementation and exposure time affects polyamine and glucose metabolism in primary liver cells isolated from Atlantic salmon. *Amino Acids, 46*(5), 1225-1233.

Anderson, C. (1996). Distribution of salmonid diseases in New Zealand. *Surveillance (Wellington), 23*(4), 23-24.

Anderson, D. P. (1992). Immunostimulants, adjuvants, and vaccine carriers in fish: applications to aquaculture. *Annual Review of Fish Diseases, 2*, 281-307.

Andreopoulos, B., An, A., Wang, X., & Schroeder, M. (2009). A roadmap of clustering algorithms: finding a match for a biomedical application. *Briefings in Bioinformatics, 10*(3), 297-314.

Ángeles Esteban, M. (2012). An overview of the immunological defenses in fish skin. *ISRN Immunology, 2012*, 1-29.

Anon. (1976). *Commercial feasibility of the establishment of a salmon hatchery on the Waitaki river: commissioned by the Otago Regional Development Council.*

AnvariFar, H., Amirkolaie, A. K., Miandare, H. K., Ouraji, H., Jalali, M. A., & Üçüncü, S. İ. (2017). Apoptosis in fish: environmental factors and programmed cell death. *Cell and tissue research, 368*(3), 425-439.

Arkoosh, M. R., Casillas, E., Clemons, E., McCain, B., & Varanasi, U. (1991). Suppression of immunological memory in juvenile chinook salmon (*Oncorhynchus tshawytscha*) from an urban estuary. *Fish & shellfish immunology, 1*(4), 261-277.

Arkoosh, M. R., Clemons, E., Myers, M., & Casillas, E. (1994). Suppression of B-cell mediated immunity in Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) after exposure to either a polycyclic aromatic hydrocan or to polychlorinated biphenyls. *Immunopharmacology and immunotoxicology, 16*(2), 293-314.

Arkoosh, M. R., & Kaattari, S. L. (1991). Development of immunological memory in rainbow trout (*Oncorhynchus mykiss*). I. An immunochemical and cellular analysis of the B cell response. *Developmental & Comparative Immunology, 15*(4), 279-293.

References

Arkoosh, M. R., Van Gaest, A. L., Strickland, S. A., Hutchinson, G. P., Krupkin, A. B., & Dietrich, J. P. (2015). Dietary exposure to individual polybrominated diphenyl ether congeners BDE-47 and BDE-99 alters innate immunity and disease susceptibility in juvenile chinook salmon. *Environmental Science and Technology, 49*(11), 6974-6981.

Arkoosh, M. R., Van Gaest, A. L., Strickland, S. A., Hutchinson, G. P., Krupkin, A. B., Hicks, M. B. R., & Dietrich, J. P. (2018). Dietary exposure to a binary mixture of polybrominated diphenyl ethers alters innate immunity and disease susceptibility in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). *Ecotoxicology and environmental safety, 163*, 96-103.

Arnemo, M., Kavaliauskis, A., & Gjøen, T. (2014). Effects of TLR agonists and viral infection on cytokine and TLR expression in Atlantic salmon (*Salmo salar*). *Developmental & Comparative Immunology, 46*(2), 139-145.

Asche, F., Cojocaru, A. L., & Roth, B. (2018). The development of large scale aquaculture production: a comparison of the supply chains for chicken and salmon. *Aquaculture, 493*, 446-455.

Asche, F., Roll, K. H., Sandvold, H. N., Sørvig, A., & Zhang, D. (2013). Salmon aquaculture: Larger companies and increased production. *Aquaculture Economics & Management, 17*(3), 322-339.

Ashley, P. J. (2007). Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science, 104*(3-4), 199-235. https://doi.org/10.1016/j.applanim.2006.09.001

Assefa, A., & Abunna, F. (2018). Maintenance of fish health in aquaculture: review of epidemiological approaches for prevention and control of infectious disease of fish. *Veterinary medicine international, 2018*.

Aste-Amezaga, M., Ma, X., Sartori, A., & Trinchieri, G. (1998). Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *The Journal of Immunology, 160*(12), 5936-5944.

Attaya, A., Jiang, Y., Secombes, C. J., & Wang, T. (2019). Distinct response of immune gene expression in peripheral blood leucocytes modulated by bacterin vaccine candidates in rainbow trout *Oncorhynchus mykiss*: A potential in vitro screening and batch testing system for vaccine development in aquaculture. *Fish & shellfish immunology, 93*, 631-640.

Aurélio, M., Faleiro, F., Lopes, V. M., Pires, V., Lopes, A. R., Pimentel, M. S., Repolho, T., Baptista, M., Narciso, L., & Rosa, R. (2013). Physiological and behavioral responses of temperate seahorses (*Hippocampus guttulatus*) to environmental warming. *Marine biology, 160*(10), 2663-2670.

Aursand, M., Gribbestad, I. S., & Martinez, I. (2008). Omega-3 Fatty Add Content of Intact Muscle of Farmed Atlantic Salmon (*Salmo salar*) Examined by ^{1}H MAS NMR Spectroscopy. In *Modern magnetic resonance* (pp. 941-945). Springer.

Aursand, M., Rainuzzo, J. R., & Grasdalen, H. (1993). Quantitative high-resolution ^{13}C and ^{1}H nuclear magnetic resonance of ω3 fatty acids from white muscle of atlantic salmon (*Salmo salar*). *Journal of the American Oil Chemists' Society, 70*(10), 971-981.

Aursand, M., Standal, I. B., Praël, A., McEvoy, L., Irvine, J., & Axelson, D. E. (2009). ^{13}C NMR pattern recognition techniques for the classification of Atlantic Salmon (*Salmo salar* L.) according to their wild, farmed, and geographical origin. *Journal of agricultural and food chemistry, 57*(9), 3444-3451.

References

Awasthi, A., Rathore, G., Pradhan, P. K., Rebello, S. C., Khan, M. Y., & Lakra, W. S. (2014). Isolation and characterization of head kidney derived macrophages of *Labeo rohita*. *Journal of Environmental Biology, 35*(5), 949.

Axelson, D. E., Standal, I. B., Martinez, I., & Aursand, M. (2009). Classification of wild and farmed salmon using Bayesian belief networks and gas chromatography-derived fatty acid distributions. *Journal of agricultural and food chemistry, 57*(17), 7634-7639.

Bachmann-Vargas, P., van Koppen, C. S. A. K., & Lamers, M. (2021). Re-framing salmon aquaculture in the aftermath of the ISAV crisis in Chile. *Marine Policy, 124*, 104358.

Bakshtansky, E. L. (1980). Introduction of pink salmon into the Kola Peninsula. *Salmon ranching, edited by John Thorpe*.

Ballantyne, J. S. (2001). Amino acid metabolism. In P. A. Wright & P. M. Anderson (Eds.), *Nitrogen excretion* (Vol. 20, pp. 77-107). Academic Press.

Bankefors, J., Kaszowska, M., Schlechtriem, C., Pickova, J., Brannas, E., Edebo, L., Kiessling, A., & Sandstrom, C. (2011). A comparison of the metabolic profile on intact tissue and extracts of muscle and liver of juvenile Atlantic salmon (*Salmo salar* L.) - Application to a short feeding study. *Food chemistry, 129*(4), 1397-1405.

Baoprasertkul, P., Peatman, E., Somridhivej, B., & Liu, Z. (2006). Toll-like receptor 3 and TICAM genes in catfish: species-specific expression profiles following infection with *Edwardsiella ictaluri*. *Immunogenetics, 58*(10), 817-830.

Bar, N. (2014). Physiological and hormonal changes during prolonged starvation in fish. *Canadian Journal of Fisheries and Aquatic Sciences, 71*(10), 1447-1458.

Barnhart, R. A. (1969). Effects of certain variables on hematological characteristics of rainbow trout. *Transactions of the American Fisheries Society, 98*(3), 411-418.

Barrett, P. V. D. (1971). The effect of diet and fasting on the serum bilirubin concentration in the rat. *Gastroenterology, 60*(4), 572-576.

Barton, B. A. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr Comp Biol, 42*(3), 517-525.

Bassity, E., & Clark, T. G. (2012). Functional identification of dendritic cells in the teleost model, rainbow trout (*Oncorhynchus mykiss*). *PLoS One, 7*(3), e33196.

Basu, M., Kumar, M. N., & Samanta, M. (2013). Toll-like receptor (TLR) 4 in mrigal (*Cirrhinus mrigala*): response to lipopolysaccharide treatment and Aeromonas hydrophila infection. *International Research Journal of Biological Sciences, 2*(4), 20-27.

Baumgarner, B. L., & Cooper, B. R. (2012). Evaluation of a tandem gas chromatography/time-of-flight mass spectrometry metabolomics platform as a single method to investigate the effect of starvation on whole-animal metabolism in rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology, 215*(10), 1627-1632.

Benfey, T. J., & Sutterlin, A. M. (1984). The haematology of triploid landlocked Atlantic salmon, Salmo solar L. *Journal of Fish Biology, 24*(3), 333-338.

Bennett, C. M., Kanki, J. P., Rhodes, J., Liu, T. X., Paw, B. H., Kieran, M. W., Langenau, D. M., Delahaye-Brown, A., Zon, L. I., Fleming, M. D., & Look, A. T. (2001). Myelopoiesis in the zebrafish, *Danio rerio*. *Blood, 98*(3), 643.

References

Berg, M. (1977). Pink salmon, *Oncorhynchus gorbuscha* (Walbaum) in Norway [occurence, spawning, experiments with farming]. *Report-Institute of Freshwater Research, Drottningsholm (Sweden)*.

Bermejo-Poza, R., De la Fuente, J., Pérez, C., de Chavarri, E. G., Diaz, M. T., Torrent, F., & Villarroel, M. (2017). Determination of optimal degree days of fasting before slaughter in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture, 473*, 272-277.

Berntssen, M. H., Ornsrud, R., Rasinger, J., Softeland, L., Lock, E. J., Kolas, K., Moren, M., Hylland, K., Silva, J., Johansen, J., & Lie, K. (2016). Dietary vitamin A supplementation ameliorates the effects of poly-aromatic hydrocarbons in Atlantic salmon (*Salmo salar*). *Aquat Toxicol, 175*, 171-183.

Biller-Takahashi, J. D., Takahashi, L. S., Marzocchi-Machado, C. M., Zanuzzo, F. S., & Urbinati, E. C. (2014). Disease resistance of pacu *Piaractus mesopotamicus* (Holmberg, 1887) fed with β-glucan. *Brazilian Journal of Biology, 74*(3), 698-703.

Biron, C. A., & Sen, G. C. (2001). Interferons and other cytokines. In D. M. Knipe, P. M. Howley, D. E. Griffin, M. Martin, B. Roizman, & S. E. Straus (Eds.), *Fields virology* (Vol. 4th, pp. 321-351). Lippincott-Raven.

Bjork, S. J., Zhang, Y. A., Hurst, C. N., Alonso-Naveiro, M. E., Alexander, J. D., Sunyer, J. O., & Bartholomew, J. L. (2014). Defenses of susceptible and resistant Chinook salmon (*Oncorhynchus tshawytscha*) against the myxozoan parasite *Ceratomyxa shasta*. *Fish & shellfish immunology, 37*(1), 87-95.

Bjørndal, T., & Tusvik, A. (2019). Economic analysis of land based farming of salmon. *Aquaculture Economics & Management, 23*(4), 449-475.

Blaxhall, P. C. (1981). A comparison of methods used for the separation of fish lymphocytes. *Journal of Fish Biology, 18*(2), 177-181.

Boccard, J., Veuthey, J. L., & Rudaz, S. (2010). Knowledge discovery in metabolomics: an overview of MS data handling. *Journal of Separation Science, 33*(3), 290-304.

Boesen, H. T., Pedersen, K., Koch, C., & Larsen, J. L. (1997). Immune response of rainbow trout (*Oncorhynchus mykiss*) to antigenic preparations from *Vibrio anguillarum* serogroup O1. *Fish and Shellfish Immunology, 7*(8), 543-553.

Boltana, S., Doñate, C., Goetz, F. W., MacKenzie, S., & Balasch, J. C. (2009). Characterization and expression of NADPH oxidase in LPS-, poly (I: C)-and zymosan-stimulated trout (*Oncorhynchus mykiss* W.) macrophages. *Fish & shellfish immunology, 26*(4), 651-661.

Boshra, H., Li, J., & Sunyer, J. O. (2006). Recent advances on the complement system of teleost fish. *Fish & shellfish immunology, 20*(2), 239-262.

Boyum, A. (1968). Separation of leukocytes from blood and bone marrow. *Scand. J. clin. lab. Invest., 21*, 77.

Braun-Nesje, R., Bertheussen, K., Kaplan, G., & Seljelid, R. (1981). Salmonid macrophages: separation, in vitro culture and characterization. *Journal of Fish Diseases, 4*(2), 141-151.

Brestenský, M., Nitrayová, S., Patrás, P., Heger, J., & Nitray, J. (2015). Branched chain amino acids and their importance in nutrition. *The Journal of Microbiology, Biotechnology and Food Sciences, 5*(2), 197.

References

Briggs, R. T., Drath, D. B., Karnovsky, M. L., & Karnovsky, M. J. (1975). Localization of NADH oxidase on the surface of human polymorphonuclear leukocytes by a new cytochemical method. *The Journal of Cell Biology, 67*(3), 566-586.

Brisse, M., & Ly, H. (2019). Comparative Structure and Function Analysis of the RIG-I-Like Receptors: RIG-I and MDA5. *Frontiers in immunology, 10*, 1586.

Broekhuizen, N., Plew, D. R., Pinkerton, M. H., & Gall, M. G. (2021). Sea temperature rise over the period 2002–2020 in Pelorus Sound, New Zealand–with possible implications for the aquaculture industry. *New Zealand Journal of Marine and Freshwater Research*, 1-19.

Brosnahan, C. L. (2020). *Diagnostic investigation into summer mortality events of farmed Chinook salmon (Oncorhynchus tshawytscha) in New Zealand: a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Massey University, Manawatū, New Zealand* Massey University.

Brosnahan, C. L., Georgiades, E., McDonald, C., Keeling, S. E., Munday, J. S., & Jones, B. (2019b). Optimisation and validation of a PCR to detect viable *Tenacibaculum maritimum* in salmon skin tissue samples. *Journal of Microbiological Methods, 159*, 186-193.

Brosnahan, C. L., Ha, H. J., Booth, K., McFadden, A. M. J., & Jones, J. B. (2017). First report of a rickettsia-like organism from farmed Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), in New Zealand. *New Zealand Journal of Marine and Freshwater Research, 51*(3), 356-369.

Brosnahan, C. L., Munday, J. S., Davie, P. S., Kennedy, L., Preece, M., Barnes, S., Jones, J. B., & McDonald, W. L. (2019c). Pathogenicity of the bacterium New Zealand rickettsia-like organism (NZ-RLO2) in Chinook salmon *Oncorhynchus tshawytscha* smolt. *Diseases of aquatic organisms, 134*(3), 175-187.

Brosnahan, C. L., Munday, J. S., Ha, H. J., Preece, M., & Jones, J. B. (2019a). New Zealand rickettsia-like organism (NZ-RLO) and *Tenacibaculum maritimum*: Distribution and phylogeny in farmed Chinook salmon (Oncorhynchus tshawytscha). *Journal of Fish Diseases, 42*(1), 85-95.

Browdy, C. L., Hulata, G., Liu, Z., Allan, G. L., Sommerville, C., Passos de Andrade, T., Pereira, R., Yarish, C., Shpigel, M., & Chopin, T. (2012). *Novel and emerging technologies: can they contribute to improving aquaculture sustainability?* Farming the waters for people and food, Phuket, Thailand. http://www.bashanfoundation.org/contributions/Liu-Z/2010.-Liu-PGCA.pdf

Bruce, T. J., & Brown, M. L. (2017). A review of immune system components, cytokines, and immunostimulants in cultured finfish species. *Open Journal of Animal Sciences, 7*(03), 267.

Brum, A., Pereira, S. A., Owatari, M. S., Chagas, E. C., Chaves, F. C. M., Mouriño, J. L. P., & Martins, M. L. (2017). Effect of dietary essential oils of clove basil and ginger on Nile tilapia (*Oreochromis niloticus*) following challenge with *Streptococcus agalactiae*. *Aquaculture, 468*, 235-243.

Brunt, J., & Austin, B. (2005). Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases, 28*(12), 693-701.

References

Brunt, J., Newaj-Fyzul, A., & Austin, B. (2007). The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases, 30*(10), 573-579.

Bugeon, J., Lefèvre, F., & Fauconneau, B. (2004). Correlated changes in skeletal muscle connective tissue and flesh texture during starvation and re-feeding in brown trout (*Salmo trutta*) reared in seawater. *Journal of the Science of Food and Agriculture, 84*(11), 1433-1441.

Buschmann, A. H., Tomova, A., Lopez, A., Maldonado, M. A., Henriquez, L. A., Ivanova, L., Moy, F., Godfrey, H. P., & Cabello, F. C. (2012). Salmon Aquaculture and Antimicrobial Resistance in the Marine Environment. *PLoS One, 7*(8), 11.

Bussolati, B., Mariano, F., Montrucchio, G., Piccoli, G., & Camussi, G. (1997). Modulatory effect of interleukin-10 on the production of platelet-activating factor and superoxide anions by human leucocytes. *Immunology, 90*(3), 440-447.

Cai, H., Chen, Y., Cui, X., Cai, S., & Chen, Z. (2014). High-resolution 1 H NMR spectroscopy of fish muscle, eggs and small whole fish via Hadamard-encoded intermolecular multiple-quantum coherence. *PLoS One, 9*(1), e86422.

Caipang, C. M. A., Hirono, I., & Aoki, T. (2003). *In vitro* inhibition of fish rhabdoviruses by Japanese flounder, *Paralichthys olivaceus* Mx. *Virology, 317*(2), 373-382.

Calder, P. C. (2006). Branched-chain amino acids and immunity. *The Journal of nutrition, 136*(1), 288S-293S.

Camargo, T. R., Bueno, G. W., Ajayi, O., & Roubach, R. (2020). Aquaculture Feed Ingredients. *FAO Aquaculture Newsletter*(61), 47-48.

Cambiaghi, A., Ferrario, M., & Masseroli, M. (2016). Analysis of metabolomic data: tools, current strategies and future challenges for omics data integration. *Briefings in Bioinformatics, 18*(3), 498-510.

Cannon, M. S., Mollenhauer, H. H., Eurell, T. E., Lewis, D. H., Cannon, A. M., & Tompkins, C. (1980). An ultrastructural study of the leukocytes of the channel catfish, *Ictalurus punctatus*. *Journal of Morphology, 164*(1), 1-23.

Caruso, G., Denaro, M. G., Caruso, R., Genovese, L., Mancari, F., & Maricchiolo, G. (2012). Short fasting and refeeding in red porgy (*Pagrus pagrus*, Linnaeus 1758): Response of some haematological, biochemical and non specific immune parameters. *Marine environmental research, 81*, 18-25.

Casanovas, P., Walker, S. P., Johnston, H., Johnston, C., & Symonds, J. E. (2021). Comparative assessment of blood biochemistry and haematology normal ranges between Chinook salmon (*Oncorhynchus tshawytscha*) from seawater and freshwater farms. *Aquaculture*, 537, 736464.

Cassatella, M. A., Della Bianca, V., Berton, G., & Rossi, F. (1985). Activation by gamma interferon of human macrophage capability to produce toxic oxygen molecules is accompanied by decreased Km of the superoxide-generating NADPH oxidase. *Biochemical and biophysical research communications, 132*(3), 908-914.

Castillo, A. H. (2020). The 4R Genome Duplication In Salmonine Fishes: Insights From Conserved Non-Coding Elements. *bioRxiv*, 2020.2009.2024.278218.

References

Castro, R., & Tafalla, C. (2015). Overview of fish immunity. In *Mucosal Health in Aquaculture* (pp. 3-54).

Cavallaro, R. A., Fuso, A., d'Erme, M., Miraglia, N., & Martire, S. (2016). Role of S-adenosylmethionine in the Modulation of Oxidative Stress-Related Neurodegeneration. *Int J Clin Nutr Diet, 2*(1), 109.

Cecchini, S., Caputo, A. R., & Mecca, F. (2005). Evaluation of in vitro endocytosis and antibody synthesis by rainbow trout head kidney cells treated with bovine lactoferrin. *Journal of Fish Biology, 66*(3), 609-617.

Cerqueira, M., Schrama, D., Silva, T. S., Colen, R., Engrola, S. A. D., Conceição, L. E. C., Rodrigues, P. M. L., & Farinha, A. P. (2020). How tryptophan levels in plant-based aquafeeds affect fish physiology, metabolism and proteome. *Journal of proteomics*, 103782.

Chandra, S. (1982). Effect of starvation on serum acid phosphatase levels of freshwater catfish *Clarias batrachus*. *Experientia, 38*(7), 827-828.

Chaudhary, D. K., Sood, N., Pradhan, P. K., Singh, A., Punia, P., Agarwal, N. K., & Rathore, G. (2012). Establishment of a macrophage cell line from adherent peripheral blood mononuclear cells of *Catla catla*. *In Vitro Cellular & Developmental Biology-Animal, 48*(6), 340-348.

Chaudhary, D. K., Sood, N., Rathore, G., Pradhan, P. K., Punia, P., Agarwal, N. K., & Jena, J. K. (2014). Establishment and characterization of macrophage cell line from thymus of *Catla catla* (Hamilton, 1822). *Aquaculture Research, 45*(2), 299-311.

Chauton, M. S., Galloway, T. F., Kjørsvik, E., Størseth, T. R., Puvanendran, V., van der Meeren, T., Karlsen, Ø., Rønnestad, I., & Hamre, K. (2015). ^{1}H NMR metabolic profiling of cod (*Gadus morhua*) larvae: potential effects of temperature and diet composition during early developmental stages. *Biology open, 4*(12), 1671-1678.

Chen, C., Zou, W., Cui, G., Tian, J., Wang, Y., & Ma, L. (2020). Ecological risk assessment of current-use pesticides in an aquatic system of Shanghai, China. *Chemosphere, 257*, 127222.

Chen, H., Liu, S., Xu, X. R., Diao, Z. H., Sun, K. F., Hao, Q. W., Liu, S. S., & Ying, G. G. (2018). Tissue distribution, bioaccumulation characteristics and health risk of antibiotics in cultured fish from a typical aquaculture area. *Journal of hazardous materials, 343*, 140-148.

Chen, R., Wang, Q., Zhao, L., Yang, S., Li, Z., Feng, Y., Chen, J., Ong, C. N., & Zhang, H. (2019). Lomatogonium Rotatum for Treatment of Acute Liver Injury in Mice: A Metabolomics Study. *Metabolites, 9*(10), 227.

Cheng, A. C., Cheng, S. A., Chen, Y. Y., & Chen, J. C. (2009). Effects of temperature change on the innate cellular and humoral immune responses of orange-spotted grouper *Epinephelus coioides* and its susceptibility to *Vibrio alginolyticus*. *Fish & shellfish immunology, 26*(5), 768-772.

Cheng, K., Wagner, L., Moazzami, A. A., Gomez-Requeni, P., Vestergren, A. S., Brannas, E., Pickova, J., & Trattner, S. (2016a). Decontaminated fishmeal and fish oil from the Baltic Sea are promising feed sources for Arctic char (*Salvelinus alpinus* L.)-studies of flesh lipid quality and metabolic profile. *European Journal of Lipid Science and Technology, 118*(6), 862-873.

References

Cheng, K., Wagner, L., Pickova, J., & Moazzami, A. A. (2016b). NMR-based metabolomics reveals compartmental metabolic heterogeneity in liver of Arctic char (*Salvelinus alpinus*). *Canadian Journal of Zoology, 94*(9), 665-669.

Cheung, W. W. L., & Frölicher, T. L. (2020). Marine heatwaves exacerbate climate change impacts for fisheries in the northeast Pacific. *Scientific Reports, 10*(1), 1-10.

Childerhose, R. J., & Trim, M. (1979). *Pacific salmon and steelhead trout [British Columbia; Great Basin and Pacific Slope States]*. University of Washington Press.

Chilmonczyk, S., & Monge, D. (1999). Flow cytometry as a tool for assessment of the fish cellular immune response to pathogens. *Fish & shellfish immunology, 9*(4), 319-333.

Chiswell, S. M., & Sutton, P. J. H. (2020). Relationships between long-term ocean warming, marine heat waves and primary production in the New Zealand region. *New Zealand Journal of Marine and Freshwater Research, 54*(4), 614-635.

Cho, S. Y., Kwon, Y. K., Nam, M., Vaidya, B., Kim, S. R., Lee, S., Kwon, J., Kim, D., & Hwang, G. S. (2017). Integrated profiling of global metabolomic and transcriptomic responses to viral hemorrhagic septicemia virus infection in olive flounder. *Fish & shellfish immunology, 71*, 220-229.

Chong, J., Soufan, O., Li, C., Caraus, L., Li, S., Bourque, G., Wishart, D. S., & Xia, J. (2018). MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Research, 46*(W1), W486-W494.

Christensen, K. A., Leong, J. S., Sakhrani, D., Biagi, C. A., Minkley, D. R., Withler, R. E., Rondeau, E. B., Koop, B. F., & Devlin, R. H. (2018). Chinook salmon (*Oncorhynchus tshawytscha*) genome and transcriptome. *PLoS One, 13*(4), e0195461.

Chuah, L. O., Effarizah, M. E., Goni, A. M., & Rusul, G. (2016). Antibiotic application and emergence of multiple antibiotic resistance (MAR) in global catfish aquaculture. *Current environmental health reports, 3*(2), 118-127.

Chung, S., & Secombes, C. J. (1988). Analysis of events occurring within teleost macrophages during the respiratory burst. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 89*(3), 539-544.

Ciepliński, M., Kasprzak, M., Grandtke, M., Steliga, A., Kamiński, P., & Jerzak, L. (2019). The effect of dipotassium EDTA and lithium heparin on hematologic values of farmed brown trout *Salmo trutta* (L.) spawners. *Aquaculture International, 27*(1), 79-87.

Cipriano, R. C., Smith, M. L., Vermeersch, K. A., Dove, A. D., & Styczynski, M. P. (2015). Differential metabolite levels in response to spawning-induced inappetence in Atlantic salmon Salmo salar. *Comp Biochem Physiol Part D Genomics Proteomics, 13*, 52-59.

Clark, T. D., Donaldson, M. R., Pieperhoff, S., Drenner, S. M., Lotto, A., Cooke, S. J., Hinch, S. G., Patterson, D. A., & Farrell, A. P. (2012). Physiological benefits of being small in a changing world: responses of coho salmon (*Oncorhynchus kisutch*) to an acute thermal challenge and a simulated capture event. *PLoS One, 7*(6), e39079.

Clauss, T. M., Dove, A. D. M., & Arnold, J. E. (2008). Hematologic disorders of fish. *Veterinary Clinics of North America: Exotic Animal Practice, 11*(3), 445-462.

Claver, J. A., & Quaglia, A. I. E. (2009). Comparative morphology, development, and function of blood cells in nonmammalian vertebrates. *Journal of exotic pet medicine, 18*(2), 87-97.

References

Clem, L. W., Sizemore, R. C., Ellsaesser, C. F., & Miller, N. W. (1985). Monocytes as accessory cells in fish immune responses. *Developmental & Comparative Immunology, 9*(4), 803-809.

Clerton, P., Troutaud, D., Verlhac, V., Gabaudan, J., & Deschaux, P. (2001). Dietary vitamin E and rainbow trout (*Oncorhynchus mykiss*) phagocyte functions: effect on gut and on head kidney leucocytes. *Fish Shellfish Immunol, 11*(1), 1-13.

Coccia, E., Imperatore, R., Orso, G., Melck, D., Varricchio, E., Volpe, M. G., & Paolucci, M. (2019). Explants of *Oncorhynchus mykiss* intestine to detect bioactive molecules uptake and metabolic effects: Applications in aquaculture. *Aquaculture, 506*, 193-204.

Conde-Sieira, M., Chivite, M., Míguez, J. M., & Soengas, J. L. (2018). Stress effects on the mechanisms regulating appetite in teleost fish. *Frontiers in Endocrinology, 9*, 631.

Conroy, D. A. (1972). Studies on the haematology of the Atlantic salmon (*Salmo salar* L.). Symp. Zool. Soc., London, G. B.

Costas, B., Conceição, L. E. C., Aragão, C., Martos, J. A., Ruiz-Jarabo, I., Mancera, J. M., & Afonso, A. (2011). Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress challenge: effects on non-specific immune parameters, plasma free amino acids and energy metabolism. *Aquaculture, 316*(1-4), 68-76.

Costas, B., Rêgo, P. C. N. P., Simões, I., Marques, J. F., Castro-Cunha, M., & Afonso, A. (2013). Cellular and humoral immune responses of Senegalese sole, *Solea senegalensis* (Kaup), following challenge with two *Photobacterium damselae* subsp. piscicida strains from different geographical origins. *Journal of Fish Diseases, 36*(6), 543-553.

Coughlin, S. S. (2014). Toward a road map for global -omics: a primer on -omic technologies. *Am J Epidemiol, 180*(12), 1188-1195.

Crippen, T. L., Bootland, L. M., Leong, J. A. C., Fitzpatrick, M. S., Schreck, C. B., & Vella, A. T. (2001). Analysis of salmonid leukocytes purified by hypotonic lysis of erythrocytes. *Journal of Aquatic Animal Health, 13*(3), 234-245.

D'Mello, J. P. F. (2003). An outline of pathways in amino acid metabolism. In J. P. F. D'Mello (Ed.), *Amino acids in animal nutrition.* (Second ed., pp. 71-86). CAB International.

Dalmo, R. A., & Seljelid, R. (1995). The immunomodulatory effect of LPS, laminaran and sulphated laminaran [β (l, 3)-D-glucan] on Atlantic salmon, *Salmo salar* L., macrophages in vitro. *Journal of Fish Diseases, 18*(2), 175-185.

Damsgård, B., Evensen, T. H., Øverli, Ø., Gorissen, M., Ebbesson, L. O. E., Rey, S., & Höglund, E. (2019). Proactive avoidance behaviour and pace-of-life syndrome in Atlantic salmon. *Royal Society open science, 6*(3), 181859.

Dannevig, B. H., Brudeseth, B. E., Gjøen, T., Rode, M., Wergeland, H. I., Evensen, Ø., & Press, C. M. (1997). Characterisation of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.). *Fish & shellfish immunology, 7*(4), 213-226.

Dannevig, B. H., Falk, K., & Namork, E. (1995). Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *Journal of General Virology, 76*(6), 1353-1359.

References

Dannevig, B. H., Lauve, A., Press, C. M., & Landsverk, T. (1994). Receptor-mediated endocytosis and phagocytosis by rainbow trout head kidney sinusoidal cells. *Fish & shellfish immunology, 4*(1), 3-18.

Das, B. K., Ellis, A. E., & Collet, B. (2009). Induction and persistence of Mx protein in tissues, blood and plasma of Atlantic salmon parr, *Salmo salar*, injected with poly I: C. *Fish & shellfish immunology, 26*(1), 40-48.

Dawber, C. (2004). *Lines in the water: a history of Greenshell mussel farming in New Zealand*. River Press for NZ Marine Farming Association.

De Boeck, G., Vlaeminck, A., Van der Linden, A., & Blust, R. (2000). The Energy Metabolism of Common Carp (*Cyprinus carpio*) When Exposed to Salt Stress: An Increase in Energy Expenditure or Effects of Starvation? *Physiological and Biochemical Zoology, 73*(1), 102-111.

Deborde, C., Hounoum, B. M., Moing, A., Maucourt, M., Jacob, D., Corraze, G., Médale, F., & Fauconneau, B. (2021). Putative imbalanced amino acid metabolism in rainbow trout long term fed a plant-based diet as revealed by ^{1}H-NMR metabolomics. *Journal of Nutritional Science, 10*.

Delorme, N. J., Venter, L., Rolton, A., & Ericson, J. A. (2021). Integrating animal health and stress assessment tools using the green-lipped mussel, *Perna canaliculus*, as a case study. *Journal of Shellfish Research*.

Desvignes, L., Quentel, C., Lamour, F., & Le Ven, A. (2002). Pathogenesis and immune response in Atlantic salmon (*Salmo salar* L.) parr experimentally infected with salmon pancreas disease virus (SPDV). *Fish and Shellfish Immunology, 12*(1), 77-95.

DeWitte-Orr, S. J., Collins, S. E., Bauer, C. M. T., Bowdish, D. M., & Mossman, K. L. (2010). An accessory to the 'Trinity': SR-As are essential pathogen sensors of extracellular dsRNA, mediating entry and leading to subsequent type I IFN responses. *PLoS pathogens, 6*(3), e1000829.

Dietrich, M. A., Samczuk, P., Ciborowski, M., Nynca, J., Parfieniuk, E., Pietrowska, K., Kowalczyk, T., Kretowski, A., & Ciereszko, A. (2019). Metabolic fingerprinting of carp and rainbow trout seminal plasma. *Aquaculture, 501*, 178-190.

Diggles, B. (2016). *Updated disease risk assessment report–relocation of salmon farms in Marlborough Sounds, New Zealand* (Prepared for the Ministry for Primary Industries. DigsFish Services Report: DF, Issue.

Doggett, T. A., & Harris, J. E. (1989). Ultrastructure of the peripheral blood leucocytes of *Oreochromis mossambicus*. *Journal of Fish Biology, 34*(5), 747-756.

Du, C. C., Yang, M. J., Li, M. Y., Yang, J., Peng, B., Li, H., & Peng, X. X. (2017). Metabolic mechanism for l-Leucine-induced metabolome to eliminate *Streptococcus iniae*. *J Proteome Res, 16*(5), 1880-1889.

Dubreil, E., Laurentie, M., Delmas, J., M., Danion, M., Morin, T., Hurtaud-Pessel, D., Viel, A., Sanders, P., & Verdon, E. (2020). Tissue distribution, metabolism, and elimination of Victoria Pure Blue BO in rainbow trout: main metabolite as an appropriate residue marker. *Chemosphere*, 127636.

Dubreil, E., Mompelat, S., Kromer, V., Guitton, Y., Danion, M., Morin, T., Hurtaud-Pessel, D., & Verdon, E. (2019). Dye residues in aquaculture products: Targeted and metabolomics mass spectrometric approaches to track their abuse. *Food chemistry, 294*, 355-367.

References

Dügenci, S. K., Arda, N., & Candan, A. (2003). Some medicinal plants as immunostimulant for fish. *Journal of ethnopharmacology, 88*(1), 99-106.

Dunkelberger, J. R., & Song, W. C. (2010). Complement and its role in innate and adaptive immune responses. *Cell research, 20*(1), 34.

Eaton, W. D. (1990). Anti-viral activity in four species of salmonids following exposure to poly inosinic: cytidylic acid. *Dis Aquat Organ, 9*(3), 193-198.

Echevarría, G., Martínez-Bebiá, M., & Zamora, S. (1997). Evolution of biometric indices and plasma metabolites during prolonged starvation in European sea bass (*Dicentrarchus labrax*, L.). *Comparative Biochemistry and Physiology Part A: Physiology, 118*(1), 111-123.

Edwards, P., Zhang, W., Belton, B., & Little, D. C. (2019). Misunderstandings, myths and mantras in aquaculture: Its contribution to world food supplies has been systematically over reported. *Marine Policy, 106*, 103547.

Eisenreich, W., Rudel, T., Heesemann, J., & Goebel, W. (2019). How Viral and intracellular bacterial pathogens reprogram the metabolism of host cells to allow their intracellular replication. *Frontiers in cellular and infection microbiology, 9*, 42-42.

Ekman, D. R., Skelton, D. M., Davis, J. M., Villeneuve, D. L., Cavallin, J. E., Schroeder, A., Jensen, K. M., Ankley, G. T., & Collette, T. W. (2015). Metabolite profiling of fish skin mucus: a novel approach for minimally-invasive environmental exposure monitoring and surveillance. *Environmental science & technology, 49*(5), 3091-3100.

El-Boshy, M., El-Ashram, A., Risha, E., Abdelhamid, F., Zahran, E., & Gab-Alla, A. (2014). Dietary fucoidan enhance the non-specific immune response and disease resistance in African catfish, *Clarias gariepinus*, immunosuppressed by cadmium chloride. *Veterinary immunology and immunopathology, 162*(3-4), 168-173.

Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Developmental & Comparative Immunology, 25*(8-9), 827-839.

Elmada, C. Z., Huang, W., Jin, M., Liang, X., Mai, K., & Zhou, Q. (2016). The effect of dietary methionine on growth, antioxidant capacity, innate immune response and disease resistance of juvenile yellow catfish (*Pelteobagrus fulvidraco*). *Aquaculture nutrition, 22*(6), 1163-1173.

Elward, K., & Gasque, P. (2003). "Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. *Molecular Immunology, 40*(2-4), 85-94.

Encarnação, P. (2016). 5 - Functional feed additives in aquaculture feeds. In S. F. Nates (Ed.), *Aquafeed Formulation* (pp. 217-237). Academic Press.

Enes, P., Panserat, S., Kaushik, S., & Oliva-Teles, A. (2009). Nutritional regulation of hepatic glucose metabolism in fish. *Fish Physiol Biochem, 35*(3), 519-539.

Erdal, J. I., & Reitan, L. J. (1992). Immune response and protective immunity after vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. *Fish and Shellfish Immunology, 2*(2), 99-108.

References

Espe, M., Hevroy, E. M., Liaset, B., Lemme, A., & El-Mowafi, A. (2008). Methionine intake affect hepatic sulphur metabolism in Atlantic salmon, *Salmo salar*. *Aquaculture, 274*(1), 132-141.

Espelid, S., Løkken, G. B., Steiro, K., & Bøgwald, J. (1996). Effects of cortisol and stress on the immune system in Atlantic salmon (*Salmo salar* L.). *Fish & shellfish immunology, 6*(2), 95-110.

Espert, L., Gongora, C., & Mechti, N. (2003). Interferon: antiviral mechanisms and viral escape. *Bulletin du cancer, 90*(2), 131-141.

Esteban, M., Muñoz, J., & Meseguer, J. (2000). Blood cells of sea bass (*Dicentrarchus labrax* L.). Flow cytometric and microscopic studies. *The Anatomical Record, 258*(1), 80-89.

Everts, B., Amiel, E., Huang, S. C.-C., Smith, A. M., Chang, C.-H., Lam, W. Y., Redmann, V., Freitas, T. C., Blagih, J., & Van Der Windt, G. J. (2014). TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKε supports the anabolic demands of dendritic cell activation. *Nature immunology, 15*(4), 323.

Faisal, M., & Ahne, W. (1990). A cell line (CLC) of adherent peripheral blood mononuclear leucocytes of normal common CARP *Cyprinus carpio*. *Developmental and Comparative Immunology, 14*(2), 255-260.

Fang, Y. Z., Yang, S., & Wu, G. (2002). Free radicals, antioxidants, and nutrition. *Nutrition, 18*(10), 872-879.

FAO. (2007). *National Aquaculture Sector Overview. New Zealand. National Aquaculture Sector Overview Fact Sheets Text by Jeffs, A.* http://www.fao.org.ezproxy.aut.ac.nz/fishery/countrysector/naso_newzealand/en.

FAO. (2010). *Fisheries and Aquaculture Department Statistics*. http://www.fao.org/3/ba0058t/ba0058t.pdf

FAO. (2014). *The State of World Fisheries and Aquaculture 2014. Opportunities and challenges*.

FAO. (2018a). *Fishery and Aquaculture Statistics Aquaculture production*. http://www.fao.org/fishery/static/Yearbook/YB2016_USBcard/root/aquaculture/yearbook_aquaculture.pdf

FAO. (2018b). *Fishery and Aquaculture Statistics. Global Aquaculture Production 1950-2016 (FishstatJ)* www.fao.org/fishery/statistics/software/fishstatj/en

FAO. (2018c). *The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals*. http://www.fao.org/3/i9540en/i9540en.pdf

FAO. (2019). *Fishery and Aquaculture Statistics. Global aquaculture production 1950-2017 (FishstatJ)*. www.fao.org/fishery/statistics/software/fishstatj/en

FAO. (2020a). *Fishery and Aquaculture Statistics. Global capture production 1950-2018 (FishstatJ)*. www.fao.org/fishery/statistics/software/fishstatj/en

FAO. (2020b). *The state of World Fisheries and Aquaculture 2020. Sustainability in action.* .

FAO/WHO. (2001). *Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria*.

References

Faroe Fish Farmers Association. (2012). *About Salmon from the Faroe Islands* Faroe Fish Farmers Association. Retrieved 22/05/2019 from http://salmon-from-the-faroe-islands.com/production.html

Fazio, F. (2019). Fish hematology analysis as an important tool of aquaculture: A review. *Aquaculture, 500*, 237-242.

Fazio, F., Saoca, C., Vazzana, I., & Piccione, G. (2017). Influence of body size on blood hemogram in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792). *Vet Med Open J, 2*(3), 91-94.

Fiehn, O., Robertson, D., Griffin, J., van der Werf, M., Nikolau, B., Morrison, N., Sumner, L. W., Goodacre, R., Hardy, N. W., & Taylor, C. (2007). The metabolomics standards initiative (MSI). *Metabolomics, 3*(3), 175-178.

Finch, G., Yilmaz, A., & Utz, M. (2016). An optimised detector for in-situ high-resolution NMR in microfluidic devices. *Journal of Magnetic Resonance, 262*, 73-80.

Fiorino, G. M., Losito, I., De Angelis, E., Arlorio, M., Logrieco, A. F., & Monaci, L. (2019). Assessing fish authenticity by direct analysis in real time-high resolution mass spectrometry and multivariate analysis: discrimination between wild-type and farmed salmon. *Food Research International, 116*, 1258-1265.

Fiorino, G. M., Losito, I., De Angelis, E., Logrieco, A. F., & Monaci, L. (2018). Direct analysis in real time coupled to high resolution mass spectrometry as a rapid tool to assess salmon (*Salmo salar*) freshness. *Journal of Mass Spectrometry, 53*(9), 781-791.

Fischer, U., Utke, K., Somamoto, T., Köllner, B., Ototake, M., & Nakanishi, T. (2006). Cytotoxic activities of fish leucocytes. *Fish & shellfish immunology, 20*(2), 209-226.

Foroutan, A., Fitzsimmons, C., Mandal, R., Piri-Moghadam, H., Zheng, J., Guo, A. C., Li, C., Guan, L. L., & Wishart, D. S. (2020). The Bovine Metabolome. *Metabolites, 10*(6), 233.

Fritsch, S. D., & Weichhart, T. (2016). Effects of interferons and viruses on metabolism. *Frontiers in immunology, 7*, 630.

Furne, M., Holen, E., Araujo, P., Lie, K. K., & Moren, M. (2013). Cytokine gene expression and prostaglandin production in head kidney leukocytes isolated from Atlantic cod (*Gadus morhua*) added different levels of arachidonic acid and eicosapentaenoic acid. *Fish & shellfish immunology, 34*(3), 770-777.

Gaber, T., Strehl, C., & Buttgereit, F. (2017). Metabolic regulation of inflammation. *Nature Reviews Rheumatology, 13*(5), 267-279.

Gahlawat, S. K., Ellis, A. E., & Collet, B. (2009). Expression of interferon and interferon - Induced genes in Atlantic salmon *Salmo salar* cell lines SHK-1 and TO following infection with Salmon AlphaVirus SAV. *Fish and Shellfish Immunology, 26*(4), 672-675.

Galeottia, M., Romano, N., Volpatti, D., Bulfon, C., Brunetti, A., Tiscar, P. G., Mosca, F., Bertoni, F., Marchetti, M. G., & Abelli, L. (2013). Innovative vaccination protocol against vibriosis in *Dicentrarchus labrax* (L.) juveniles: Improvement of immune parameters and protection to challenge. *Vaccine, 31*(8), 1224-1230.

Gall, W. E., Beebe, K., Lawton, K. A., Adam, K. P., Mitchell, M. W., Nakhle, P. J., Ryals, J. A., Milburn, M. V., Nannipieri, M., Camastra, S., Natali, A., & Ferrannini, E. (2010). alpha-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population. *PLoS One, 5*(5), e10883.

Gallage, S., Katagiri, T., Endo, M., & Maita, M. (2017). Comprehensive evaluation of immunomodulation by moderate hypoxia in S. agalactiae vaccinated Nile tilapia. *Fish & shellfish immunology, 66*, 445-454.

Ganassin, R. C., & Bols, N. C. (1996). Development of long-term rainbow trout spleen cultures that are haemopoietic and produce dendritic cells. *Fish & shellfish immunology, 6*(1), 17-34.

Ganassin, R. C., & Bols, N. C. (1998). Development of a monocyte/macrophage-like cell line, RTS11, from rainbow trout spleen. *Fish & shellfish immunology, 8*(6), 457-476.

Gaoping, C., Ying, Q., Xiaowen, H., Pengkun, W., Liya, S., Xuhan, Y., Jie, J., Juan, Z., Hang, W., Lei, F., Yuhua, S., Shengying, Q., & Chunling, W. (2020). Serum Metabolomic Profiling Based on Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometry: Do the Dysfunctions of Metabolic Pathways Reveal a Universal Risk of Oxidative Stress in Schizophrenia? *Antioxidants & Redox Signaling, 33*(10), 679-688.

Garcia, L. G., Lemaire, S., Kahl, B. C., Becker, K., Proctor, R. A., Tulkens, P. M., & Van Bambeke, F. (2012). Influence of the protein kinase C activator phorbol myristate acetate on the intracellular activity of antibiotics against hemin-and menadione-auxotrophic small-colony variant mutants of *Staphylococcus aureus* and their wild-type parental strain in human THP-1 cells. *Antimicrobial Agents and Chemotherapy, 56*(12), 6166-6174.

Gaspar, C., Silva-Marrero, J. I., Salgado, M. C., Baanante, I. V., & Metón, I. (2018). Role of upstream stimulatory factor 2 in glutamate dehydrogenase gene transcription. *Journal of molecular endocrinology, 60*(3), 247-259.

Gavery, M. R., & Roberts, S. B. (2017). Epigenetic considerations in aquaculture. *PeerJ, 5*, e4147.

George, J. C., & Don, S. E. (1978). Fine structure and metabolic adaptation of red and white muscles in tuna. *Environmental Biology of Fishes, 3*(2), 185-191.

Ghidini, S., Varrà, M. O., Dall'Asta, C., Badiani, A., Ianieri, A., & Zanardi, E. (2019b). Rapid authentication of European sea bass (*Dicentrarchus labrax* L.) according to production method, farming system, and geographical origin by near infrared spectroscopy coupled with chemometrics. *Food chemistry, 280*, 321-327.

Ghidini, S., Varrà, M. O., & Zanardi, E. (2019a). Approaching authenticity issues in fish and seafood products by qualitative spectroscopy and chemometrics. *Molecules, 24*(9), 1812.

Gibson, D. (2021). *The salmon you buy in the future may be farmed on land*. BBC News. Retrieved 07/09/2021 from https://www.bbc.com/news/business-56829129

Gillis, K., Chowdhury, K., Clor, J., & Tyagarajan, K. (2016). *Simplified methods for algal characterization using microcapillary flow cytometry*.

Gillis, K., Chowdhury, K., Clor, J., & Tyagarajan, K. (2016). *Simplified methods for algal characterization using microcapillary flow cytometry*.

Gillis, T. E., & Ballantyne, J. S. (1996). The effects of starvation on plasma free amino acid and glucose concentrations in lake sturgeon. *Journal of Fish Biology, 49*(6), 1306-1316.

Giraudeau, P. (2020). NMR-based metabolomics and fluxomics: Developments and future prospects. *Analyst, 145*(7), 2457-2472.

Goel, G., Makkar, H. P. S., Francis, G., & Becker, K. (2007). Phorbol esters: structure, biological activity, and toxicity in animals. *International Journal of Toxicology, 26*(4), 279-288.

Goetz, F. W., Iliev, D. B., McCauley, L. A. R., Liarte, C. Q., Tort, L. B., Planas, J. V., & MacKenzie, S. (2004). Analysis of genes isolated from lipopolysaccharide-stimulated rainbow trout (*Oncorhynchus mykiss*) macrophages. *Molecular Immunology, 41*(12), 1199-1210.

Goldansaz, S. A., Guo, A. C., Sajed, T., Steele, M. A., Plastow, G. S., & Wishart, D. S. (2017). Livestock metabolomics and the livestock metabolome: A systematic review. *PLoS One, 12*(5), e0177675.

Gomez, D., Sunyer, J. O., & Salinas, I. (2013). The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. *Fish & shellfish immunology, 35*(6), 1729-1739.

Gonçalves, D. B., Santos, C. S. P., Pinho, T., Queirós, R., Vaz, P. D., Bloore, M., Satta, P., Kovács, Z., Casal, S., & Hoffmann, I. (2020). Near Infrared Reflectance Spectroscopy Coupled to Chemometrics as a Cost-Effective, Rapid, and Non-Destructive Tool for Fish Fraud Control: Monitoring Source, Condition, and Nutritional Value of Five Common Whitefish Species. *Journal of AOAC International, 104*(1), 53-60.

Gong, Q., Yang, D., Jiang, M., Zheng, J., & Peng, B. (2020). l-aspartic acid promotes fish survival against *Vibrio alginolyticus* infection through nitric oxide-induced phagocytosis. *Fish & shellfish immunology, 97*, 359-366.

Gong, Y., Feng, S., Li, S., Zhang, Y., Zhao, Z., Hu, M., Xu, P., & Jiang, Y. (2017). Genome-wide characterization of Toll-like receptor gene family in common carp (*Cyprinus carpio*) and their involvement in host immune response to *Aeromonas hydrophila* infection. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 24*, 89-98.

Goodacre, R., Vaidyanathan, S., Dunn, W. B., Harrigan, G. G., & Kell, D. B. (2004). Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends in Biotechnology, 22*(5), 245-252.

Gordon, I. (2018). Livestock production increasingly influences wildlife across the globe. *Animal, 12*(s2), s372-s382.

Graham, S., & Secombes, C. J. (1988). The production of a macrophage-activating factor from rainbow trout *Salmo gairdneri* leucocytes. *Immunology, 65*(2), 293-297.

Grandiosa, R., Bouwman, M. L., Young, T., Mérien, F., & Alfaro, A. C. (2018). Effect of antiaggregants on the in vitro viability, cell count and stability of abalone (*Haliotis iris*) haemocytes. *Fish & shellfish immunology, 78*, 131-139.

Grandiosa, R., Mérien, F., Krish, P., & Alfaro, A. C. (2016). Innovative application of classic and newer techniques for the characterization of haemocytes in the New Zealand black-footed abalone (*Haliotis iris*). *Fish Shellfish Immunol, 48*, 175-184.

Grandiosa, R., Mérien, F., Young, T., Nguyen, T. V., Gutierrez, N., Kitundu, E., & Alfaro, A. C. (2018). Multi-strain probiotics enhance immune responsiveness and alters metabolic profiles in the New Zealand black-footed abalone (*Haliotis iris*). *Fish & shellfish immunology, 82*, 330-338.

Graves, P. R., & Haystead, T. A. J. (2002). Molecular biologist's guide to proteomics. *Microbiology and molecular biology reviews, 66*(1), 39-63.

Grayfer, L., & Belosevic, M. (2009). Molecular characterization, expression and functional analysis of goldfish (*Carassius aurutus* L.) interferon gamma. *Developmental & Comparative Immunology, 33*(2), 235-246.

Grayfer, L., Hodgkinson, J. W., & Belosevic, M. (2014). Antimicrobial responses of teleost phagocytes and innate immune evasion strategies of intracellular bacteria. *Developmental & Comparative Immunology, 43*(2), 223-242.

Grayfer, L., Kerimoglu, B., Yaparla, A., Hodgkinson, J. W., Xie, J., & Belosevic, M. (2018). Mechanisms of fish macrophage antimicrobial immunity. *Frontiers in immunology, 9, 1105*.

Gribbestad, I. S., Aursand, M., & Martinez, I. (2005). High-resolution ^{1}H magnetic resonance spectroscopy of whole fish, fillets and extracts of farmed Atlantic salmon (Salmo salar) for quality assessment and compositional analyses. *Aquaculture, 250*(1-2), 445-457.

Gross, K. A., Morrison, R. N., Butler, R., & Nowak, B. F. (2004). Atlantic salmon, *Salmo salar* L., previously infected with *Neoparamoeba sp* are not resistant to re-infection and have suppressed phagocyte function. *Journal of Fish Diseases, 27*(1), 47-56.

Guijarro, J. A., Cascales, D., García-Torrico, A. I., García-Domínguez, M., & Méndez, J. (2015). Temperature-dependent expression of virulence genes in fish-pathogenic bacteria. *Frontiers in microbiology, 6*, 700.

Haghighi, M., Sharif Rohani, M., Pourmoghim, H., Samadi, M., Tavoli, M., Eslami, M., & Yusefi, R. (2017). Enhancement of immune responses of rainbow trout (*Oncorhynchus mykiss*) fed a diet supplemented with Aloe vera extract. *Iranian Journal of Fisheries Sciences, 17*(3), 884-896.

Hajirezaee, S., Rafieepour, A., & Shafiei, S. (2019). A NMR-based metabonomic study on the ameliorating effects of Ginkgo biloba extract in rainbow trout, *Oncorhynchus mykiss* exposed to organophosphate pesticide, diazinon. *Aquaculture, 513*, 734450.

Hall, J. C. (1998). Glycine. *Journal of Parenteral and Enteral Nutrition, 22*(6), 393-398.

Halver, J. E., Delong, D. C., & Mertz, E. T. (1957). Nutrition of salmonoid fishes. V. Classification of essential amino acids for Chinook salmon. *J Nutr, 63*(1), 95-105.

Han, P., Wang, S., Zhang, Q., Zhang, S., Shao, R., Xu, W., Zhang, W., Xu, Q., Wei, Q., & Qi, Z. (2018). Molecular characterization and expression analysis of TLR1 and TLR4 from the endangered fish Dabry's sturgeon (*Acipenser dabryanus*). *Developmental & Comparative Immunology, 86*, 180-188.

Hano, T., Ito, M., Ito, K., & Uchida, M. (2021). Alterations of stool metabolome, phenome, and microbiome of the marine fish, red sea bream, *Pagrus major*, following exposure to phenanthrene: A non-invasive approach for exposure assessment. *Science of The Total Environment, 752*, 141796.

Hansen, A. K. G., Kortner, T. M., Krasnov, A., Björkhem, I., Penn, M., & Krogdahl, Å. (2020). Choline supplementation prevents diet induced gut mucosa lipid accumulation in post-smolt Atlantic salmon (*Salmo salar* L.). *Bmc Veterinary Research, 16*(1), 32.

Hardie, L. J., Marsden, M. J., Fletcher, T. C., & Secombes, C. J. (1993). In vitro addition of vitamin C affects rainbow trout lymphocyte responses. *Fish & shellfish immunology, 3*(3), 207-219.

Härdig, J., & Höglund, L. B. (1983). On accuracy in estimating fish blood variables. *Comparative Biochemistry and Physiology Part A: Physiology, 75*(1), 35-40.

Hardy, C. J. (1989). *Establishment and operation of the Silverstream [New Zealand] fish hatchery, 1964 to 1982*. MAFFish.

Haugland, G. T., Jordal, A. E. O., & Wergeland, H. I. (2012). Characterization of small, mononuclear blood cells from salmon having high phagocytic capacity and ability to differentiate into dendritic like cells. *PLoS One, 7*(11), e49260.

Haugland, G. T., Rønneseth, A., & Wergeland, H. I. (2014). Flow cytometry analyses of phagocytic and respiratory burst activities and cytochemical characterization of leucocytes isolated from wrasse (*Labrus bergylta* A.). *Fish & shellfish immunology, 39*(1), 51-60.

Hawlisch, H., & Köhl, J. (2006). Complement and Toll-like receptors: key regulators of adaptive immune responses. *Molecular Immunology, 43*(1-2), 13-21.

Hedfors, I. A., Bakke, H., Skjødt, K., & Grimholt, U. (2012). Antibodies recognizing both IgM isotypes in Atlantic salmon. *Fish and Shellfish Immunology, 33*(5), 1199-1206.

Heming, T. A., & Paleczny, E. J. (1987). Compositional changes in skin mucus and blood serum during starvation of trout. *Aquaculture, 66*(3-4), 265-273.

Heude, C., Lemasson, E., Elbayed, K., & Piotto, M. (2015). Rapid Assessment of Fish Freshness and Quality by ^{1}H HR-MAS NMR Spectroscopy. *Food Analytical Methods, 8*(4), 907-915.

Hine, P. M. (1992). The granulocytes of fish. *Fish & shellfish immunology, 2*(2), 79-98.

Hodgkinson, J., Grayfer, L., & Belosevic, M. (2015). Biology of bony fish macrophages. *Biology, 4*(4), 881-906.

Hoeger, B., Van Den Heuvel, M. R., Hitzfeld, B. C., & Dietrich, D. R. (2004). Effects of treated sewage effluent on immune function in rainbow trout (*Oncorhynchus mykiss*). *Aquatic toxicology, 70*(4), 345-355.

Holen, E., Lie, K. K., Araujo, P., & Olsvik, P. A. (2012). Pathogen recognition and mechanisms in Atlantic cod (*Gadus morhua)* head kidney cells: bacteria (LPS) and virus (poly I: C) signals through different pathways and affect distinct genes. *Fish & shellfish immunology, 33*(2), 267-276.

Holen, E., Winterthun, S., Du, Z. Y., & Krøvel, A. V. (2011). Inhibition of p38 MAPK during cellular activation modulate gene expression of head kidney leukocytes isolated from Atlantic salmon (*Salmo salar*) fed soy bean oil or fish oil based diets. *Fish & shellfish immunology, 30*(1), 397-405.

Holland, M. C. H., & Lambris, J. D. (2002). The complement system in teleosts. *Fish & shellfish immunology, 12*(5), 399-420.

Honda, M., & Suzuki, N. (2020). Toxicities of Polycyclic Aromatic Hydrocarbons for Aquatic Animals. *International Journal of Environmental Research and Public Health, 17*(4), 1363.

Hong, S., Li, R., Xu, Q., Secombes, C. J., & Wang, T. (2013). Two types of TNF-α exist in teleost fish: phylogeny, expression, and bioactivity analysis of type-II TNF-α3 in Rainbow trout *Oncorhynchus mykiss*. *The Journal of Immunology, 191*(12), 5959.

Hopkins, C. L., & Todd, P. R. (1991). *Monosex and triploid salmon stocks*.

Hu, Y., Carpio, Y., Scott, C., Alnabulsi, A., Alnabulsi, A., Wang, T., Liu, F., Monte, M., Wang, T., & Secombes, C. J. (2019). Induction of IL-22 protein and IL-22-producing cells in rainbow trout Oncorhynchus mykiss. *Developmental & Comparative Immunology, 101*, 103449.

Hu, Y., Maisey, K., Subramani, P. A., Liu, F., Flores-Kossack, C., Imarai, M., Secombes, C. J., & Wang, T. (2018). Characterisation of rainbow trout peripheral blood leucocytes prepared by hypotonic lysis of erythrocytes, and analysis of their phagocytic activity, proliferation and response to PAMPs and proinflammatory cytokines. *Developmental & Comparative Immunology, 88*, 104-113.

Huang, H. Y., Chen, Y. C., Wang, P. C., Tsai, M. A., Yeh, S. C., Liang, H. J., & Chen, S. C. (2014). Efficacy of a formalin-inactivated vaccine against Streptococcus iniae infection in the farmed grouper *Epinephelus coioides* by intraperitoneal immunization. *Vaccine, 32*(51), 7014-7020.

Huang, S. M., Xu, F., Lam, S. H., Gong, Z., & Ong, C. N. (2013). Metabolomics of developing zebrafish embryos using gas chromatography-and liquid chromatography-mass spectrometry. *Molecular BioSystems, 9*(6), 1372-1380.

Huang, Y., Zhang, Y., Zheng, J., Wang, L., Qin, Q., & Huang, X. (2020). Metabolic profiles of fish nodavirus infection in vitro: RGNNV induced and exploited cellular fatty acid synthesis for virus infection. *Cellular microbiology, 22*(9), e13216.

Hvas, M., Folkedal, O., & Oppedal, F. (2020). Fish welfare in offshore salmon aquaculture. *Reviews in Aquaculture, 13(2), 836-852*.

Hwang, S. D., Ohtani, M., Hikima, J., Jung, T. S., Kondo, H., Hirono, I., & Aoki, T. (2012). Molecular cloning and characterization of Toll-like receptor 3 in Japanese flounder, *Paralichthys olivaceus*. *Dev Comp Immunol, 37*(1), 87-96.

Ibarz, A., Martín-Pérez, M., Blasco, J., Bellido, D., de Oliveira, E., & Fernández-Borràs, J. (2010). Gilthead sea bream liver proteome altered at low temperatures by oxidative stress. *Proteomics, 10*(5), 963-975.

Idriss, H. T., & Naismith, J. H. (2000). TNFα and the TNF receptor superfamily: Structure-function relationship (s). *Microscopy research and technique, 50*(3), 184-195.

Irarrázaval, F., & Bustos-Gallardo, B. (2019). Global Salmon Networks: Unpacking Ecological Contradictions at the Production Stage. *Economic Geography, 95*(2), 159-178.

Irianto, A., & Austin, B. (2002). Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases, 25*(6), 333-342.

Iversen, A., Asche, F., Hermansen, Ø., & Nystøyl, R. (2020). Production cost and competitiveness in major salmon farming countries 2003–2018. *Aquaculture, 522*, 735089.

Iversen, A., Hermansen, Ø., Nystøyl, R., Rolland, K. H., & Garshol, L. D. (2019). Konkurranseevne for norsk oppdrettslaks: Kostnader og kostnadsdrivere i Norge og konkurrentland. *Nofima rapportserie*.

Iwanaga, S. (2002). The molecular basis of innate immunity in the horseshoe crab. *Current Opinion in Immunology, 14*(1), 87-95.

Jääskeläinen, E., Jakobsen, L. M. A., Hultman, J., Eggers, N., Bertram, H. C., & Björkroth, J. (2019). Metabolomics and bacterial diversity of packaged yellowfin tuna (*Thunnus albacares*) and salmon (*Salmo salar*) show fish species-specific spoilage development during chilled storage. *International journal of food microbiology, 293*, 44-52.

Jacobsen, D. W., & Hannibal, L. (2019). Redox signaling in inherited diseases of metabolism. *Current Opinion in Physiology, 9*, 48-55.

Jamalzadeh, H. R., & Ghomi, M. R. (2009). Hematological parameters of Caspian salmon *Salmo trutta* caspius associated with age and season. *Marine and Freshwater Behaviour and Physiology, 42*(1), 81-87.

Jang, S. I., Hardie, L. J., & Secombes, C. J. (1995). Elevation of rainbow trout *Oncorhynchus mykiss* macrophage respiratory burst activity with macrophage-derived supernatants. *Journal of leukocyte biology, 57*(6), 943-947.

Jarak, I., Tavares, L., Palma, M., Rito, J., Carvalho, R. A., & Viegas, I. (2018). Response to dietary carbohydrates in European seabass (*Dicentrarchus labrax*) muscle tissue as revealed by NMR-based metabolomics. *Metabolomics, 14*(7), 95.

Jasour, M. S., Wagner, L., Sundekilde, U. K., Larsen, B. K., Greco, I., Orlien, V., Olsen, K., Rasmussen, H. T., Hjermitslev, N. H., & Hammershøj, M. (2017). A comprehensive approach to assess feather meal as an alternative protein source in aquafeed. *Journal of agricultural and food chemistry, 65*(48), 10673-10684.

Jensen, I., Albuquerque, A., Sommer, A. I., & Robertsen, B. (2002b). Effect of poly I:C on the expression of Mx proteins and resistance against infection by infectious salmon anaemia virus in Atlantic salmon. *Fish & shellfish immunology, 13*(4), 311-326.

Jensen, I., Larsen, R., & Robertsen, B. (2002c). An antiviral state induced in Chinook salmon embryo cells (CHSE-214) by transfection with the double-stranded RNA poly I: C. *Fish & shellfish immunology, 13*(5), 367-378.

Jensen, I., & Robertsen, B. (2002a). Effect of double-stranded RNA and interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anemia virus and infectious pancreatic necrosis virus. *Fish & shellfish immunology, 13*(3), 221-241.

Jha, A. K., Huang, S. C. C., Sergushichev, A., Lampropoulou, V., Ivanova, Y., Loginicheva, E., Chmielewski, K., Stewart, K. M., Ashall, J., Everts, B., Pearce, E. J., Driggers, E. M., & Artyomov, M. N. (2015). Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity, 42*(3), 419-430.

Jiang, M., Yang, L., Chen, Z., Lai, S., Zheng, J., & Peng, B. (2020). Exogenous maltose enhances Zebrafish immunity to levofloxacin-resistant *Vibrio alginolyticus*. *Microbial biotechnology*.

Jiang, N., Tan, N. S., Ho, B., & Ding, J. L. (2007). Respiratory protein–generated reactive oxygen species as an antimicrobial strategy. *Nature immunology, 8*(10), 1114-1122.

Jiao, S., Nie, M., Song, H., Xu, D., & You, F. (2020). Physiological responses to cold and starvation stresses in the liver of yellow drum (*Nibea albiflora*) revealed by LC-MS metabolomics. *Science of The Total Environment, 715*, 136940.

Jin, Y., Tian, L. X., Xie, S. W., Guo, D. Q., Yang, H. J., Liang, G. Y., & Liu, Y. J. (2015). Interactions between dietary protein levels, growth performance, feed utilization, gene

expression and metabolic products in juvenile grass carp (*Ctenopharyngodon idella*). *Aquaculture, 437*, 75-83.

Joffre, O., & Verdegem, M. (2019). Feeding both pond and fish: A pathway to ecological intensification of aquaculture systems. *INFOFISH International*, (3/2019), 55-58. https://digitalarchive.worldfishcenter.org/bitstream/handle/20.500.12348/3420/4384.pdf

Johansson, P., Wang, T., Collet, B., Corripio-Miyar, Y., Monte, M. M., Secombes, C. J., & Zou, J. (2016). Identification and expression modulation of a C-type lectin domain family 4 homologue that is highly expressed in monocytes/macrophages in rainbow trout (*Oncorhynchus mykiss*). *Developmental & Comparative Immunology, 54*(1), 55-65.

Johnson, E. C. (2017). Aspartic Acid. In M. Caplan & R. Bradshaw (Eds.), *Reference Module in Biomedical Sciences*. Elsevier.

Jokinen, I. E., Markkula, E. S., Salo, H. M., Kuhn, P., Nikoskelainen, S., Arts, M. T., & Browman, H. I. (2008). Exposure to increased ambient ultraviolet B radiation has negative effects on growth, condition and immune function of juvenile Atlantic salmon (*Salmo salar*). *Photochemistry and Photobiology, 84*(5), 1265-1271.

Jokinen, I. E., Salo, H. M., Markkula, E., Rikalainen, K., Arts, M. T., & Browman, H. I. (2011). Additive effects of enhanced ambient ultraviolet B radiation and increased temperature on immune function, growth and physiological condition of juvenile (parr) Atlantic Salmon, *Salmo salar*. *Fish & shellfish immunology, 30*(1), 102-108.

Jørgensen, J. B., Johansen, A., Stenersen, B., & Sommer, A. I. (2001). CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (*Salmo salar* L.) leucocytes to produce supernatants with antiviral activity. *Developmental & Comparative Immunology, 25*(4), 313-321.

Jørgensen, J. B., Johansen, L. H., Steiro, K., & Johansen, A. (2003). CpG DNA Induces Protective Antiviral Immune Responses in Atlantic Salmon (*Salmo salar* L.). *Journal of Virology, 77*(21), 11471.

Jørgensen, J. B., & Robertsen, B. (1995). Yeast β-glucan stimulates respiratory burst activity of Atlantic salmon (*Salmo salar* L.) macrophages. *Developmental & Comparative Immunology, 19*(1), 43-57.

Jóźwiak, Z., & PaŁecz, D. (1988). Influence of heat treatment on osmotic fragility of carp erythrocytes. *International journal of radiation biology, 54*(2), 299-303.

Judycka, S., Nynca, J., Hliwa, P., & Ciereszko, A. (2021). Characteristics and cryopreservation of semen of sex-reversed females of Salmonid fish. *International Journal of Molecular Sciences, 22*(2), 964.

Julin, K., Johansen, L. H., Sommer, A. I., & Jørgensen, J. B. (2015). Persistent infections with infectious pancreatic necrosis virus (IPNV) of different virulence in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases, 38*(11), 1005-1019.

Jun, J. W., Kang, J. W., Giri, S. S., Yun, S., Kim, H. J., Kim, S. G., Kim, S. W., Han, S. J., Kwon, J., & Oh, W. T. (2020). Immunostimulation by starch hydrogel-based oral vaccine using formalin-killed cells against edwardsiellosis in Japanese eel, *Anguilla japonica*. *Vaccine, 38*(22), 3847-3853.

Kaattari, S. L., & Irwin, M. J. (1985). Salmonid spleen and anterior kidney harbor populations of lymphocytes with different B cell repertoires. *Developmental & Comparative Immunology, 9*(3), 433-444.

Kaattari, S. L., Irwin, M. J., Yui, M. A., Tripp, R. A., & Parkins, J. S. (1986). Primary in vitro stimulation of antibody production by rainbow trout lymphocytes. *Veterinary immunology and immunopathology, 12*(1-4), 29-38.

Kaattari, S. L., & Yui, M. A. (1987). Polyclonal activation of salmonid B lymphocytes. *Developmental & Comparative Immunology, 11*(1), 155-165.

Kadowaki, T., Yasui, Y., Takahashi, Y., Kohchi, C., Soma, G. I., & Inagawa, H. (2009). Comparative immunological analysis of innate immunity activation after oral administration of wheat fermented extract to teleost fish. *Anticancer Research, 29*(11), 4871-4877.

Kaleeswaran, B., Ilavenil, S., & Ravikumar, S. (2012). Changes in biochemical, histological and specific immune parameters in *Catla catla* (Ham.) by Cynodon dactylon (L.). *Journal of King Saud University-Science, 24*(2), 139-152.

Kales, S., Parks-Dely, J., Schulte, P., & Dixon, B. (2006). Beta-2-microglobulin gene expression is maintained in rainbow trout and Atlantic salmon kept at low temperatures. *Fish & shellfish immunology, 21*(2), 176-186.

Kalgraff, C. A. K., Wergeland, H. I., & Pettersen, E. F. (2011). Flow cytometry assays of respiratory burst in Atlantic salmon (*Salmo salar* L.) and in Atlantic cod (*Gadus morhua* L.) leucocytes. *Fish & shellfish immunology, 31*(3), 381-388.

Kammerer, B. D., Cech Jr, J. J., & Kültz, D. (2010). Rapid changes in plasma cortisol, osmolality, and respiration in response to salinity stress in tilapia (*Oreochromis mossambicus*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 157*(3), 260-265.

Karakach, T. K., Huenupi, E. C., Soo, E. C., Walter, J. A., & Afonso, L. O. B. (2009). ^{1}H-NMR and mass spectrometric characterization of the metabolic response of juvenile Atlantic salmon (*Salmo salar*) to long-term handling stress. *Metabolomics, 5*(1), 123-137.

Karrow, N. A., Boermans, H. J., Dixon, D. G., Hontella, A., Solomon, K. R., Whyte, J. J., & Bols, N. C. (1999). Characterizing the immunotoxicity of creosote to rainbow trout (*Oncorhynchus mykiss*): A microcosm study. *Aquatic toxicology, 45*(4), 223-239.

Katakura, F., Takizawa, F., Yoshida, M., Yamaguchi, T., Araki, K., Tomana, M., Nakao, M., Moritomo, T., & Nakanishi, T. (2009). Co-culture of carp (*Cyprinus carpio*) kidney haematopoietic cells with feeder cells resulting in long-term proliferation of T-cell lineages. *Veterinary immunology and immunopathology, 131*(1), 127-136.

Kavaliauskis, A., Arnemo, M., Kim, S. H., Ulanova, L., Speth, M., Novoa, B., Dios, S., Evensen, Ø., Griffiths, G. W., & Gjøen, T. (2015). Use of Poly (I: C) stabilized with chitosan as a vaccine-adjuvant against viral hemorrhagic septicemia virus infection in zebrafish. *Zebrafish, 12*(6), 421-431.

Kawahara, E., Oshima, S. I., & Nomura, S. (1990). Separation of Lymphocytes from the Peripheral Blood of Coho Salmon *Oncorhynchus kisutch*. *Nippon Suisan Gakkaishi, 56*(2), 363-363.

Kayansamruaj, P., Pirarat, N., Hirono, I., & Rodkhum, C. (2014). Increasing of temperature induces pathogenicity of Streptococcus agalactiae and the up-regulation of inflammatory related genes in infected Nile tilapia (*Oreochromis niloticus*). *Veterinary microbiology, 172*(1), 265-271.

Kelly, B., & O'Neill, L. A. J. (2015). Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell research, 25*(7), 771-784.

Kenyon, W., & Davies, D. (2018). *Salmon Farming in Scotland* (SPICe Briefing, Issue. S. P. I. C. (SPICe). https://sp-bpr-en-prod-cdnep.azureedge.net/published/2018/2/13/Salmon-Farming-in-Scotland/SB%2018-12%20rev.pdf

Kepka, M., Szwejser, E., Pijanowski, L., Verburg-van Kemenade, B. M. L., & Chadzinska, M. (2015). A role for melatonin in maintaining the pro- and anti-inflammatory balance by influencing leukocyte migration and apoptosis in carp. *Developmental & Comparative Immunology, 53*(1), 179-190.

Kibenge, F. S. (2019). Emerging viruses in aquaculture. *Current opinion in virology, 34*, 97-103.

Kiessling, A., Ruohonen, K., & Bjørnevik, M. (2006). Muscle fibre growth and quality in fish.

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol, 8*(6), e1000412.

Kim, D. H., & Austin, B. (2006). Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics. *Fish & shellfish immunology, 21*(5), 513-524.

Kim, H. J., Oseko, N., Nishizawa, T., & Yoshimizu, M. (2009). Protection of rainbow trout from infectious hematopoietic necrosis (IHN) by injection of infectious pancreatic necrosis virus (IPNV) or Poly(I:C). *Diseases of aquatic organisms, 83*(2), 105-113.

Kiron, V., Takeuchi, T., & Watanabe, T. (1994). The osmotic fragility of erythrocytes in rainbow trout under different dietary fatty acid status. *Fisheries Science, 60*(1), 93-95.

Kitao, Y., Kono, T., Korenaga, H., Iizasa, T., Nakamura, K., Savan, R., & Sakai, M. (2009). Characterization and expression analysis of type I interferon in common carp *Cyprinus carpio* L. *Molecular Immunology, 46*(13), 2548-2556.

Kobayashi, M., & Shimizu, S. (1999). [Nicotinic acid and nicotinamide]. *Nihon Rinsho, Japanese journal of clinical medicine, 57*(10), 2211-2217.

Kokushi, E., Uno, S., & Koyama, J. (2011). Assessing the effects of starvation on freshwater carp *Cyprinus carpio* L. by ^{1}H-NMR metabolomics approach. *Nippon Suisan Gakkaishi, 77*(1), 75-83.

Köllner, B., Fischer, U., Rombout, J. H. W. M., Taverne-Thiele, J. J., & Hansen, J. D. (2004). Potential involvement of rainbow trout thrombocytes in immune functions: a study using a panel of monoclonal antibodies and RT-PCR. *Developmental & Comparative Immunology, 28*(10), 1049-1062.

Kortet, R., Taskinen, J., Sinisalo, T., & Jokinen, I. (2003). Breeding-related seasonal changes in immunocompetence, health state and condition of the cyprinid fish, *Rutilus rutilus*, L. *Biological Journal of the Linnean Society, 78*(1), 117-127.

Korytář, T., Thi, H. D., Takizawa, F., & Köllner, B. (2013). A multicolour flow cytometry identifying defined leukocyte subsets of rainbow trout (*Oncorhynchus mykiss*). *Fish & shellfish immunology, 35*(6), 2017-2019.

Kullgren, A., Jutfelt, F., Fontanillas, R., Sundell, K., Samuelsson, L., Wiklander, K., Kling, P., Koppe, W., Larsson, D. G. J., Bjornsson, B. T., & Jonsson, E. (2013). The impact of temperature on the metabolome and endocrine metabolic signals in Atlantic salmon

(*Salmo salar*). *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 164*(1), 44-53.

Kullgren, A., Samuelsson, L. M., Larsson, D. G. J., Björnsson, B. T., & Bergman, E. J. (2010). A metabolomics approach to elucidate effects of food deprivation in juvenile rainbow trout (*Oncorhynchus mykiss*). *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 299*(6), R1440-R1448.

Kumar, S., Raman, R. P., Prasad, K. P., Srivastava, P. P., Kumar, S., & Rajendran, K. V. (2017). Modulation of innate immune responses and induction of oxidative stress biomarkers in *Pangasianodon hypophthalmus* following an experimental infection with dactylogyrid monogeneans. *Fish & shellfish immunology, 63*, 334-343.

Kumari, K. (2020). Pesticides toxicity in fishes: A review. *Journal of Entomology and Zoology Studies, 8*(5), 1640-1642.

Kwasek, K., Thorne-Lyman, A. L., & Phillips, M. (2020). Can human nutrition be improved through better fish feeding practices? a review paper. *Critical reviews in food science and nutrition*, 1-14.

Lai, R., Liu, H., Jakovlić, I., Zhan, F., Wei, J., Yang, P., & Wang, W. (2016). Molecular cloning and expression of toll-like receptor 4 (tlr4) in the blunt snout bream (*Megalobrama amblycephala*). *Developmental & Comparative Immunology, 59*, 63-76.

Lane, H. C. (1979). Progressive changes in haematology and tissue water of sexually mature trout, *Salmo gairdneri* Richardson during the autumn and winter. *Journal of Fish Biology, 15*(4), 425-436.

Langevin, C., Boudinot, P., & Collet, B. (2019). IFN signaling in inflammation and viral infections: new insights from fish models. *Viruses, 11*(3), 302.

Larsen, R., Røkenes, T. P., & Robertsen, B. (2004). Inhibition of infectious pancreatic necrosis virus replication by Atlantic salmon Mx1 protein. *Journal of Virology, 78*(15), 7938-7944.

Law, C. S., Rickard, G. J., Mikaloff-Fletcher, S. E., Pinkerton, M. H., Behrens, E., Chiswell, S. M., & Currie, K. (2018). Climate change projections for the surface ocean around New Zealand. *New Zealand Journal of Marine and Freshwater Research, 52*(3), 309-335.

Lazado, C. C., Pedersen, L. F., Kirste, K. H., Soleng, M., Breiland, M. W., & Timmerhaus, G. (2020). Oxidant-induced modifications in the mucosal transcriptome and circulating metabolome of Atlantic salmon. *Aquatic toxicology, 227*, 105625.

Lear, W. H. (1980). The Pink salmon transplant experiment in Newfoundland. In J. E. Thorpe (Eds.), *Salmon ranching* (pp. 213-243). Academic Press.

Lee, P. T., Zou, J., Holland, J. W., Martin, S. A. M., Kanellos, T., & Secombes, C. J. (2013). Identification and characterization of TLR7, TLR8a2, TLR8b1 and TLR8b2 genes in Atlantic salmon (*Salmo salar*). *Developmental & Comparative Immunology, 41*(2), 295-305.

Lee, W. N. P., & Go, V. L. W. (2005). Nutrient-gene interaction: tracer-based metabolomics. *The Journal of nutrition, 135*(12), 3027S-3032S.

Lei, Z., Huhman, D. V., & Sumner, L. W. (2011). Mass spectrometry strategies in metabolomics. *Journal of Biological Chemistry, 286*(29), 25435-25442.

Leng, X., Zhou, H., Tan, Q., Du, H., Wu, J., Liang, X., He, S., & Wei, Q. (2019). Integrated metabolomic and transcriptomic analyses suggest that high dietary lipid levels facilitate ovary development through the enhanced arachidonic acid metabolism, cholesterol biosynthesis and steroid hormone synthesis in Chinese sturgeon (*Acipenser sinensis*). *British Journal of Nutrition, 122*(11), 1230-1241.

Leonardi, M. O., & Klempau, A. E. (2003). Artificial photoperiod influence on the immune system of juvenile rainbow trout (*Oncorhynchus mykiss*) in the Southern Hemisphere. *Aquaculture, 221*(1-4), 581-591.

Levine, R. L., Berlett, B. S., Moskovitz, J., Mosoni, L., & Stadtman, E. R. (1999). Methionine residues may protect proteins from critical oxidative damage. *Mechanisms of ageing and development, 107*(3), 323-332.

Li, L., Maitra, U., Singh, N., & Gan, L. (2010). Molecular mechanism underlying LPS-induced generation of reactive oxygen species in macrophages. *The FASEB Journal, 24*, 422-3.

Li, M., Zhang, M., Ma, Y., Ye, R., Wang, M., Chen, H., Xie, D., Dong, Y., Ning, L., You, C., Wang, S., & Li, Y. (2020). Dietary supplementation with n-3 high unsaturated fatty acids decreases serum lipid levels and improves flesh quality in the marine teleost golden pompano *Trachinotus ovatus*. *Aquaculture, 516*, 734632.

Li, S. (2017). *Evaluation and Improvement of Current Computational Tools for Metabolomics Data Analysis* Auckland University of Technology].

Li, S., Pozhitkov, A., Ryan, R. A., Manning, C. S., Brown-Peterson, N., & Brouwer, M. (2010). Constructing a fish metabolic network model. *Genome Biology, 11*(11), R115.

Li, W., Tang, X., Xing, J., Sheng, X., & Zhan, W. (2014). Proteomic analysis of differentially expressed proteins in *Fenneropenaeus chinensis* hemocytes upon white spot syndrome virus infection. *PLoS One, 9*(2), e89962.

Li, Y., Lu, Z., Zhan, F., Yang, M., Zhao, L., Shi, F., Li, J., Lin, L., & Qin, Z. (2021). Nrf2 modulates host defense during antibacterial immunity response in grass carp (*Ctenopharyngodon idellus*). *Aquaculture*, 536, 736474.

Liepke, C., Baxmann, S., Heine, C., Breithaupt, N., Ständker, L., & Forssmann, W. G. (2003). Human hemoglobin-derived peptides exhibit antimicrobial activity: a class of host defense peptides. *Journal of Chromatography B, 791*(1-2), 345-356.

Lieschke, G. J., & Trede, N. S. (2009). Fish immunology. *Current Biology, 19*(16), R678-R682.

Lin, C. Y., Anderson, B. S., Phillips, B. M., Peng, A. C., Clark, S., Voorhees, J., Wu, H.-D. I., Martin, M. J., McCall, J., Todd, C. R., Hsieh, F., Crane, D., Viant, M. R., Sowby, M. L., & Tjeerdema, R. S. (2009). Characterization of the metabolic actions of crude versus dispersed oil in salmon smolts via NMR-based metabolomics. *Aquatic toxicology, 95*(3), 230-238.

Liu, L., Liang, X. F., Fang, J., & Li, J. (2017). The differentia of nitrogen utilization between fast growth individuals and slow growth individuals in hybrid of *Siniperca chuatsi* (♀)× *Siniperca scherzeri* (♂) mandarin fish fed minced prey fish. *Aquaculture Research, 48*(8), 4590-4595.

Liu, M. L., Zheng, P., Liu, Z., Xu, Y., Mu, J., Guo, J., Huang, T., Meng, H. Q., & Xie, P. (2014). GC-MS based metabolomics identification of possible novel biomarkers for schizophrenia in peripheral blood mononuclear cells. *Molecular BioSystems, 10*(9), 2398-2406.

Liu, P. F., Du, Y. S., Meng, L. J., Li, X., & Liu, Y. (2016). Metabolic profiling in kidneys of Atlantic salmon infected with *Aeromonas salmonicida* based on ^{1}H-NMR. *Fish & shellfish immunology, 58*, 292-301.

Liu, S., He, L., & Yao, K. (2018). The antioxidative function of alpha-ketoglutarate and its applications. *BioMed research international, 2018*.

Liu, X., Steele, J. C., & Meng, X. Z. (2017). Usage, residue, and human health risk of antibiotics in Chinese aquaculture: A review. *Environmental Pollution, 223*, 161-169.

Lockhart, K., Bowden, T. J., & Ellis, a., A. E. (2004). Poly I: C-induced Mx responses in Atlantic salmon parr, post-smolts and growers. *Fish & shellfish immunology, 17*(3), 245-254.

Lockhart, K., Gahlawat, S. K., Soto-Mosquera, D., Bowden, T. J., & Ellis, A. E. (2004). IPNV carrier Atlantic salmon growers do not express Mx mRNA and poly I:C-induced Mx response does not cure the carrier state. *Fish Shellfish Immunol, 17*(4), 347-352.

Lockhart, K., McBeath, A. J. A., Collet, B., Snow, M., & Ellis, A. E. (2007). Expression of Mx mRNA following infection with IPNV is greater in IPN-susceptible Atlantic salmon post-smolts than in IPN-resistant Atlantic salmon parr. *Fish & shellfish immunology, 22*(3), 151-156.

Loh, R. (2007). *Swim bladder - structure, function, disorders and treatment* (Vol. 2007 AVA Annual Conference). Australian Veterinary Association.

López-Mas, L., Claret, A., Reinders, M. J., Banovic, M., Krystallis, A., & Guerrero, L. (2020). Farmed or wild fish? Segmenting European consumers based on their beliefs. *Aquaculture, 532*, 735992.

López-Ruiz, A., Esteban, M. A., & Meseguer, J. (1992). Blood cells of the gilthead seabream (Sparus aurata L.): light and electron microscopic studies. *The Anatomical Record, 234*(2), 161-171.

Lowery, M. S., & Somero, G. N. (1990). Starvation effects on protein synthesis in red and white muscle of the barred sand bass, *Paralabrax nebulifer*. *Physiological Zoology, 63*(3), 630-648.

Lozano, I., Díaz, N. F., Muñoz, S., & Riquelme, C. (2018). Antibiotics in Chilean Aquaculture: A Review. In S. Savic (Ed.), *Antibiotic Use in Animals*. InTechOpen.

Lucas-Torres, C., Huber, G., Ichikawa, A., Nishiyama, Y., & Wong, A. (2018). HR-μMAS NMR-based metabolomics: localized metabolic profiling of a garlic clove with μg tissues. *Analytical chemistry, 90*(22), 13736-13743.

Lulijwa, R., Alfaro, A. C., Merien, F., Burdass, M., Meyer, J., Venter, L., & Young, T. (2020a). Metabolic and immune responses of Chinook salmon (*Oncorhynchus tshawytscha*) smolts to a short-term Poly (I: C) challenge. *Journal of Fish Biology, 96*(3), 731-746.

Lulijwa, R., Alfaro, A. C., Merien, F., Burdass, M., Meyer, J., Venter, L., & Young, T. (2020b). Polyinosinic:polycytidylic acid in vivo enhances Chinook salmon (*Oncorhynchus tshawytscha*) immunity and alters the fish metabolome. *Aquaculture International, 28,* 2437.

Lulijwa, R., Alfaro, A. C., Merien, F., Burdass, M., Venter, L., & Young, T. (2019b). In vitro immune response of chinook salmon (*Oncorhynchus tshawytscha*) peripheral blood

mononuclear cells stimulated by bacterial lipopolysaccharide. *Fish & shellfish immunology, 94*, 190-198.

Lulijwa, R., Alfaro, A. C., Merien, F., Burdass, M., Young, T., Meyer, J., Nguyen, T. V., & Trembath, C. (2019a). Characterisation of Chinook salmon (*Oncorhynchus tshawytscha*) blood and validation of flow cytometry cell count and viability assay kit. *Fish & shellfish immunology, 88*, 179-188.

Lulijwa, R., Alfaro, A. C., Merien, F., Meyer, J., & Young, T. (2019c). Advances in salmonid fish immunology: A review of methods and techniques for lymphoid tissue and peripheral blood leucocyte isolation and application. *Fish & shellfish immunology, 95*, 44-80.

Ma, R., Meng, Y., Zhang, W., & Mai, K. (2020). Comparative study on the organoleptic quality of wild and farmed large yellow croaker *Larimichthys crocea*. *Journal of Oceanology and Limnology, 38*(1), 260-274.

Macaulay, G., Wright, D., Oppedal, F., & Dempster, T. (2020). Buoyancy matters: Establishing the maximum neutral buoyancy depth of Atlantic salmon. *Aquaculture, 519*, 734925.

MacKenzie, S., Iliev, D., Liarte, C., Koskinen, H., Planas, J. V., Goetz, F. W., Mölsä, H., Krasnov, A., & Tort, L. (2006). Transcriptional analysis of LPS-stimulated activation of trout (*Oncorhynchus mykiss*) monocyte/macrophage cells in primary culture treated with cortisol. *Molecular Immunology, 43*(9), 1340-1348.

MacKenzie, S., Planas, J. V., & Goetz, F. W. (2003). LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. *Developmental & Comparative Immunology, 27*(5), 393-400.

Madaro, A., Olsen, R. E., Kristiansen, T. S., Ebbesson, L. O. E., Nilsen, T. O., Flik, G., & Gorissen, M. (2015). Stress in Atlantic salmon: response to unpredictable chronic stress. *Journal of Experimental Biology, 218*(16), 2538-2550.

Madeira, D., Narciso, L., Cabral, H. N., Vinagre, C., & Diniz, M. S. (2013). Influence of temperature in thermal and oxidative stress responses in estuarine fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 166*(2), 237-243.

Magnadottir, B. (2010). Immunological control of fish diseases. *Marine Biotechnology, 12*(4), 361-379.

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish & shellfish immunology, 20*(2), 137-151.

Magnuson, J. T., Cryder, Z., Andrzejczyk, N. E., Harraka, G., Wolf, D., Gan, J. J., & Schlenk, D. (2020b). Metabolomic profiles in brains of juvenile steelhead (*Oncorhynchus mykiss*) following bifenthrin treatment. *Environmental science & technology*.

Magnuson, J. T., Giroux, M., Cryder, Z., Gan, J., & Schlenk, D. (2020a). The use of non-targeted metabolomics to assess the toxicity of bifenthrin to juvenile Chinook salmon (*Oncorhynchus tshawytscha*). *Aquatic toxicology*, 105518.

Molnár, J & Pal, M. (2020). Applying Single Cell Protein as Functional Foods. *Journal of Microbiology, Immunology and Biotechnology 7*, 33-35.

Mahmoud, M. M. A., El-Lamie, M. M. M., Kilany, O. E., & Dessouki, A. A. (2018). Spirulina (*Arthrospira platensis*) supplementation improves growth performance, feed utilization,

immune response, and relieves oxidative stress in Nile tilapia (*Oreochromis niloticus*) challenged with *Pseudomonas fluorescens*. *Fish and Shellfish Immunology, 72*, 291-300.

Malcorps, W., Kok, B., van't Land, M., Fritz, M., van Doren, D., Servin, K., van der Heijden, P., Palmer, R., Auchterlonie, N. A., & Rietkerk, M. (2019). The sustainability conundrum of fishmeal substitution by plant ingredients in shrimp feeds. *Sustainability, 11*(4), 1212.

Malekar, V. C., Morton, J. D., Hider, R. N., Cruickshank, R. H., Hodge, S., & Metcalf, V. J. (2018). Effect of elevated temperature on membrane lipid saturation in Antarctic notothenioid fish. *PeerJ, 6*, e4765-e4765.

Mansour, A. T., & Esteban, M. Á. (2017). Effects of carbon sources and plant protein levels in a biofloc system on growth performance, and the immune and antioxidant status of Nile tilapia (*Oreochromis niloticus*). *Fish & shellfish immunology, 64*, 202-209.

Marc, A. M., Quentel, C., Severe, A., Le Bail, P. Y., & Boeuf, G. (1995). Changes in some endocrinological and non-specific immunological parameters during seawater exposure in the brown trout. *Journal of Fish Biology, 46*(6), 1065-1081.

Marchand, J., Martineau, E., Guitton, Y., Dervilly-Pinel, G., & Giraudeau, P. (2017). Multidimensional NMR approaches towards highly resolved, sensitive and high-throughput quantitative metabolomics. *Current Opinion in Biotechnology, 43*, 49-55.

Marine Scotland. (2012). *Sea Lice: Control and regulation*. https://www2.gov.scot/Resource/0039/00399444.pdf

Markkula, S. E., Karvonen, A., Salo, H. M., Tellervo, V. E., & Jokinen, I. E. (2007). Ultraviolet B irradiation affects resistance of rainbow trout (*Oncorhynchus mykiss*) against bacterium *Yersinia ruckeri* and trematode Diplostomum spathaceum. *Photochemistry and Photobiology, 83*(5), 1263-1269.

Markkula, S. E., Salo, H. M., Rikalainen, K., & Jokinen, I. E. (2006). Different sensitivity of carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) to the immunomodulatory effects of UVB irradiation. *Fish & shellfish immunology, 21*(1), 70-79.

Markkula, S. E., Salo, H. M., Rikalainen, K., & Jokinen, I. E. (2009). Long-term UVB Irradiation Affects the Immune Functions of Carp (*Cyprinus carpio*) and Rainbow Trout (*Oncorhynchus mykiss*). *Photochemistry and Photobiology, 85*(1), 347-352.

Marsden, M. J., Cox, D., & Secombes, C. J. (1994). Antigen-induced release of macrophage activating factor from rainbow trout *Oncorhynchus mykiss* leucocytes. *Veterinary immunology and immunopathology, 42*(2), 199-208.

Marsden, M. J., Hamdani, S. H., & Secombes, C. J. (1995). Proliferative responses of rainbow trout, *Oncorhynchusmykiss*, T and B cells to antigens of *Aeromonas salmonicida*. *Fish & shellfish immunology, 5*(3), 199-210.

Marsden, M. J., & Secombes, C. J. (1997). The influence of vaccine preparations on the induction of antigen specific responsiveness in rainbow trout *Oncorhynchus mykiss*. *Fish and Shellfish Immunology, 7*(7), 455-469.

Marsden, M. J., Vaughan, L. M., Foster, T. J., & Secombes, C. J. (1996). A live (delta aroA) *Aeromonas salmonicida* vaccine for furunculosis preferentially stimulates T-cell responses relative to B-cell responses in rainbow trout (*Oncorhynchus mykiss*). *Infection and immunity, 64*(9), 3863-3869.

Martin, S. A. M., & Król, E. (2017). Nutrigenomics and immune function in fish: new insights from omics technologies. *Developmental & Comparative Immunology, 75*, 86-98.

Martin, S. A. M., Zou, J., Houlihan, D. F., & Secombes, C. J. (2007). Directional responses following recombinant cytokine stimulation of rainbow trout (*Oncorhynchus mykiss*) RTS-11 macrophage cells as revealed by transcriptome profiling. *BMC Genomics, 8*(1), 150.

Martinez-Rubio, L., Morais, S., Evensen, Ø., Wadsworth, S., Vecino, J. G., Ruohonen, K., Bell, J. G., & Tocher, D. R. (2013). Effect of functional feeds on fatty acid and eicosanoid metabolism in liver and head kidney of Atlantic salmon (*Salmo salar* L.) with experimentally induced Heart and Skeletal Muscle Inflammation. *Fish & shellfish immunology, 34*(6), 1533-1545.

Martinez, R., Carpio, Y., Morales, A., Lugo, J. M., Herrera, F., Zaldívar, C., Carrillo, O., Arenal, A., Pimentel, E., & Estrada, M. P. (2016). Oral administration of the growth hormone secretagogue-6 (GHRP-6) enhances growth and non-specific immune responses in tilapia (*Oreochromis* sp.). *Aquaculture, 452*, 304-310.

Martins, G. P., Espe, M., Zhang, Z., Guimarães, I. G., & Holen, E. (2019). Surplus arginine reduced lipopolysaccharide induced transcription of proinflammatory genes in Atlantic salmon head kidney cells. *Fish & shellfish immunology, 86*, 1130-1138.

Martyniuk, C. J., Mehinto, A. C., & Denslow, N. D. (2020). Organochlorine pesticides: Agrochemicals with potent endocrine-disrupting properties in fish. *Molecular and Cellular Endocrinology, 507*, 110764.

Maruhenda Egea, F. C., Toledo-Guedes, K., Sanchez-Jerez, P., Ibanco-Cañete, R., Uglem, I., & Saether, B. S. (2015). A Metabolomic Approach to Detect Effects of Salmon Farming on Wild Saithe (*Pollachius virens*) Populations. *Journal of agricultural and food chemistry, 63*(49), 10717-10726.

Mathias, J. R., Dodd, M. E., Walters, K. B., Yoo, S. K., Ranheim, E. A., & Huttenlocher, A. (2009). Characterization of zebrafish larval inflammatory macrophages. *Developmental & Comparative Immunology, 33*(11), 1212-1217.

McBeath, A. J. A., Snow, M., Secombes, C. J., Ellis, A. E., & Collet, B. (2007). Expression kinetics of interferon and interferon-induced genes in Atlantic salmon (*Salmo salar*) following infection with infectious pancreatic necrosis virus and infectious salmon anaemia virus. *Fish & shellfish immunology, 22*(3), 230-241.

McDowall, R. M. (1988). *Diadromy in fishes: migrations between freshwater and marine environments*. Croom Helm.

McDowall, R. M. (1990). New Zealand freshwater fishes: a natural history and guide.

McDowall, R. M. (1991). Freshwater fisheries research in New Zealand: processes, projects, and people. *New Zealand Journal of Marine and Freshwater Research, 25*(4), 393-413.

McDowall, R. M. (1994). The origins of New Zealand's chinook salmon, *Oncorhynchus tshawytscha*. *Marine Fisheries Review, 56*(1), 1-7.

McNab, F., Mayer-Barber, K., Sher, A., Wack, A., & O'garra, A. (2015). Type I interferons in infectious disease. *Nature Reviews Immunology, 15*(2), 87.

Meador, J. P., Bettcher, L. F., Ellenberger, M. C., & Senn, T. D. (2020). Metabolomic profiling for juvenile Chinook salmon exposed to contaminants of emerging concern. *Science of*

The Total Environment, 747, 141097. https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.141097

Mejía, T. J., Montiel, T., & Massieu, L. (2006). D-β-Hydroxybutyrate prevents glutamate-mediated lipoperoxidation and neuronal damage elicited during glycolysis inhibition in vivo. *Neurochemical research, 31*(12), 1399-1408.

Mekuchi, M., Sakata, K., Yamaguchi, T., Koiso, M., & Kikuchi, J. (2017). Trans-omics approaches used to characterise fish nutritional biorhythms in leopard coral grouper (*Plectropomus leopardus*). *Scientific Reports, 7*(1), 9372.

Melingen, G. O., Pettersen, E. F., & Wergeland, H. I. (2002). Leucocyte populations and responses to immunization and photoperiod manipulation in Atlantic salmon (*Salmo salar* L.) 0+ smolt. *Aquaculture, 214*(1-4), 381-396.

Melis, R., Sanna, R., Braca, A., Bonaglini, E., Cappuccinelli, R., Slawski, H., Roggio, T., Uzzau, S., & Anedda, R. (2017). Molecular details on gilthead sea bream (*Sparus aurata*) sensitivity to low water temperatures from ^{1}H-NMR metabolomics. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 204*, 129-136.

Merien, F. (2016). A Journey with elie Metchnikoff: From innate cell Mechanisms in infectious Diseases to Quantum Biology. *Frontiers in public health, 4*, 125.

Meyer, B. H., Scholtz, H. E., Schall, R., Muller, F. O., Hundt, H. K., & Maree, J. S. (1995). The effect of fasting on total serum bilirubin concentrations. *British journal of clinical pharmacology, 39*(2), 169-171.

Midtlyng, P. J., Reitan, L. J., Lillehaug, A., & Ramstad, A. (1996). Protection, immune responses and side effects in atlantic salmon (*Salmo salar* L.) vaccinated against furunculosis by different procedures. *Fish and Shellfish Immunology, 6*(8), 599-613.

Millipore, E. M. D. (2012). Precise and Accurate Counts and Viability Measurements Across Multiple Cell Lines Using the Muse™ Cell Count & Viability Assay. *BioTechniques, 52*(3), 200-203.

Milston, R. H., Vella, A. T., Crippen, T. L., Fitzpatrick, M. S., Leong, J. A. C., & Schreck, C. B. (2003, Aug). In vitro detection of functional humoral immunocompetence in juvenile chinook salmon (*Oncorhynchus tshawytscha*) using flow cytometry. *Fish & shellfish immunology, 15*(2), 145-158.

Miranda, C. D., Godoy, F. A., & Lee, M. R. (2018). Current Status of the Use of Antibiotics and the Antimicrobial Resistance in the Chilean Salmon Farms. *Frontiers in microbiology, 9,* 1284.

Misumi, I., Vella, A. T., Leong, J. A. C., Nakanishi, T., & Schreck, C. B. (2005). p,p′-DDE depresses the immune competence of chinook salmon (*Oncorhynchus tshawytscha*) leukocytes. *Fish & shellfish immunology, 19*(2), 97-114.

Misumi, I., Yada, T., Leong, J. C., & Schreck, C. B. (2009). The effect of in vitro exposure to tributyltin on the immune competence of Chinook Salmon (*Oncorhynchus tshawytscha*) leukocytes. *Archives of environmental contamination and toxicology, 56*(2), 229-237.

Moe, E., Håvardstun, K., Frøseth, K. A., Amundsen, A. P., Øyvind, N., Skage, M., Åsland, J. E., & Helsengreen, M. B. (2017). *The Norwegian aquaculture analysis 2017* (The aquaculture industry at a glance 2016 and 2017, Issue. https://mb.cision.com/Public/12397/2457990/818c800a18b3c888.pdf.

Monjo, A. L., Poynter, S. J., & DeWitte-Orr, S. J. (2017). CHSE-214: A model for studying extracellular dsRNA sensing in vitro. *Fish & shellfish immunology, 68*, 266-271.

Monte, M. M., Wang, T., Collet, B., Zou, J., & Secombes, C. J. (2015). Molecular characterisation of four class 2 cytokine receptor family members in rainbow trout, *Oncorhynchus mykiss*. *Developmental and Comparative Immunology, 48*(1), 43-54.

Montero, D., Lalumera, G., Izquierdo, M. S., Caballero, M. J., Saroglia, M., & Tort, L. (2009). Establishment of dominance relationships in gilthead sea bream *Sparus aurata* juveniles during feeding: effects on feeding behaviour, feed utilization and fish health. *Journal of Fish Biology, 74*(4), 790-805.

Morais, C. L. M., Lima, K. M. G., Singh, M., & Martin, F. L. (2020). Tutorial: multivariate classification for vibrational spectroscopy in biological samples. *Nature Protocols, 15*(7), 2143-2162.

Morera, D., Roher, N., Ribas, L., Balasch, J. C., Doñate, C., Callol, A., Boltaña, S., Roberts, S., Goetz, G., & Goetz, F. W. (2011). RNA-Seq reveals an integrated immune response in nucleated erythrocytes. *PLoS One, 6*(10), e26998.

Morgan, A. L., Thompson, K. D., Auchinachie, N. A., & Migaud, H. (2008). The effect of seasonality on normal haematological and innate immune parameters of rainbow trout *Oncorhynchus mykiss* L. *Fish & shellfish immunology, 25*(6), 791-799.

Morin, G., Pinel, K., Dias, K., Seiliez, I., & Beaumatin, F. (2020). RTH-149 Cell Line, a Useful Tool to Decipher Molecular Mechanisms Related to Fish Nutrition. *Cells, 9*(8), 1754.

Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology, 8*(12), 958-969.

MOWI. (2020). *Salmon Farming Industry Handbook 2020*. https://mowi.com/it/wp-content/uploads/sites/16/2020/06/Mowi-Salmon-Farming-Industry-Handbook-2020.pdf

MPI. (2019). *The New Zealand Government Aquaculture Strategy 2025*. https://www.mpi.govt.nz/dmsdocument/15895-the-governments-aquaculture-strategy-to-2025

Mumford, S., Heidel, J., Smith, C., Morrison, J., MacConnell, B., & Blazer, V. (2007). *Fish histology and histopathology Manual* (U.S. Fish & Wildlife Service-National ConservationTraining Center., Issue. https://training.fws.gov/resources/course-resources/fish-histology/Fish_Histology_Manual_v4.pdf

Munang'andu, H. M., Galindo-Villegas, J., & David, L. (2018). Teleosts genomics: Progress and prospects in disease prevention and control. *International Journal of Molecular Sciences, 19*(4), 1083.

Munro, L. A., & Wallace, I. S. (2018). *Marine Scotland Science. Scottish fish farm production survey 2017*. T. S. Government.

Murray, A. L., Pascho, R. J., Alcorn, S. W., Fairgrieve, W. T., Shearer, K. D., & Roley, D. (2003). Effects of various feed supplements containing fish protein hydrolysate or fish processing by-products on the innate immune functions of juvenile coho salmon (*Oncorhynchus kisutch*). *Aquaculture, 220*(1), 643-653.

Myrick, C. A., & Cech, J. J. (2001). Temperature effects on Chinook salmon and steelhead: a review focusing on California's Central Valley populations. Bay-Delta Modeling Forum.

Nakano, T., Shirakawa, H., Yeo, G., Devlin, R. H., & Soga, T. (2019). Metabolome Profiling of Growth Hormone Transgenic Coho Salmon by Capillary Electrophoresis Time-of-Flight Mass Spectrometry. In *Oceanography Challenges to Future Earth* (pp. 223-234). Springer.

Nath, S., Kales, S., Fujiki, K., & Dixon, B. (2006). Major histocompatibility class II genes in rainbow trout (*Oncorhynchus mykiss*) exhibit temperature dependent downregulation. *Immunogenetics, 58*(5-6), 443-453.

National Research Council. (2011). *Nutrient requirements of fish and shrimp*. National Academies Press, USA.

Naylor, R. L., Hardy, R. W., Buschmann, A. H., Bush, S. R., Cao, L., Klinger, D. H., Little, D. C., Lubchenco, J., Shumway, S. E., & Troell, M. (2021). A 20-year retrospective review of global aquaculture. *Nature, 591*(7851), 551-563.

Ndong, D., Chen, Y. Y., Lin, Y. H., Vaseeharan, B., & Chen, J. C. (2007). The immune response of tilapia *Oreochromis mossambicus* and its susceptibility to *Streptococcus iniae* under stress in low and high temperatures. *Fish & shellfish immunology, 22*(6), 686-694.

Nestor, G., Bankefors, J., Schlechtriem, C., Brännäs, E., Pickova, J., & Sandström, C. (2010). High-resolution ^{1}H magic angle spinning NMR spectroscopy of intact Arctic char (*Salvelinus alpinus*) muscle. Quantitative analysis of n-3 fatty acids, EPA and DHA. *Journal of agricultural and food chemistry, 58*(20), 10799-10803.

Neumann, N. F., Fagan, D., & Belosevic, M. (1995). Macrophage activating factor(s) secreted by mitogen stimulated goldfish kidney leukocytes synergize with bacterial lipopolysaccharide to induce nitric oxide production in teleost macrophages. *Developmental & Comparative Immunology, 19*(6), 473-482.

Neumann, N. F., Stafford, J. L., Barreda, D., Ainsworth, A. J., & Belosevic, M. (2001). Antimicrobial mechanisms of fish phagocytes and their role in host defense. *Developmental & Comparative Immunology, 25*(8), 807-825.

New Zealand Marine Department. (1885-1927). *Annual report on fisheries*.

Nguyen, T. M., Mandiki, S. N. M., Salomon, J. M. A. J., Baruti, J. B., Thi, N. T. T., Nguyen, T. H., Nhu, T. Q., & Kestemont, P. (2021). Pro- and anti-inflammatory responses of common carp Cyprinus carpio head kidney leukocytes to E.coli LPS as modified by different dietary plant oils. *Developmental & Comparative Immunology, 114*, 103828.

Nguyen, T. V., Alfaro, A. C., Merien, F., Lulijwa, R., & Young, T. (2018). Copper-induced immunomodulation in mussel (*Perna canaliculus*) haemocytes. *Metallomics, 10*(7), 965-978.

Nguyen, T. V., Alfaro, A. C., Young, T., & Merien, F. (2019). Tissue-specific immune responses to Vibrio sp. infection in mussels (*Perna canaliculus*): A metabolomics approach. *Aquaculture, 500*, 118-125.

Nguyen, T. V., Alfaro, A. C., Young, T., Ravi, S., & Merien, F. (2018). Metabolomics Study of Immune Responses of New Zealand Greenshell™ Mussels (*Perna canaliculus*) Infected with Pathogenic *Vibrio sp*. *Marine Biotechnology, 20*(3), 396-409.

Nishizawa, T., Takami, I., Kokawa, Y., & Yoshimizu, M. (2009). Fish immunization using a synthetic double-stranded RNA Poly(I:C), an interferon inducer, offers protection against RGNNV, a fish nodavirus. *Dis Aquat Organ, 83*(2), 115-122.

References

Norman, R., Brosnahan, C. L., Fischer, J., Frazer, J., Johnston, C., Jones, B., Keeling, S., Pande, A., Pulford, D., & Rawdon, T. (2013). Salmon mortality investigation. *MPI Technical Paper 2013/19*.

Norwegian Veterinary Institute. (2016). *Use of Antibiotics in Norwegian Aquaculture* (22 - 2016). T. N. V. Institute. https://naturvernforbundet.no/getfile.php/.../Barents%202033%20I%20Jacobsen.pdf

Norwegian Veterinary Institute. (2018). *The Health Situation in Norwegian Aquaculture 2017*. N. V. Institute. www.vetinst.no:fiskehelserapporten/

Norwegian Veterinary Institute. (2019). *The Health Situation in Norwegian Aquaculture 2018* (Norwegian Veterinary Institute, annual summary of fish health in Norway, Issue. N. V. Institute. www.vetinst.no:fiskehelserapporten/

Novoa, B., Figueras, A., Ashton, I., & Secombes, C. J. (1996). In vitro studies on the regulation of rainbow trout (*Oncorhynchus mykiss*) macrophage respiratory burst activity. *Developmental & Comparative Immunology, 20*(3), 207-216.

Nya, E. J., & Austin, B. (2009). Use of garlic, Allium sativum, to control *Aeromonas hydrophila* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases, 32*(11), 963-970.

Nya, E. J., & Austin, B. (2010). Use of bacterial lipopolysaccharide (LPS) as an immunostimulant for the control of *Aeromonas hydrophila* infections in rainbow trout *Oncorhynchus mykiss* (Walbaum). *Journal of applied microbiology, 108*(2), 686-694.

Nygaard, R., Husgard, S., Sommer, A. I., Leong, J. A. C., & Robertsen, B. (2000). Induction of Mx protein by interferon and double-stranded RNA in salmonid cells. *Fish & shellfish immunology, 10*(5), 435-450.

NZ Salmon Farmers Association INC. (n.d.-a). *Farming regions*. Retrieved 09/08/2017 from http://www.salmon.org.nz/new-zealand-salmon-farming/farming-regions/

NZ Salmon Farmers Association INC. (n.d.-b). *Ocean and Freshwater Farming*. Retrieved 13/06/2017 from http://www.salmon.org.nz/new-zealand-salmon-farming/ocean-farming/

NZKS. (2021). *NZ King Salmon Farms*. Retrieved 29/09/2021 from https://www.kingsalmon.co.nz/seawater//

NZKS. (2019). *Annual Report FY19. New Zealand King Salmon Ltd; Nelson, New Zealand, 2019*.

O'Neill, L. A. J., & Pearce, E. J. (2016). Immunometabolism governs dendritic cell and macrophage function. *Journal of Experimental Medicine, 213*(1), 15-23.

Ogle, J. D., Ogle, C. K., Noel, J. G., Hurtubise, P., & Alexander, J. W. (1985). Studies on the binding of C3b-coated microspheres to human neutrophils. *Journal of Immunological Methods, 76*(1), 47-62.

Oidtmann, B., Dixon, P., Way, K., Joiner, C., & Bayley, A. E. (2018). Risk of waterborne virus spread–review of survival of relevant fish and crustacean viruses in the aquatic environment and implications for control measures. *Reviews in Aquaculture, 10*(3), 641-669.

Oliveira, T. M. B. F., Ribeiro, F. W. P., Sousa, C. P., Salazar-Banda, G. R., de Lima-Neto, P., Correia, A. N., & Morais, S. (2020). Current overview and perspectives on carbon-based

(bio) sensors for carbamate pesticides electroanalysis. *TrAC Trends in Analytical Chemistry, 124*, 115779.

Olsvik, P. A., Berntssen, M. H. G., & Softeland, L. (2015). Modifying effects of vitamin E on chlorpyrifos toxicity in Atlantic salmon. *PLoS One, 10*(3), e0119250.

Olsvik, P. A., Berntssen, M. H. G., & Softeland, L. (2017a). In vitro toxicity of pirimiphos-methyl in Atlantic salmon hepatocytes. *Toxicology in Vitro, 39*, 1-14.

Olsvik, P. A., Skjærven, K. H., & Søfteland, L. (2017b). Metabolic signatures of bisphenol A and genistein in Atlantic salmon liver cells. *Chemosphere, 189*, 730-743.

Olsvik, P. A., & Søfteland, L. (2018). Metabolic effects of p,p′-DDE on Atlantic salmon hepatocytes. *Journal of Applied Toxicology, 38*(4), 489-503.

Oshiumi, H., Tsujita, T., Shida, K., Matsumoto, M., Ikeo, K., & Seya, T. (2003). Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. *Immunogenetics, 54*(11), 791-800.

Øverland, H. S., Pettersen, E. F., Rønneseth, A., & Wergeland, H. I. (2010). Phagocytosis by B-cells and neutrophils in Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.). *Fish & shellfish immunology, 28*(1), 193-204.

Overton, K., Oppedal, F., Stien, L. H., Moltumyr, L., Wright, D. W., & Dempster, T. (2019). Thermal delousing with cold water: Effects on salmon lice removal and salmon welfare. *Aquaculture, 505*, 41-46.

Owen, E. (2019). *These are the leading land-based salmon farms in the world right now*. Aslak Berge. Retrieved 07/09/2021 from https://salmonbusiness.com/these-are-the-leading-land-based-salmon-farms-in-the-world-right-now/

Palma, M., Tavares, L. C., Rito, J., Henriques, L. F., Silva, J. G., Ozório, R., Pardal, M. A., Magnoni, L. J., & Viegas, I. (2019). Metabolic effects of dietary glycerol supplementation in muscle and liver of European seabass and Rainbow trout by ^{1}H-NMR metabolomics. *Metabolites, 9*(10), 202.

Palti, Y., Rodriguez, M. F., Gahr, S. A., Purcell, M. K., Rexroad, C. E., & Wiens, G. D. (2010). Identification, characterization and genetic mapping of TLR1 loci in rainbow trout (*Oncorhynchus mykiss*). *Fish & shellfish immunology, 28*(5), 918-926.

Pan, J., Tristram-Nagle, S., Kučerka, N., & Nagle, J. F. (2008). Temperature dependence of structure, bending rigidity, and bilayer interactions of dioleoylphosphatidylcholine bilayers. *Biophysical journal, 94*(1), 117-124.

Panigrahi, A., Kiron, V., Kobayashi, T., Puangkaew, J., Satoh, S., & Sugita, H. (2004). Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. *Veterinary immunology and immunopathology, 102*(4), 379-388.

Paredes, M., Gonzalez, K., Figueroa, J., & Montiel-Eulefi, E. (2013). Immunomodulatory effect of prolactin on Atlantic salmon (*Salmo salar*) macrophage function. *Fish Physiology and Biochemistry, 39*(5), 1215-1221.

Passantino, L., Altamura, M., Cianciotta, A., Patruno, R., Tafaro, A., Jirillo, E., & Passantino, G. F. (2002). Fish immunology. I. Binding and engulfment of Candida albicans by erythrocytes of rainbow trout (*Salmo gairdneri* Richardson). *Immunopharmacology and immunotoxicology, 24*(4), 665-678.

Passantino, L., Tafaro, A., Altamura, M., Arena, R., Passantino, G. F., & Jirillo, E. (2002). Fish immunology. II. Morphologycal and cytochemical characterization and phagocytic activities of head kidney macrophages from rainbow trout (*Salmo gairdneri* Richardson). *Immunopharmacology and immunotoxicology, 24*(4), 679-691.

Patel, D., & Witt, S. N. (2017). Ethanolamine and phosphatidylethanolamine: partners in health and disease. *Oxidative medicine and cellular longevity, 2017*, 4829180-4829180.

Pattnaik, M. (2020). Effect of Organochlorine Pesticides on Living Organisms and Environment. *ChemicalScienceReviewandLetters, 35*(9), 682-686.

Paulsen, A. M. (2020). *Trichlorfon, a salmon lice pesticide, and the effect on metabolism and inflammation in Atlantic salmon (Salmo salar, L.)* (Publication Number 117) The University of Bergen]. Bergen, Norway.

Paulsen, S. M., Engstad, R. E., & Robertsen, B. (2001). Enhanced lysozyme production in Atlantic salmon (*Salmo salar* L.) macrophages treated with yeast β-glucan and bacterial lipopolysaccharide. *Fish & shellfish immunology, 11*(1), 23-37.

Peña, B., Isla, A., Haussmann, D., & Figueroa, J. (2016). Immunostimulatory effect of salmon prolactin on expression of Toll-like receptors in *Oncorhynchus mykiss* infected with *Piscirickettsia salmonis*. *Fish Physiology and Biochemistry, 42*(2), 509-516.

Peñaranda, M. D., Jensen, I., Tollersrud, L. G., Bruun, J. A., & Jørgensen, J. B. (2019). Profiling the Atlantic Salmon IgM+ B cell surface proteome: novel information on teleost fish B cell protein repertoire and identification of potential B cell markers. *Frontiers in immunology, 10*, 37.

Peng, B., Li, H., & Peng, X. X. (2015b). Functional metabolomics: from biomarker discovery to metabolome reprogramming. *Protein & cell, 6*(9), 628-637.

Peng, B., Ma, Y. M., Zhang, J. Y., & Li, H. (2015c). Metabolome strategy against *Edwardsiella tarda* infection through glucose-enhanced metabolic modulation in tilapias. *Fish & shellfish immunology, 45*(2), 869-876.

Peng, B., Su, Y. B., Li, H., Han, Y., Guo, C., Tian, Y. M., & Peng, X. X. (2015a). Exogenous alanine and/or glucose plus kanamycin kills antibiotic-resistant bacteria. *Cell metabolism, 21*(2), 249-262.

Pens, M. N. (2017). *Atlantic Salmon British Columbia, Canada - Seafood Watch*. M. B. Aquarium. https://www.seafoodwatch.org/-/m/sfw/pdf/reports/s/mba_seafoodwatch_farmedbcsalmon_report.pdf

Peres, H., Santos, S., & Oliva-Teles, A. (2013). Selected plasma biochemistry parameters in gilthead seabream (*Sparus aurata*) juveniles. *Journal of Applied Ichthyology, 29*(3), 630-636.

Petropoulos, I. K., Thompson, K. D., Morgan, A., Dick, J. R., Tocher, D. R., & Bell, J. G. (2009). Effects of substitution of dietary fish oil with a blend of vegetable oils on liver and peripheral blood leucocyte fatty acid composition, plasma prostaglandin E2 and immune parameters in three strains of Atlantic salmon (*Salmo salar*). *Aquaculture nutrition, 15*(6), 596-607.

Pettersen, F. E., Bjerknes, R., & Wergeland, H. I. (2000). Studies of Atlantic salmon (*Salmo salar* L.) blood, spleen and head kidney leucocytes using specific monoclonal antibodies,

immunohistochemistry and flow cytometry. *Fish & shellfish immunology, 10*(8), 695-710.

Pettersen, F. E., Bjørløw, I., Hagland, T. J., & Wergeland, H. I. (2005). Effect of seawater temperature on leucocyte populations in Atlantic salmon post-smolts. *Veterinary immunology and immunopathology, 106*(1-2), 65-76.

Pettersen, F. E., Fyllingen, I., Kavlie, A., Maaseide, N. P., Glette, J., Endresen, C., & Wergeland, H. I. (1995). Monoclonal antibodies reactive with serum IgM and leukocytes from Atlantic salmon (*Salmo salar* L.). *Fish & shellfish immunology, 5*(4), 275-287.

Pettersen, F. E., Ingerslev, H. C., Stavang, V., Egenberg, M., & Wergeland, H. I. (2008). A highly phagocytic cell line TO from Atlantic salmon is CD83 positive and M-CSFR negative, indicating a dendritic-like cell type. *Fish & shellfish immunology, 25*(6), 809-819.

Pettersen, F. E., Ulvenes, M., Melingen, G. O., & Wergeland, H. I. (2003). Peripheral blood and head kidney leucocyte populations during out-of-season (0+) parr-smolt transformation and seawater transfer of Atlantic salmon (*Salmo salar* L.). *Fish & shellfish immunology, 15*(5), 373-385.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research, 29*(9), e45-e45.

Phang, J. M., Liu, W., & Zabirnyk, O. (2010). Proline metabolism and microenvironmental stress. *Annual review of nutrition, 30*, 441-463.

Phelan, P. E., Mellon, M. T., & Kim, C. H. (2005). Functional characterization of full-length TLR3, IRAK-4, and TRAF6 in zebrafish (*Danio rerio*). *Mol Immunol, 42*(9), 1057-1071.

Phu, T. M., Phuong, N. T., Dung, T. T., Hai, D. M., Son, V. N., Rico, A., Clausen, J. H., Madsen, H., Murray, F. J., & Dalsgaard, A. (2016). An evaluation of fish health-management practices and occupational health hazards associated with Pangasius catfish (*Pangasianodon hypophthalmus*) aquaculture in the Mekong Delta, Vietnam. *Aquaculture Research, 47*(9), 2778-2794.

Pierrard, M. A., Roland, K., Kestemont, P., Dieu, M., Raes, M., & Silvestre, F. (2012). Fish peripheral blood mononuclear cells preparation for future monitoring applications. *Analytical biochemistry, 426*(2), 153-165.

Pietsch, C., Vogt, R., Neumann, N. F., & Kloas, W. (2008). Production of nitric oxide by carp (*Cyprinus carpio* L.) kidney leukocytes is regulated by cyclic 3′,5′-adenosine monophosphate. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, 150*(1), 58-65.

Pike, A. W. (1989). Sea lice - Major pathogens of farmed atlantic salmon. *Parasitology Today, 5*(9), 291-297.

Pinu, F. R., Beale, D. J., Paten, A. M., Kouremenos, K., Swarup, S., Schirra, H. J., & Wishart, D. (2019). Systems biology and multi-omics integration: viewpoints from the metabolomics research community. *Metabolites, 9*(4), 76.

Plant, K. P., Harbottle, H., & Thune, R. L. (2005). Poly I:C induces an antiviral state against Ictalurid Herpesvirus 1 and Mx1 transcription in the channel catfish (*Ictalurus punctatus*). *Dev Comp Immunol, 29*(7), 627-635.

References

Podo, F. (1999). Tumour phospholipid metabolism. *NMR in Biomedicine: An International Journal Devoted to the Development and Application of Magnetic Resonance In Vivo, 12*(7), 413-439.

Prado, E., Eklouh-Molinier, C., Enez, F., Blay, C., Dupont-Nivet, M., Labbé, L., Petit, V., Moréac, A., Taupier, G., & Guemene, D. (2020). Prediction of fatty acids in the rainbow trout *Oncorhynchus mykiss*: a Raman scattering spectroscopy approach. 2020 22nd International Conference on Transparent Optical Networks (ICTON),

Pridgeon, J. W., & Klesius, P. H. (2012). Major bacterial diseases in aquaculture and their vaccine development. *Anim. Sci. Rev, 7*, 1-16.

Purcell, M. K., Kurath, G., Garver, K. A., Herwig, R. P., & Winton, J. R. (2004). Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. *Fish Shellfish Immunol, 17*(5), 447-462.

Qiao, Q., Le Manach, S., Sotton, B., Huet, H., Duvernois-Berthet, E., Paris, A., Duval, C., Ponger, L., Marie, A., Blond, A., Mathéron, L., Vinh, J., Bolbach, G., Djediat, C., Bernard, C., Edery, M., & Marie, B. (2016). Deep sexual dimorphism in adult medaka fish liver highlighted by multi-omic approach. *Scientific Reports, 6*(1), 32459.

Qin, Q. W., Ototake, M., Noguchi, K., Soma, G. I., Yokomizo, Y., & Nakanishi, T. (2001). Tumor necrosis factor alpha (TNFα)-like factor produced by macrophages in rainbow trout, *Oncorhynchus mykiss*. *Fish & shellfish immunology, 11*(3), 245-256.

Quiniou, S. M. A., Boudinot, P., & Bengtén, E. (2013). Comprehensive survey and genomic characterization of Toll-like receptors (TLRs) in channel catfish, *Ictalurus punctatus*: identification of novel fish TLRs. *Immunogenetics, 65*(7), 511-530.

Rabah, S. (2005). Light microscope study of *Oncorhynchus kisutch* muscle development. *Egyptian Journal of Aquatic Research, 31*(1), 303-3013.

Rabinovitch, M. (1995). Professional and non-professional phagocytes: an introduction. *Trends in cell biology, 5*(3), 85-87.

Racicot, J. G., Gaudet, M., & Leray, C. (1975). Blood and liver enzymes in rainbow trout (*Salmo gairdneri* Rich.) with emphasis on their diagnostic use: Study of CCl4 toxicity and a case of Aeromonas infection. *Journal of Fish Biology, 7*(6), 825-835.

Radhakrishnan, M. V. (2010). Immunological effect of cadmium in Heteropneustes fossilis Bloch. *Global Veterinaria, 4*(6), 544-547.

Rahimi, R., Hajirezaee, S., & Pordanjani, H. R. (2020). A ^{1}H-NMR-based molecular study of anesthesia in fish. *Aquaculture, 520*, 734995.

Rahman, M. Z., & Begum, B. A. (2005). Serum total protein, albumin and A/G ratio in different grades of protein energy malnutrition. *Mymensingh medical journal: MMJ, 14*(1), 38-40.

Rajab, S. A. S. (2020). *An Integrated Metabolomic and Transcriptomic Approach for Understanding White Muscle Growth Regulation in Hybrid Striped Bass Aquaculture* North Carolina State University].

Rangel-Huerta, O. D., Ivanova, L., Hytterød, S., Moen, A., Uhlig, S., Hagen, A. G., Olstad, K., & Fæste, C. K. (2020). Integrated Omics in salmon skin mucus for determining consequences of chloramine treatment against parasite infestation. *Journal of Integrated Omics, 10*(3), 5-17.

Raposo de Magalhães, C. S. F., Carrilho, R., Schrama, D., Cerqueira, M., da Costa, A. M. R., & Rodrigues, P. M. (2020c). Mid-infrared spectroscopic screening of metabolic alterations in stress-exposed gilthead seabream (*Sparus aurata*). *Scientific Reports, 10*(1), 1-9.

Raposo de Magalhães, C. S. F., Cerqueira, M. A. C., Schrama, D., Moreira, M. J. V., Boonanuntanasarn, S., & Rodrigues, P. M. L. (2020b). A Proteomics and other Omics approach in the context of farmed fish welfare and biomarker discovery. *Reviews in Aquaculture, 12*(1), 122-144.

Raposo de Magalhães, C. S. F., Schrama, D., Farinha, A. P., Revets, D., Kuehn, A., Planchon, S., Rodrigues, P. M., & Cerqueira, M. (2020a). Protein changes as robust signatures of fish chronic stress: a proteomics approach to fish welfare research. *BMC Genomics, 21*, 1-16.

Rauta, P. R., Nayak, B., & Das, S. (2012). Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. *Immunology letters, 148*(1), 23-33.

Ray, C. S., Singh, B., Jena, I., Behera, S., & Ray, S. (2017). Low alkaline phosphatase (ALP) in adult population an indicator of zinc (Zn) and magnesium (Mg) deficiency. *Current Research in Nutrition and Food Science Journal, 5*(3), 347-352.

Readman, G. D., Owen, S. F., Murrell, J. C., & Knowles, T. G. (2013). Do fish perceive anaesthetics as aversive? *PLoS One, 8*(9), e73773.

Rebello, S. C., Rathore, G., Punia, P., Sood, N., & Elangovan, V. (2014). Development and characterization of a continuous macrophage cell line, LRTM, derived from thymus of *Labeo rohita* (Hamilton 1822). *In Vitro Cellular and Developmental Biology - Animal, 50*(1), 22-38.

Rebl, A., & Goldammer, T. (2018). Under control: The innate immunity of fish from the inhibitors' perspective. *Fish & shellfish immunology, 77*, 328-349.

Rebl, A., Korytář, T., Borchel, A., Bochert, R., Strzelczyk, J. E., Goldammer, T., & Verleih, M. (2020). The synergistic interaction of thermal stress coupled with overstocking strongly modulates the transcriptomic activity and immune capacity of rainbow trout (*Oncorhynchus mykiss*). *Scientific Reports, 10*(1), 1-15.

Rebl, A., Siegl, E., Köllner, B., Fischer, U., & Seyfert, H. M. (2007). Characterization of twin toll-like receptors from rainbow trout (*Oncorhynchus mykiss*): evolutionary relationship and induced expression by *Aeromonas salmonicida salmonicida*. *Developmental & Comparative Immunology, 31*(5), 499-510.

Rebl, A., Verleih, M., Köbis, J. M., Kühn, C., Wimmers, K., Köllner, B., & Goldammer, T. (2013). Transcriptome profiling of gill tissue in regionally bred and globally farmed rainbow trout strains reveals different strategies for coping with thermal stress. *Marine Biotechnology, 15*(4), 445-460.

Rebl, A., Verleih, M., Nipkow, M., Altmann, S., Bochert, R., & Goldammer, T. (2018). Gradual and acute temperature rise induces crossing endocrine, metabolic, and immunological pathways in Maraena whitefish (*Coregonus maraena*). *Frontiers in Genetics, 9*, 241.

Rehberger, K., Werner, I., Hitzfeld, B., Segner, H., & Baumann, L. (2017). 20 Years of fish immunotoxicology–what we know and where we are. *Critical reviews in toxicology, 47*(6), 516-542.

References

Řehulka, J. (2000). Influence of astaxanthin on growth rate, condition, and some blood indices of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture, 190*(1), 27-47.

Řehulka, J., Minařík, B., & Řehulková, E. (2004). Red blood cell indices of rainbow trout *Oncorhynchus mykiss* (Walbaum) in aquaculture. *Aquaculture Research, 35*(6), 529-546.

Reid, G. K., Gurney-Smith, H. J., Marcogliese, D. J., Knowler, D., Benfey, T., Garber, A. F., Forster, I., Chopin, T., Brewer-Dalton, K., & Moccia, R. D. (2019). Climate change and aquaculture: considering biological response and resources. *Aquaculture Environment Interactions, 11*, 569-602.

Reitan, L. J., & Thuvander, A. (1991). In vitro stimulation of salmonid leucocytes with mitogens and with *Aeromonas salmonicida*. *Fish & shellfish immunology, 1*(4), 297-307.

Repine, J. E., White, J. G., Clawson, C. C., & Holmes, B. M. (1974). Effects of phorbol myristate acetate on the metabolism and ultrastructure of neutrophils in chronic granulomatous disease. *The Journal of clinical investigation, 54*(1), 83-90.

Revina, O., Revins, V., Cīrule, D., & Valdovska, A. (2019). The effect of dietary β-glucans supplements on the haematological parameters of sea trout. *Vet Med, 1*, 247-251.

Richter, A., & Kolmes, S. A. (2005). Maximum temperature limits for Chinook, coho, and chum salmon, and steelhead trout in the Pacific Northwest. *Reviews in Fisheries Science, 13*(1), 23-49.

Rieger, A. M., & Barreda, D. R. (2011). Antimicrobial mechanisms of fish leukocytes. *Developmental & Comparative Immunology, 35*(12), 1238-1245.

Rios, F. S., Oba, E. T., Fernandes, M. N., Kalinin, A. L., & Rantin, F. T. (2005). Erythrocyte senescence and haematological changes induced by starvation in the neotropical fish traíra, *Hoplias malabaricus* (Characiformes, Erythrinidae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 140*(3), 281-287.

Robertsen, B., Bergan, V., Røkenes, T., Larsen, R., & Albuquerque, A. (2003). Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. *Journal of Interferon & Cytokine Research, 23*(10), 601-612.

Robertsen, B., Trobridge, G., & Leongt, J. A. (1997). Molecular cloning of double-stranded RNA inducible Mx genes from Atlantic salmon (*Salmo salar* L.). *Developmental & Comparative Immunology, 21*(5), 397-412.

Rodriguez, M. F., Wiens, G. D., Purcell, M. K., & Palti, Y. (2005). Characterization of Toll-like receptor 3 gene in rainbow trout (*Oncorhynchus mykiss*). *Immunogenetics, 57*(7), 510-519.

Roland, K., Kestemont, P., Hénuset, L., Pierrard, M. A., Raes, M., Dieu, M., & Silvestre, F. (2013). Proteomic responses of peripheral blood mononuclear cells in the European eel (*Anguilla anguilla*) after perfluorooctane sulfonate exposure. *Aquatic toxicology, 128*, 43-52.

Rombout, J. H. W. M., Huttenhuis, H. B. T., Picchietti, S., & Scapigliati, G. (2005). Phylogeny and ontogeny of fish leucocytes. *Fish & shellfish immunology, 19*(5), 441-455.

Rombout, J. H. W. M., Yang, G., & Kiron, V. (2014). Adaptive immune responses at mucosal surfaces of teleost fish. *Fish & shellfish immunology, 40*(2), 634-643.

References

Romero, A., Manríquez, R., Alvarez, C., Gajardo, C., Vásquez, J., Kausel, G., Monrás, M., Olavarría, V. H., Yáñez, A., & Enríquez, R. (2012). Prolactin-releasing peptide is a potent mediator of the innate immune response in leukocytes from Salmo salar. *Veterinary immunology and immunopathology, 147*(3-4), 170-179.

Rønneseth, A., Haugland, G. T., & Wergeland, H. I. (2013). Flow cytometry detection of infectious pancreatic necrosis virus (IPNV) within subpopulations of Atlantic salmon (*Salmo salar* L.) leucocytes after vaccination and during the time course of experimental infection. *Fish and Shellfish Immunology, 34*(5), 1294-1305.

Rønneseth, A., Pettersen, E. F., & Wergeland, H. I. (2006). Neutrophils and B-cells in blood and head kidney of Atlantic salmon (*Salmo salar* L.) challenged with infectious pancreatic necrosis virus (IPNV). *Fish & shellfish immunology, 20*(4), 610-620.

Roques, S., Deborde, C., Guimas, L., Marchand, Y., Richard, N., Jacob, D., Skiba-Cassy, S., Moing, A., & Fauconneau, B. (2020a). Integrative Metabolomics for Assessing the Effect of Insect (*Hermetia illucens*) Protein Extract on Rainbow Trout Metabolism. *Metabolites, 10*(3), 83.

Roques, S., Deborde, C., Richard, N., Marchand, Y., Larroquet, L., Prigent, S., Skiba-Cassy, S., Moing, A., & Fauconneau, B. (2020b). Proton-NMR Metabolomics of Rainbow Trout Fed a Plant-Based Diet Supplemented with Graded Levels of a Protein-Rich Yeast Fraction Reveal Several Metabolic Processes Involved in Growth. *The Journal of nutrition, 150*(9), 2268-2277.

Roques, S., Deborde, C., Richard, N., Sergent, L., Kurz, F., Skiba-Cassy, S., Fauconneau, B., & Moing, A. (2018b). Characterizing alternative feeds for rainbow trout (*O. mykiss*) by ^{1}H-NMR metabolomics. *Metabolomics, 14*(12), 155.

Roques, S., Deborde, C., Richard, N., Skiba-Cassy, S., Moing, A., & Fauconneau, B. (2018a). ^{1}H-NMR metabolomic investigation of the effect of alternative diets on rainbow trout plasma profiles. World Aquaculture Society 2018, Montpellier, France.

Roques, S., Deborde, C., Richard, N., Skiba-Cassy, S., Moing, A., & Fauconneau, B. (2018c). Metabolomics and fish nutrition: a review in the context of sustainable feed development. *Reviews in Aquaculture, 12*(2), 261-282.

Roy, S., Kumar, V., Kumar, V., & Behera, B. K. (2017). Acute phase proteins and their potential role as an indicator for fish health and in diagnosis of fish diseases. *Protein and peptide letters, 24*(1), 78-89.

Ruyter, B., Sissener, N. H., Østbye, T. K., Simon, C. J., Krasnov, A., Bou, M., Sanden, M., Nichols, P. D., Lutfi, E., & Berge, G. M. (2019). n-3 Canola oil effectively replaces fish oil as a new safe dietary source of DHA in feed for juvenile Atlantic salmon. *British Journal of Nutrition, 122*(12), 1329-1345.

Sadhu, N., Sharma, S. R. K., Joseph, S., Dube, P., & Philipose, K. K. (2014). Chronic stress due to high stocking density in open sea cage farming induces variation in biochemical and immunological functions in Asian seabass (*Lates calcarifer*, Bloch). *Fish Physiology and Biochemistry, 40*(4), 1105-1113.

Sahoo, S., Banu, H., Prakash, A., & Tripathi, G. (2021). Immune System of Fish: An Evolutionary Perspective. In *Antimicrobial Immune Response*. IntechOpen.

Saint-Jean, S. R., & Pérez-Prieto, S. I. (2007). Effects of salmonid fish viruses on Mx gene expression and resistance to single or dual viral infections. *Fish & shellfish immunology, 23*(2), 390-400.

Sakai, D. K. (1981). Separation of lymphocytes from the peripheral blood of rainbow trout and goldfish. *Bulletin of the Japanese Society of Scientific Fisheries, 47*(10), 1281-1288.

Sakai, D. K. (1992). Repertoire of complement in immunological defense mechanisms of fish. *Annual Review of Fish Diseases, 2*, 223-247.

Sakai, M., Kobayashi, M., & Yoshida, T. (1995). Activation of rainbow trout, *Oncorhynchus mykiss*, phagocytic cells by administration of bovine lactoferrin. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 110*(4), 755-759.

Sakai, T., Gotoh, O., Noguchi, M., & Kawatsu, H. (1985). Changes in Bilirubin Contents and Composition in the Bile of Starved and CCl4 Injected Carp Studies on bilirubin metabolism in fish-III. *Fish Pathology, 20*(4), 469-473.

Sakamaki, K., & Satou, Y. (2009). Caspases: evolutionary aspects of their functions in vertebrates. *Journal of Fish Biology, 74*(4), 727-753.

Salati, F., Hamaguchi, M., & Kusuda, R. (1987). Immune response of red sea bream to *Edwardsiella tarda* antigens. *Fish Pathology, 22*(2), 93-98.

Salazar, J. H. (2014). Overview of urea and creatinine. *Laboratory Medicine, 45*(1), e19-e20.

Salinas, I., Lockhart, K., Bowden, T. J., Collet, B., Secombes, C. J., & Ellis, A. E. (2004). An assessment of immunostimulants as Mx inducers in Atlantic salmon (*Salmo salar* L.) parr and the effect of temperature on the kinetics of Mx responses. *Fish & shellfish immunology, 17*(2), 159-170.

Salinas, I., & Miller, R. D. (2015). Comparative phylogeny of the mucosa-associated lymphoid tissue. In: J. Mestecky, W. Strober, M. Russell, H. Cheroutre, B. N. Lambrecht, & B. Kelsall (Eds.), *Mucosal Immunology* (pp 145–159). Elsevier.

Salinas, I., & Parra, D. (2015). Fish mucosal immunity: intestine. *Mucosal Health in Aquaculture*, 135-158.

Salinas, I., Zhang, Y. A., & Sunyer, J. O. (2011). Mucosal immunoglobulins and B cells of teleost fish. *Developmental & Comparative Immunology, 35*(12), 1346-1365.

Salinger, M. J., Diamond, H. J., Behrens, E., Fernandez, D., Fitzharris, B. B., Herold, N., Johnstone, P., Kerckhoffs, H., Mullan, A. B., & Parker, A. K. (2020). Unparalleled coupled ocean-atmosphere summer heatwaves in the New Zealand region: drivers, mechanisms and impacts. *Climatic Change, 162*, 485-506.

Salinger, M. J., Renwick, J., Behrens, E., Mullan, A. B., Diamond, H. J., Sirguey, P., Smith, R. O., Trought, M. C. T., Alexander, L., Cullen, N. J., Fitzharris, B. B., Hepburn, C. D., Parker, A. K., & Sutton, P. J. (2019). The unprecedented coupled ocean-atmosphere summer heatwave in the New Zealand region 2017/18: drivers, mechanisms and impacts. *Environmental Research Letters, 14*(4), 044023.

Salo, H. M., Jokinen, I. E., Markkula, S. E., & Aaltonen, T. M. (2000). Ultraviolet B irradiation modulates the immune system of fish (*Rutilus rutilus*, Cyprinidae) II: Blood. *Photochemistry and Photobiology, 71*(1), 65-70.

Samaï, H. C., Rioult, D., Bado-Nilles, A., Delahaut, L., Jubréaux, J., Geffard, A., Porcher, J. M., & Betoulle, S. (2018). Procedures for leukocytes isolation from lymphoid tissues and

consequences on immune endpoints used to evaluate fish immune status: A case study on roach (*Rutilus rutilus*). *Fish & shellfish immunology, 74*, 190-204.

Samuel, C. E. (2001). Antiviral actions of interferons. *Clinical Microbiology Reviews, 14*(4), 778-809.

Samuelsen, O. B., Lunestad, B. T., Farestveit, E., Grefsrud, E. S., Hannisdal, R., Holmelid, B., Tjensvoll, T., & Agnalt, A. L. (2014). Mortality and deformities in European lobster (*Homarus gammarus*) juveniles exposed to the anti-parasitic drug teflubenzuron. *Aquatic toxicology, 149*, 8-15.

Samuelsson, L. M., Björlenius, B., Förlin, L., & Larsson, D. G. J. (2011). Reproducible ^{1}H-NMR-based metabolomic responses in fish exposed to different sewage effluents in two separate studies. *Environmental science & technology, 45*(4), 1703-1710.

Samuelsson, L. M., Forlin, L., Karlsson, G., Adolfsson-Erici, M., & Larsson, D. G. J. (2006). Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. *Aquat Toxicol, 78*(4), 341-349.

Samuelsson, L. M., & Larsson, D. G. J. (2008). Contributions from metabolomics to fish research. *Molecular BioSystems, 4*(10), 974-979.

Sandnes, K., Lie, Ø., & Waagbø, R. (1988). Normal ranges of some blood chemistry parameters in adult farmed Atlantic salmon, *Salmo salar*. *Journal of Fish Biology, 32*(1), 129-136.

Sangrador-Vegas, A., Martin, S. A. M., O'Dea, P. G., & Smith, T. J. (2000). Cloning and characterization of the rainbow trout (*Oncorhynchus mykiss*) type II interleukin-1 receptor cDNA. *European journal of biochemistry, 267*(24), 7031-7037.

Saray, P., Roytrakul, S., Pangeson, T., & Phetrungnapha, A. (2018). Comparative proteomic analysis of hepatopancreas in *Macrobrachium rosenbergii* responded to Poly (I:C). *Fish & shellfish immunology, 75*, 164-171.

Sarkar, M., Barari, S., Mandal, D., Nandankar, U., Basu, A., Mohanty, T., & Ray, S. (1999). The Effect of Anti-coagulants on the Osmotic Fragility of Erythrocytes in the Yak (*Poephagus grunniens*). *The Veterinary Journal, 157*(1), 91-93.

Sarvin, B., Lagziel, S., Sarvin, N., Mukha, D., Kumar, P., Aizenshtein, E., & Shlomi, T. (2020). Fast and sensitive flow-injection mass spectrometry metabolomics by analyzing sample-specific ion distributions. *Nature communications, 11*(1), 3186.

Savage, A. G. (1983). The ultrastructure of the blood cells of the pike *Esox lucius* L. *Journal of Morphology, 178*(2), 187-206.

Scharsack, J. P., Steinhagen, D., Leibold, W., Rabe, U., Körting, W., & Schuberth, H. J. (2001). Flow cytometric analysis of mitogen-induced activation of rainbow trout (*Oncorhynchus mykiss*) peripheral blood leucocytes. *Journal of Veterinary Medicine, Series B, 48*(5), 331-339.

Schiøtz, B. L., Bækkevold, E. S., Poulsen, L. C., Mjaaland, S., & Gjøen, T. (2009). Analysis of host- and strain-dependent cell death responses during infectious salmon anemia virus infection in vitro. *Virology journal, 6*(1), 91.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C T method. *Nature Protocols, 3*(6), 1101.

Schock, T. B., Newton, S., Brenkert, K., Leffler, J., & Bearden, D. W. (2012). An NMR-based metabolomic assessment of cultured cobia health in response to dietary manipulation. *Food chemistry, 133*(1), 90-101.

Schoggins, J. W., & Rice, C. M. (2011). Interferon-stimulated genes and their antiviral effector functions. *Current opinion in virology, 1*(6), 519-525.

Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-γ: an overview of signals, mechanisms and functions. *Journal of leukocyte biology, 75*(2), 163-189.

Scotland, M. (2019). Scottish Fish Farm Production Survey.

Scott, D. (1984). Origin of the New Zealand sockeye salmon, *Oncorhynchus nerka* (Walbaum). *Journal of the Royal Society of New Zealand, 14*(3), 245-249.

Scott, D., Hewitson, J., & Fraser, J. C. (1978). Origins of Rainbow-trout, *Salmo-gairdneri* Richardson, in New Zealand. *California fish and game, 64*(3), 210-218.

Scott, D., & Poynter, M. (1991). Upper temperature limits for trout in New Zealand and climate change. *Hydrobiologia, 222*(2), 147-151.

Secombes, C. J. (1990). Isolation of salmonid macrophages and analysis of their killing activity. *Techniques in fish immunology.*, 137-154.

Secombes, C. J. (2016). What's new in fish cytokine research? *Fish & shellfish immunology, 53*, 1-3.

Segner, H., Dölle, A., & Böhm, R. (1997). Ketone Body Metabolism in the Carp *Cyprinus carpio*: Biochemical and ^{1}H-NMR Spectroscopical Analysis. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 116*(2), 257-262.

Seibel, H., Baßmann, B., & Rebl, A. (2021). Blood Will Tell: What Hematological Analyses Can Reveal About Fish Welfare. *Frontiers in Veterinary Science, 8*, 194.

Séité, S., Mourier, A., Camougrand, N., Salin, B., Figueiredo-Silva, A. C., Fontagné-Dicharry, S., Panserat, S., & Seiliez, I. (2018). Dietary methionine deficiency affects oxidative status, mitochondrial integrity and mitophagy in the liver of rainbow trout (*Oncorhynchus mykiss*). *Scientific Reports, 8*(1), 10151.

Semple, S. L., Vo, N. T. K., Poynter, S. J., Li, M., Heath, D. D., DeWitte-Orr, S. J., & Dixon, B. (2018). Extracellular dsRNA induces a type I interferon response mediated via class A scavenger receptors in a novel Chinook salmon derived spleen cell line. *Developmental & Comparative Immunology, 89*, 93-101.

Seppola, M., Larsen, A. N., Steiro, K., Robertsen, B., & Jensen, I. (2008). Characterisation and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.). *Molecular Immunology, 45*(4), 887-897.

Sepulcre, M. P., Alcaraz-Pérez, F., López-Muñoz, A., Roca, F. J., Meseguer, J., Cayuela, M. L., & Mulero, V. (2009). Evolution of lipopolysaccharide (LPS) recognition and signaling: Fish TLR4 does not recognize LPS and negatively regulates NF-κB activation. *Journal of Immunology, 182*(4), 1836-1845.

Sha, Z., Wang, L., Sun, L., Chen, Y., Zheng, Y., Xin, M., Li, C., & Chen, S. (2017). Isolation and characterization of monocyte/macrophage from peripheral blood of half smooth tongue sole (*Cynoglossus semilaevis*). *Fish & shellfish immunology, 65*(Supplement C), 256-266.

Sharifuzzaman, S. M., & Austin, B. (2010). Kocuria SM1 controls vibriosis in rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Journal of applied microbiology, 108*(6), 2162-2170.

Shen, G., Huang, Y., Dong, J., Wang, X., Cheng, K. K., Feng, J., Xu, J., & Ye, J. (2018). Metabolic Effect of Dietary Taurine Supplementation on Nile Tilapia (*Oreochromis niloticus*) Evaluated by NMR-Based Metabolomics. *Journal of agricultural and food chemistry, 66*(1), 368-377.

Shen, G., Wang, S., Dong, J., Feng, J., Xu, J., Xia, F., Wang, X., & Ye, J. (2019). Metabolic Effect of Dietary Taurine Supplementation on Grouper (*Epinephelus coioides*): A ^{1}H-NMR-Based Metabolomics Study. *Molecules, 24*(12), 2253.

Shen, Y., Wang, D., Zhao, J., & Chen, X. (2018). Fish red blood cells express immune genes and responses. *Aquaculture and Fisheries, 3*(1), 14-21.

Shepherd, C. J., & Jackson, A. J. (2013). Global fishmeal and fish-oil supply: inputs, outputs and marketsa. *Journal of Fish Biology, 83*(4), 1046-1066.

Sheridan, M. A., Woo, N. Y. S., & Bern, H. A. (1985). Changes in the rates of glycogenesis, glycogenolysis, lipogenesis, and lipolysis in selected tissues of the coho salmon (*Oncorhynchus kisutch*) associated with parr-smolt transformation. *Journal of Experimental Zoology, 236*(1), 35-44.

Shi, Q., Lei, Z., Cheng, G., Li, D., Wang, Q., Luo, S., Yang, H., & Jia, H. (2018). Mitochondrial ROS activate interleukin-1β expression in allergic rhinitis. *Oncology letters, 16*(3), 3193-3200.

Shtrichman, R., & Samuel, C. E. (2001). The role of gamma interferon in antimicrobial immunity. *Current opinion in microbiology, 4*(3), 251-259.

Shumilina, E., Ciampa, A., Capozzi, F., Rustad, T., & Dikiy, A. (2015). NMR approach for monitoring post-mortem changes in Atlantic salmon fillets stored at 0 and 4°C. *Food chemistry, 184*, 12-22.

Shumilina, E., Dykyy, A., & Dikiy, A. (2018). Development of a statistical model to detect quality and storage conditions of Atlantic salmon. *Food chemistry, 258*, 381-386.

Shumilina, E., Møller, I. A., & Dikiy, A. (2020). Differentiation of fresh and thawed Atlantic salmon using NMR metabolomics. *Food chemistry, 314*, 126227.

Shumilina, E., Slizyte, R., Mozuraityte, R., Dykyy, A., Stein, T. A., & Dikiy, A. (2016). Quality changes of salmon by-products during storage: Assessment and quantification by NMR. *Food chemistry, 211*, 803-811.

Silva, T. S., da Costa, A. M. R., Conceicao, L. E. C., Dias, J. P., Rodrigues, P. M. L., & Richard, N. (2014). Metabolic fingerprinting of gilthead seabream (*Sparus aurata*) liver to track interactions between dietary factors and seasonal temperature variations. *PeerJ, 2*, 19, e527.

Simon, S. A., Advani, S., & McIntosh, T. J. (1995). Temperature dependence of the repulsive pressure between phosphatidylcholine bilayers. *Biophysical journal, 69*(4), 1473-1483.

Sirimanapong, W., Thompson, K. D., Kledmanee, K., Thaijongrak, P., Collet, B., Ooi, E. L., & Adams, A. (2014). Optimisation and standardisation of functional immune assays for striped catfish (*Pangasianodon hypophthalmus*) to compare their immune response to

live and heat killed *Aeromonas hydrophila* as models of infection and vaccination. *Fish & shellfish immunology, 40*(2), 374-383.

Skugor, S., Grisdale-Helland, B., Refstie, S., Afanasyev, S., Vielma, J., & Krasnov, A. (2011). Gene expression responses to restricted feeding and extracted soybean meal in Atlantic salmon (*Salmo salar* L.). *Aquaculture nutrition, 17*(5), 505-517.

Slater, C. H., & Schreck, C. B. (1993). Testosterone alters the immune response of Chinook salmon, *Oncorhynchus tshawytscha*. *General and comparative endocrinology, 89*(2), 291-298.

Smith, V. J., Fernandes, J. M. O., Jones, S. J., Kemp, G. D., & Tatner, M. F. (2000). Antibacterial proteins in rainbow trout, *Oncorhynchus mykiss*. *Fish and Shellfish Immunology, 10*(3), 243-260.

Snyder, M., & Gerstein, M. (2003). Defining genes in the genomics era. *Science, 300*(5617), 258-260.

Snyder, S. M., Pulster, E. L., Wetzel, D. L., & Murawski, S. A. (2015). PAH exposure in Gulf of Mexico demersal fishes, post-Deepwater Horizon. *Environmental science & technology, 49*(14), 8786-8795.

Softeland, L., Berntssen, M. H. G., Kirwan, J. A., Storseth, T. R., Viant, M. R., Torstensen, B. E., Waagbo, R., & Olsvik, P. A. (2016). Omega-3 and alpha-tocopherol provide more protection against contaminants in novel feeds for Atlantic salmon (*Salmo salar* L.) than omega-6 and gamma tocopherol. *Toxicology Reports, 3*, 211-224.

Softeland, L., Kirwan, J. A., Hori, T. S. F., Storseth, T. R., Sommer, U., Berntssen, M. H. G., Viant, M. R., Rise, M. L., Waagbo, R., Torstensen, B. E., Booman, M., & Olsvik, P. A. (2014). Toxicological effect of single contaminants and contaminant mixtures associated with plant ingredients in novel salmon feeds. *Food and Chemical Toxicology, 73*, 157-174.

Solanky, K. S., Burton, I. W., MacKinnon, S. L., Walter, J. A., & Dacanay, A. (2005). Metabolic changes in Atlantic salmon exposed to Aeromonas salmonicida detected by 1H-nuclear magnetic resonance spectroscopy of plasma. *Diseases of aquatic organisms, 65*(2), 107-114.

Solem, S. T., Jørgensen, J. B., & Robertsen, B. (1995). Stimulation of respiratory burst and phagocytic activity in Atlantic salmon (*Salmo salar* L.) macrophages by lipopolysaccharide. *Fish & shellfish immunology, 5*(7), 475-491.

Sørensen, K. K., Sveinbjørnsson, B., Dalmo, R. A., Smedsrød, B., & Bertheussen, K. (1997). Isolation, cultivation and characterization of head kidney macrophages from Atlantic cod, *Gadus morhua* L. *Journal of Fish Diseases, 20*(2), 93-107.

Soto, L., Lagos, A. F., Isla, A., Haussmann, D., & Figueroa, J. (2016). Immunostimulatory effects of prolactin on TLR1 and TLR5M in SHK-1 cells infected with Piscirickettsia salmonisÃ‚Â *Diseases of aquatic organisms, 118*(3), 237-245.

Stafford, J., Neumann, N. F., & Belosevic, M. (1999). Inhibition of macrophage activity by mitogen-induced goldfish leukocyte deactivating factor. *Developmental & Comparative Immunology, 23*(7-8), 585-596.

Stafford, J. L., McLauchlan, P. E., Secombes, C. J., Ellis, A. E., & Belosevic, M. (2001). Generation of primary monocyte-like cultures from rainbow trout head kidney leukocytes. *Developmental and Comparative Immunology, 25*(5-6), 447-459.

Statistics Canada. (2017). *Table 32-10-0107-01 Aquaculture, production and value*. Fisheries and Oceans Canada. Retrieved 22/05/2019 from http://www.dfo-mpo.gc.ca/stats/aqua/aqua17-eng.htm

Steiner, K., Heasman, K., Laroche, O., Pochon, X., Preece, M., Bowman, J. P., Walker, S. P., & Symonds, J. E. (2020). The microbiome of Chinook salmon (*Oncorhynchus tshawytscha*) in a recirculation aquaculture system. *Aquaculture*, 736227.

Stenberg, O. K., Holen, E., Piemontese, L., Liland, N. S., Lock, E. J., Espe, M., & Belghit, I. (2019). Effect of dietary replacement of fish meal with insect meal on in vitro bacterial and viral induced gene response in Atlantic salmon (*Salmo salar*) head kidney leukocytes. *Fish & shellfish immunology, 91*, 223-232.

Stenton-Dozey, J. M. E., Heath, P., Ren, J. S., & Zamora, L. N. (2020). New Zealand aquaculture industry: research, opportunities and constraints for integrative multitrophic farming. *New Zealand Journal of Marine and Freshwater Research*, 1-21.

Stilo, F., Bicchi, C., Reichenbach, S. E., & Cordero, C. (2021). Comprehensive two-dimensional gas chromatography as a boosting technology in food-omic investigations. *Journal of Separation Science*.

Strandskog, G., Villoing, S., Iliev, D. B., Thim, H. L., Christie, K. E., & Jørgensen, J. B. (2011). Formulations combining CpG containing oliogonucleotides and poly I: C enhance the magnitude of immune responses and protection against pancreas disease in Atlantic salmon. *Developmental & Comparative Immunology, 35*(11), 1116-1127.

Strober, W. (2001). Trypan Blue Exclusion Test of Cell Viability. In *Current Protocols in Immunology*. John Wiley & Sons, Inc. https://doi.org/10.1002/0471142735.ima03bs111

Stultz, C., Jaramillo, R., Teehan, P., & Dorman, F. (2020). nComprehensive Two-Dimensional Gas Chromatography Thermodynamic Modeling and Selectivity Evaluation for the Separation of Polychlorinated Dibenzo-p-dioxins and Dibenzofurans in Fish Tissue Matrix. *Journal of Chromatography A*, 461311.

Subramaniam, S., Stansberg, C., Olsen, L., Zou, J., Secombes, C. J., & Cunningham, C. (2002). Cloning of a Salmo salar interleukin-1 receptor-like cDNA. *Developmental & Comparative Immunology, 26*(5), 415-431.

Sugamata, R., Suetake, H., Kikuchi, K., & Suzuki, Y. (2009). Teleost B7 expressed on monocytes regulates T cell responses. *The Journal of Immunology, 182*(11), 6799-6806.

Sugimoto, M., Kawakami, M., Robert, M., Soga, T., & Tomita, M. (2012). Bioinformatics tools for mass spectroscopy-based metabolomic data processing and analysis. *Current bioinformatics, 7*(1), 96-108.

Suhre, K., Meisinger, C., Döring, A., Altmaier, E., Belcredi, P., Gieger, C., Chang, D., Milburn, M. V., Gall, W. E., & Weinberger, K. M. (2010). Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. *PLoS One, 5*(11), e13953.

Sun, B., Skjæveland, I., Svingerud, T., Zou, J., Jørgensen, J., & Robertsen, B. (2011). Antiviral activity of salmonid gamma interferon against infectious pancreatic necrosis virus and salmonid alphavirus and its dependency on type I interferon. *Journal of Virology, 85*(17), 9188-9198.

Sun, Y. C., Han, S. C., Yao, M. Z., Wang, Y. M., Geng, L. W., Wang, P., Lu, W. H., & Liu, H. B. (2020). High-throughput metabolomics method based on liquid chromatography-mass spectrometry: insights into the underlying mechanisms of salinity-alkalinity exposures-induced metabolites changes in *Barbus capito*. *Journal of Separation Science*.

Sutton, P. J. H., & Bowen, M. (2019). Ocean temperature change around New Zealand over the last 36 years. *New Zealand Journal of Marine and Freshwater Research, 53*(3), 305-326.

Svingerud, T., Solstad, T., Sun, B., Nyrud, M. L., Kileng, O., Greiner-Tollersrud, L., & Robertsen, B. (2012). Atlantic salmon type I IFN subtypes show differences in antiviral activity and cell-dependent expression: evidence for high IFNb/IFNc-producing cells in fish lymphoid tissues. *J Immunol, 189*(12), 5912-5923.

Swain, B., Samanta, M., Basu, M., Panda, P., Sahoo, B. R., Maiti, N. K., Mishra, B. K., & Eknath, A. E. (2012). Molecular characterization, inductive expression and mechanism of interleukin-10 gene induction in the Indian major carp, catla (*Catla catla*). *Aquaculture Research, 43*(6), 897-907.

Swanson, M. G., Keshari, K. R., Tabatabai, Z. L., Simko, J. P., Shinohara, K., Carroll, P. R., Zektzer, A. S., & Kurhanewicz, J. (2008). Quantification of choline- and ethanolamine-containing metabolites in human prostate tissues using ^{1}H-HR-MAS total correlation spectroscopy. *Magnetic resonance in medicine, 60*(1), 33-40.

Takami, I., Kwon, S. R., Nishizawa, T., & Yoshimizu, M. (2010). Protection of Japanese flounder Paralichthys olivaceus from viral hemorrhagic septicemia (VHS) by Poly(I:C) immunization. *Dis Aquat Organ, 89*(2), 109-115. https://doi.org/10.3354/dao02185

Tanekhy, M. (2016). The role of Toll-like Receptors in innate immunity and infectious diseases of teleost. *Aquaculture Research, 47*(5), 1369-1391.

Tang, M., Xu, X., Li, S., Wu, T., Yan, Y., Gu, W., Wang, W., Jiang, Y., & Meng, Q. (2019). The metabolic responses of crucian carp blood to Cyprinid herpesvirus 2 infection. *Aquaculture, 498*, 72-82.

Taylor, J. F., Needham, M. P., North, B. P., Morgan, A., Thompson, K., & Migaud, H. (2007). The influence of ploidy on saltwater adaptation, acute stress response and immune function following seawater transfer in non-smolting rainbow trout. *General and comparative endocrinology, 152*(2-3), 314-325.

Tekedar, H. C., Blom, J., Kalindamar, S., Nho, S., Karsi, A., & Lawrence, M. L. (2020). Comparative genomics of the fish pathogens *Edwardsiella ictaluri* 93-146 and *Edwardsiella piscicida* C07-087. *Microbial Genomics, 6*(2).

Tewary, A., & Patra, B. C. (2008, Sep). Use of vitamin C as an immunostimulant. Effect on growth, nutritional quality, and immune response of *Labeo rohita* (Ham.). *Fish Physiol Biochem, 34*(3), 251-259.

The Fish Site. (2021). *Artec favourites for 20,000-tonne Korean salmon farm contract*. Hatch Accelerator Holding Limited. Retrieved 07/09/2021 from https://thefishsite.com/articles/artec-favourites-for-20-000-tonne-korean-salmon-farm-contract

Thim, H. L., Iliev, D. B., Christie, K. E., Villoing, S., McLoughlin, M. F., Strandskog, G., & Jørgensen, J. B. (2012). Immunoprotective activity of a Salmonid Alphavirus Vaccine: comparison of the immune responses induced by inactivated whole virus antigen formulations based on CpG class B oligonucleotides and poly I: C alone or combined with an oil adjuvant. *Vaccine, 30*(32), 4828-4834.

Thisse, C., & Zon, L. I. (2002). Organogenesis--heart and bood formation from the Zebrafish point of view. *Science, 295*(5554), 457-462.

Thomas, P. T., & Woo, P. T. K. (1990). In vivo and in vitro cell-mediated immune responses of rainbow trout, Oncorhynchus mykiss (Walbaum), against Cryptobia salmositica Katz, 1951 (Sarcomastigophora: Kinetoplastida). *Journal of Fish Diseases, 13*(6), 423-433.

Thomson, G. M. (1922). *The naturalization of plants and animals in New Zealand* (G. M. Thomson, Ed.). Cambridge University Press.

Thuvander, A. (1989). Cadmium exposure of rainbow trout, *Salmo gairdneri* Richardson: effects on immune functions. *Journal of Fish Biology, 35*(4), 521-529.

Thuvander, A., & Caristein, M. (1991). Sublethal exposure of rainbow trout (*Oncorhynchus mykiss*) to polychlorinated biphenyls: effect on the humoral immune response to *Vibrio anguillarum*. *Fish and Shellfish Immunology, 1*(2), 77-86.

Thuvander, A., Norrgren, L., & Fossum, C. (1987). Phagocytic cells in blood from rainbow trout, *Salmo gairdneri* (Richardson), characterized by flow cytometry and electron microscopy. *Journal of Fish Biology, 31*(2), 197-208.

Thuvander, A., Wiss, E., & Norrgren, L. (1993). Sublethal exposure of rainbow trout (*Oncorhynchus mykiss*) to Clophen A50: effects on cellular immunity. *Fish & shellfish immunology, 3*(2), 107-117.

Tian, H. Y., Zhang, D. D., Xu, C., Wang, F., & Liu, W. B. (2015). Effects of light intensity on growth, immune responses, antioxidant capability and disease resistance of juvenile blunt snout bream *Megalobrama amblycephala*. *Fish & shellfish immunology, 47*(2), 674-680.

Tillitt, D. E., Giesy, J. P., & Fromm, P. O. (1988). In vitro mitogenesis of peripheral blood lymphocytes from rainbow trout. *Comparative Biochemistry and Physiology A, 89*, 25-35.

Todd, P. R. (1987). Salmon farming in New Zealand. Proceedings of the New Zealand Society of Animal Production,

Tomalty, K. M. H., Meek, M. H., Stephens, M. R., Rincón, G., Fangue, N. A., May, B. P., & Baerwald, M. R. (2015). Transcriptional response to acute thermal exposure in juvenile chinook salmon determined by RNAseq. *G3: Genes, Genomes, Genetics, 5*(7), 1335-1349.

Torgersen, J. S., Koppang, E. O., Stien, L. H., Kohler, A., Pedersen, M. E., & Mørkøre, T. (2014). Soft texture of Atlantic salmon fillets is associated with glycogen accumulation. *PLoS One, 9*(1), e85551.

Totland, C., Seland, J. G., Steinkopf, S., & Nerdal, W. (2017). Analysis of wild and farmed salmon using 13 C solid-state NMR and MRI directly on fillet tissue. *Analytical Methods, 9*(8), 1290-1296.

Townsend, C. R. (1996). Invasion biology and ecological impacts of brown trout *Salmo trutta* in New Zealand. *Biological Conservation, 78*(1), 13-22.

Trede, N. S., & Zon, L. I. (1998). Development of T-cells during fish embryogenesis. *Developmental & Comparative Immunology, 22*(3), 253-263.

Tretter, L., & Adam-Vizi, V. (2005). Alpha-ketoglutarate dehydrogenase: a target and generator of oxidative stress. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 360*(1464), 2335-2345.

Tripathi, G., & Verma, P. (2003). Starvation-induced impairment of metabolism in a freshwater catfish. *Zeitschrift für Naturforschung C, 58*(5-6), 446-451.

Tucker, L. E. B. (2014). *Chinook salmon Oncorhynchus tshawytscha. Seafood Watch® Report.* . https://www.mpi.govt.nz/dmsdocument/17464/direct

Turner, M. A., Viant, M. R., Teh, S. J., & Johnson, M. L. (2007). Developmental rates, structural asymmetry, and metabolic fingerprints of steelhead trout (*Oncorhynchus mykiss*) eggs incubated at two temperatures. *Fish Physiology and Biochemistry, 33*(1), 59-72.

Ulvestad, J. S., Kumari, J., Seternes, T., Chi, H., & Dalmo, R. A. (2018). Studies on the effects of LPS, ß-glucan and metabolic inhibitors on the respiratory burst and gene expression in Atlantic salmon macrophages. *Journal of Fish Diseases, 41*(7), 1117-1127.

Unrein, J. R. (2014). Early self-sorting behavior in Chinook salmon is correlated with variation in growth, behavior and morphology later in life.

Unwin, M. J., Field-Dodgson, M. S., Lucas, D. H., & Hawke, S. P. (1989). Experimental releases of coded-wire tagged juvenile chinook salmon (*Oncorhynchus tshawytscha*) from the Glenariffe Salmon Research Station, 1982–83 to 1984–85. *Fisheries Technical Report, 10*.

Uribe, C., Folch, H., Enriquez, R., & Moran, G. (2011). Innate and adaptive immunity in teleost fish: a review. *Veterinarni Medicina, 56*(10), 486-503.

Vallejo, A. N., Ellsaesser, C. F., Miller, N. W., & Clem, L. W. (1991). Spontaneous development of functionally active long-term monocytelike cell lines from channel catfish. *In Vitro Cellular and Developmental Biology - Animal, 27 A*(4), 279-286.

van Beek, T. A. (2021). Low-field benchtop NMR spectroscopy: status and prospects in natural product analysis†. *Phytochemical Analysis, 32*(1), 24-37.

Van Doan, H., Hoseinifar, S. H., Tapingkae, W., Tongsiri, S., & Khamtavee, P. (2016). Combined administration of low molecular weight sodium alginate boosted immunomodulatory, disease resistance and growth enhancing effects of *Lactobacillus plantarum* in Nile tilapia (*Oreochromis niloticus*). *Fish and Shellfish Immunology, 58*, 678-685.

Van Doan, H., Tapingkae, W., Moonmanee, T., & Seepai, A. (2016). Effects of low molecular weight sodium alginate on growth performance, immunity, and disease resistance of tilapia, *Oreochromis niloticus*. *Fish and Shellfish Immunology, 55*, 186-194.

van Meer, G., Voelker, D. R., & Feigenson, G. W. (2008). Membrane lipids: where they are and how they behave. *Nature reviews Molecular cell biology, 9*(2), 112-124.

Van Scoy, A. R., Lin, C. Y., Anderson, B. S., Philips, B. M., Martin, M. J., McCall, J., Todd, C. R., Crane, D., Sowby, M. L., Viant, M. R., & Tjeerdema, R. S. (2010). Metabolic responses produced by crude versus dispersed oil in Chinook salmon pre-smolts via NMR-based metabolomics. *Ecotoxicology and environmental safety, 73*(5), 710-717.

Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science, 324*(5930), 1029-1033.

References

Vargas-Chacoff, L., Arjona, F. J., Ruiz-Jarabo, I., García-Lopez, A., Flik, G., & Mancera, J. M. (2020). Water temperature affects osmoregulatory responses in gilthead sea bream (*Sparus aurata* L.). *Journal of thermal biology, 88*, 102526.

Vargas-Chacoff, L., Regish, A. M., Weinstock, A., & McCormick, S. D. (2018). Effects of elevated temperature on osmoregulation and stress responses in Atlantic salmon *Salmo salar* smolts in fresh water and seawater. *Journal of Fish Biology, 93*(3), 550-559.

Vázquez, R. I., Ita-Nagy, D., & Kahhat, R. (2021). Microplastics in fisheries and aquaculture: implications to food sustainability and safety. *Current Opinion in Green and Sustainable Chemistry*, 100464.

Viant, M. R., Pincetich, C. A., & Tjeerdema, R. S. (2006). Metabolic effects of dinoseb, diazinon and esfenvalerate in eyed eggs and alevins of Chinook salmon (Oncorhynchus tshawytscha) determined by ^{1}H-NMR metabolomics. *Aquatic toxicology, 77*(4), 359-371.

Viant, M. R., Werner, I., Rosenblum, E. S., Gantner, A. S., Tjeerdema, R. S., & Johnson, M. L. (2003). Correlation between heat-shock protein induction and reduced metabolic condition in juvenile steelhead trout (*Oncorhynchus mykiss*) chronically exposed to elevated temperature. *Fish Physiology and Biochemistry, 29*(2), 159-171.

Vikeså, V., Nankervis, L., & Hevrøy, E. M. (2017). High dietary energy level stimulates growth hormone receptor and feed utilization in large Atlantic salmon (*Salmo salar* L.) under hypoxic conditions. *Aquaculture nutrition, 23*(6), 1193-1203.

Villas-Boas, S. G., Nielsen, J., Smedsgaard, J., Hansen, M. A. E., & Roessner-Tunali, U. (2007). *Metabolome analysis: an introduction* (Vol. 24). John Wiley & Sons.

Villas-Boas, S. G., Smart, K. F., Sivakumaran, S., & Lane, G. A. (2011). Alkylation or Silylation for Analysis of Amino and Non-Amino Organic Acids by GC-MS? *Metabolites, 1*(1), 3-20.

Vinagre, C., Madeira, D., Narciso, L., Cabral, H. N., & Diniz, M. S. (2012). Impact of climate change on coastal versus estuarine nursery areas: cellular and whole-animal indicators in juvenile seabass *Dicentrarchus labrax*. *Marine Ecology Progress Series, 464*, 237-243.

Volkoff, H. (2019). Fish as models for understanding the vertebrate endocrine regulation of feeding and weight. *Molecular and Cellular Endocrinology, 497*, 110437.

Vosylienė, M. Z. (1999). The effect of heavy metals on haematological indices of fish (survey). *Acta Zoologica Lituanica, 9*(2), 76-82.

Waagbø, R. (1994). The impact of nutritional factors on the immune system in Atlantic salmon, *Salmo salar* L.: a review. *Aquaculture Research, 25*(2), 175-197.

Wade, N. M., Clark, T. D., Maynard, B. T., Atherton, S., Wilkinson, R. J., Smullen, R. P., & Taylor, R. S. (2019). Effects of an unprecedented summer heatwave on the growth performance, flesh colour and plasma biochemistry of marine cage-farmed Atlantic salmon (*Salmo salar*). *Journal of thermal biology, 80*, 64-74.

Wagner, L., Gómez-Requeni, P., Moazzami, A. A., Lundh, T., Vidakovic, A., Langeland, M., Kiessling, A., & Pickova, J. (2019). ^{1}H-NMR-Based Metabolomics and Lipid Analyses Revealed the Effect of Dietary Replacement of Microbial Extracts or Mussel Meal with Fish Meal to Arctic Charr (*Salvelinus alpinus*). *Fishes, 4*(3), 46.

Wagner, L., Trattner, S., Pickova, J., Gomez-Requeni, P., & Moazzami, A. A. (2014). ^{1}H-NMR-based metabolomics studies on the effect of sesamin in Atlantic salmon (*Salmo salar*). *Food chemistry, 147*, 98-105.

Wang, H., Wang, J., Ning, C., Zheng, X., Fu, J., Wang, A., Zhang, Q., & Liu, J. F. (2017). Genome-wide DNA methylation and transcriptome analyses reveal genes involved in immune responses of pig peripheral blood mononuclear cells to poly I:C. *Scientific Reports, 7*(1), 9709-9709.

Wang, J., He, R. Z., Lu, G. L., Luo, H. L., Lu, D. Q., & Li, A. X. (2018b). Vaccine-induced antibody level as the parameter of the influence of environmental salinity on vaccine efficacy in Nile tilapia. *Fish & shellfish immunology, 82*, 522-530.

Wang, J., Lu, D. Q., Jiang, B., Luo, H. L., Lu, G. L., & Li, A. X. (2018a). The effect of intermittent hypoxia under different temperature on the immunomodulation in *Streptococcus agalactiae* vaccinated Nile tilapia (*Oreochromis niloticus*). *Fish & shellfish immunology, 79*, 181-192.

Wang, Q., Cheng, L., Liu, J., Li, Z., Xie, S., & De Silva, S. S. (2015). Freshwater aquaculture in PR China: trends and prospects. *Reviews in Aquaculture, 7*(4), 283-302.

Wang, R., Neumann, N. F., Shen, Q., & Belosevic, M. (1995). Establishment and characterization of a macrophage cell line from the goldfish. *Fish & shellfish immunology, 5*(5), 329-346.

Wang, S. Y., Fodjo, E. K., Kong, C., & Yu, H. J. (2020). Multi-Residue Screening of Pesticides in Aquaculture Waters through Ultra-High-Performance Liquid Chromatography-Q/Orbitrap Mass Spectrometry. *Water, 12*(5), 1238.

Wang, T., Hu, Y., Wangkahart, E., Liu, F., Wang, A., Zahran, E., Maisey, K. R., Liu, M., Xu, Q., Imarai, M., & Secombes, C. J. (2018). Interleukin (IL)-2 is a key regulator of T helper 1 and T helper 2 cytokine expression in fish: functional characterization of two divergent IL2 paralogs in salmonids. *Frontiers in immunology, 9*(1683).

Wang, W., Wu, Z., Dai, Z., Yang, Y., Wang, J., & Wu, G. (2013). Glycine metabolism in animals and humans: implications for nutrition and health. *Amino Acids, 45*(3), 463-477.

Warr, G. W., & Simon, R. C. (1983). The mitogen response potential of lymphocytes from the rainbow trout (*Salmo gairdneri*) re-examined. *Developmental & Comparative Immunology, 7*(2), 379-384.

Watson, A. M., Napolitano, M. P., Schock, T. B., Bowden, J. A., Frost, J., Yost, J., & Denson, M. R. (2020). Evaluation of graded levels of soy oil as a fish oil replacement in high soy protein feeds for juvenile red drum, *Sciaenops ocellatus*. *Aquaculture, 529*, 735627.

Watson, L. J., Shechmeister, I. L., & Jackson, L. L. (1963). The hematology of goldfish, *Carassius auratus*. *Cytologia, 28*(2), 118-130.

Wayne, R. (2018). *ImageJ Java application* . In (Version Version 1.52a) National Institutes of Health, USA. https://imagej.nih.gov/ij/

Weinstein, I. B., Lee, L. S., Fisher, P. B., Mufson, A., & Yamasaki, H. (1979). Action of phorbol esters in cell culture: mimicry of transformation, altered differentiation, and effects on cell membranes. *Journal of supramolecular structure, 12*(2), 195-208.

Weissmann, G., Troll, W., van Duuren, B. L., & Sessa, G. (1968). Studies on lysosomes—X: Effects of tumor-promoting agents upon biological and artificial membrane systems. *Biochemical Pharmacology, 17*(12), 2421-2434.

Wen, C. M. (2016). Development and characterization of a cell line from tilapia head kidney with melanomacrophage characteristics. *Fish and Shellfish Immunology, 49*, 442-449.

Wen, X., Hu, Y., Zhang, X., Wei, X., Wang, T., & Yin, S. (2019). Integrated application of multi-omics provides insights into cold stress responses in pufferfish Takifugu fasciatus. *BMC Genomics, 20*(1), 563.

Wergeland, H. I., & Jakobsen, R. A. (2001). A salmonid cell line (TO) for production of infectious salmon anaemia virus (ISAV). *Diseases of aquatic organisms, 44*(3), 183-190.

Wessel, Ø., Olsen, C. M., Rimstad, E., & Dahle, M. K. (2015). Piscine orthoreovirus (PRV) replicates in Atlantic salmon (*Salmo salar* L.) erythrocytes ex vivo. *Veterinary Research, 46*(1), 26.

Weyts, F. A. A., Rombout, J. H. W. M., Flik, G., & Verburg-Van Kemenade, B. M. L. (1997). A common carp (*Cyprinus carpio* L.) leucocyte cell line shares morphological and functional characteristics with macrophages. *Fish & shellfish immunology, 7*(2), 123-133.

Whang, I., Lee, Y., Lee, S., Oh, M. J., Jung, S. J., Choi, C. Y., Lee, W. S., Kim, H. S., Kim, S. J., & Lee, J. (2011). Characterization and expression analysis of a goose-type lysozyme from the rock bream *Oplegnathus fasciatus*, and antimicrobial activity of its recombinant protein. *Fish & shellfish immunology, 30*(2), 532-542.

Whitfield, P. D., German, A. J., & Noble, P. J. M. (2004). Metabolomics: an emerging post-genomic tool for nutrition. *British Journal of Nutrition, 92*(4), 549-555.

Whitney, J. E., Al-Chokhachy, R., Bunnell, D. B., Caldwell, C. A., Cooke, S. J., Eliason, E. J., Rogers, M., Lynch, A. J., & Paukert, C. P. (2016). Physiological basis of climate change impacts on North American inland fishes. *Fisheries, 41*(7), 332-345.

Whyte, S. K. (2007). The innate immune response of finfish–a review of current knowledge. *Fish & shellfish immunology, 23*(6), 1127-1151.

Wilson, R. P. (2003). Amino Acids and Proteins. In J. E. Halver & R. W. Hardy (Eds.), *Fish Nutrition (Third Edition)* (pp. 143-179). Academic Press.

Wishart, D. S., Feunang, Y. D., Marcu, A., Guo, A. C., Liang, K., Vazquez-Fresno, R., Sajed, T., Johnson, D., Li, C., Karu, N., Sayeeda, Z., Lo, E., Assempour, N., Berjanskii, M., Singhal, S., Arndt, D., Liang, Y., Badran, H., Grant, J., Serra-Cayuela, A., Liu, Y., Mandal, R., Neveu, V., Pon, A., Knox, C., Wilson, M., Manach, C., & Scalbert, A. (2018,). HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Research, 46*(D1), D608-d617.

Witeska, M. (2013). Erythrocytes in teleost fishes: a review. *Zoology and Ecology, 23*(4), 275-281.

Witeska, M. (2015). Anemia in teleost fishes. *Bull. Eur. Ass. Fish Pathol, 35*(4), 149.

Workenhe, S. T., Kibenge, M. J. T., Wright, G. M., Wadowska, D. W., Groman, D. B., & Kibenge, F. S. B. (2008). Infectious salmon anaemia virus replication and induction of alpha interferon in Atlantic salmon erythrocytes. *Virology journal, 5*(1), 36.

Worldometers. (2019). *Current World Population*. Retrieved 06 April 2019 from http://www.worldometers.info/world-population/

References

Wu, D., Sanin, D. E., Everts, B., Chen, Q., Qiu, J., Buck, M. D., Patterson, A., Smith, A. M., Chang, C. H., & Liu, Z. (2016). Type 1 interferons induce changes in core metabolism that are critical for immune function. *Immunity, 44*(6), 1325-1336.

Wu, L., Kong, L., Yang, Y., Bian, X., Wu, S., Li, B., Yin, X., Mu, L., Li, J., & Ye, J. (2019). Effects of cell differentiation on the phagocytic activities of IgM+ B cells in a teleost fish. *Frontiers in immunology, 10*, 2225.

Wu, M., Guo, L., Zhu, K. C., Guo, H. Y., Liu, B., Jiang, S. G., & Zhang, D. C. (2018). Genomic structure and molecular characterization of Toll-like receptors 1 and 2 from golden pompano *Trachinotus ovatus* (Linnaeus, 1758) and their expression response to three types of pathogen-associated molecular patterns. *Developmental & Comparative Immunology, 86*, 34-40.

Wu, N., Yang, M., Gaur, U., Xu, H., Yao, Y., & Li, D. (2016). Alpha-Ketoglutarate: Physiological Functions and Applications. *Biomol Ther (Seoul), 24*(1), 1-8.

Wu, P. F., Long, L. H., Zeng, J. H., Guan, X. L., Zhou, J., Jin, Y., Ni, L., Wang, F., Chen, J. G., & Xie, N. (2012). Protection of l-methionine against H2O2-induced oxidative damage in mitochondria. *Food and Chemical Toxicology, 50*(8), 2729-2735.

Xiao, L., Lin, H., Yang, M., Chen, S., An, W., Wu, X., Liu, H., Li, D., Yan, Y., & Hu, J. (2018). The first instance of HPR-deleted ISAV detection in eviscerated, fresh salmon at a Chinese entry-exit port. *Aquaculture, 485*, 220-224.

Xu, D., Wang, J., Guo, C., Peng, X. X., & Li, H. (2019). Elevated biosynthesis of palmitic acid is required for zebrafish against *Edwardsiella tarda* infection. *Fish & shellfish immunology, 92*, 508-518.

Xu, G., Du, F., Li, Y., Nie, Z., & Xu, P. (2016). Integrated application of transcriptomics and metabolomics yields insights into population-asynchronous ovary development in Coilia nasus. *Scientific Reports, 6*, 31835.

Xu, H., Zhang, X., Li, H., Li, C., Huo, X. J., Hou, L. P., & Gong, Z. (2018). Immune response induced by major environmental pollutants through altering neutrophils in zebrafish larvae. *Aquatic toxicology, 201*, 99-108.

Xu, Y., A., S. D., Parken, C. K., Ritchie, L. M., Patterson, D. A., & Fu, C. (2020). Climate effects on size-at-age and growth rate of Chinook Salmon (*Oncorhynchus tshawytscha*) in the Fraser River, Canada. *Fisheries Oceanography, 29*(5), 381-395.

Yada, T. (2009). Effects of insulin-like growth factor-1 on non-specific immune functions in rainbow trout. *Zoological Science, 26*(5), 338-343.

Yada, T., Abe, M., & Miyamoto, K. (2019). Down-regulation of corticosteroid receptor in leucocytes of stressed rainbow trout. *General and comparative endocrinology, 280*, 54-61.

Yada, T., Azuma, T., & Takagi, Y. (2001). Stimulation of non-specific immune functions in seawater-acclimated rainbow trout, *Oncorhynchus mykiss*, with reference to the role of growth hormone. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology, 129*(2-3), 695-701.

Yada, T., Muto, K., Azuma, T., & Ikuta, K. (2004). Effects of prolactin and growth hormone on plasma levels of lysozyme and ceruloplasmin in rainbow trout. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology, 139*(1-3), 57-63.

Yang, M. J., Xu, D., Yang, D. X., Li, L., Peng, X. X., Chen, Z. G., & Li, H. (2020). Malate enhances survival of zebrafish against *Vibrio alginolyticus* infection in the same manner as taurine. *Virulence, 11*(1), 349-364.

Yang, Y., & Sauve, A. A. (2016). NAD(+) metabolism: Bioenergetics, signaling and manipulation for therapy. *Biochimica et biophysica acta, 1864*(12), 1787-1800.

Yao, J., Wang, Z., Guo, L., Xu, X., Liu, L., Xu, L., Song, S., Xu, C., & Kuang, H. (2020). Advances in immunoassays for organophosphorus and pyrethroid pesticides. *TrAC Trends in Analytical Chemistry, 131*, 116022.

Yarahmadi, P., Miandare, H. K., Fayaz, S., & Caipang, C. M. A. (2016). Increased stocking density causes changes in expression of selected stress- and immune-related genes, humoral innate immune parameters and stress responses of rainbow trout (*Oncorhynchus mykiss*). *Fish & shellfish immunology, 48*, 43-53.

Yasumiishi, E. M., Farley, E. V. J., Maselko, J., Aydin, K. Y., Kearney, K. A., Hermann, A. J., Ruggerone, G. T., Howard, K. G., & Strasburger, W. W. (2019). Differential north–south response of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) marine growth to ecosystem change in the eastern Bering Sea, 1974–2010. *ICES Journal of Marine Science, 77*(1), 216-229.

Yi, S., Liu, L. F., Zhou, L. F., Zhao, B. W., Wang, W. M., & Gao, Z. X. (2020). Screening of Biomarkers Related to Ovarian Maturation and Spawning in Blunt Snout Bream (*Megalobrama amblycephala*) Based on Metabolomics and Transcriptomics. *Marine Biotechnology, 22*, 180-193.

Younes, A. F., Cerrato, R. M., & Nye, J. A. (2020). Overwintering survivorship and growth of young-of-the-year black sea bass Centropristis striata. *PLoS One, 15*(8), e0236705.

Young, T., & Alfaro, A. C. (2018). Metabolomic strategies for aquaculture research: a primer. *Reviews in Aquaculture, 10*(1), 26-56.

Young, T., Kesarcodi-Watson, A., Alfaro, A. C., Merien, F., Nguyen, T. V., Mae, H., Le, D. V., & Villas-Boas, S. (2017). Differential expression of novel metabolic and immunological biomarkers in oysters challenged with a virulent strain of OsHV-1. *Dev Comp Immunol, 73*, 229-245.

Young, T., Walker, S. P., Alfaro, A. C., Fletcher, L. M., Murray, J. S., Lulijwa, R., & Symonds, J. (2019). Impact of acute handling stress, anaesthesia, and euthanasia on fish plasma biochemistry: implications for veterinary screening and metabolomic sampling. *Fish Physiology and Biochemistry, 45*(4), 1485-1494.

Yu, T. C., Sinnhuber, R. O., & Hendricks, J. D. (1979). Effect of steroid hormones on the growth of juvenile coho salmon *(Oncorhynchus kisutch*). *Aquaculture, 16*(4), 351-359.

Yudkoff, M., Daikhin, Y., Nissim, I., Horyn, O., Luhovyy, B., Luhovyy, B., Lazarow, A., & Nissim, I. (2005). Brain amino acid requirements and toxicity: the example of leucine. *J Nutr, 135*(6 Suppl), 1531s-1538s.

Yui, M. A., & Kaattari, S. L. (1987). *Vibrio anguillarum* antigen stimulates mitogenesis and polyclonal activation of salmonid lymphocytes. *Developmental & Comparative Immunology, 11*(3), 539-549.

Zahl, I. H., Kiessling, A., Samuelsen, O. B., & Olsen, R. E. (2010). Anesthesia induces stress in Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*). *Fish Physiology and Biochemistry, 36*(3), 719-730.

Zahl, I. H., Samuelsen, O., & Kiessling, A. (2012). Anaesthesia of farmed fish: implications for welfare. *Fish Physiology and Biochemistry, 38*(1), 201-218.

Zaidi, B. R., & Imam, S. H. (2008). Biodegradability. In S. E. Jørgensen & B. D. Fath (Eds.), *Encyclopedia of Ecology* (pp. 357-366). Academic Press.

Zamorano, R. M. (1991). Salmon farming in Chile. Proceedings of the Special Session on Salmonid Aquaculture, World Aquaculture Society, February 16, 1989, , Los Angeles, USA.

Zapata, A., Diez, B., Cejalvo, T., Gutiérrez-de Frías, C., & Cortés, A. (2006). Ontogeny of the immune system of fish. *Fish & shellfish immunology, 20*(2), 126-136.

Zelikoff, J. T., Bowser, D., Squibb, K. S., & Frenkel, K. (1995). Immunotoxicity of low level cadmium exposure in fish: an alternative animal model for immunotoxicological studies. *Journal of Toxicology and Environmental Health, Part A Current Issues, 45*(3), 235-248.

Zeng, J., Guo, Y., Han, Y., Li, Z., Yang, Z., Chai, Q., Wang, W., Zhang, Y., & Fu, C. (2021). A Review of the Discriminant Analysis Methods for Food Quality Based on Near-Infrared Spectroscopy and Pattern Recognition. *Molecules, 26*(3), 749.

Zha, H., Lewis, G., Alfaro, A. C., Wang, S., Dong, Y., Grandiosa, R., & Jeffs, A. (2017). Immune status of the spiny lobster *Jasus edwardsii* with tail fan necrosis. *Diseases of aquatic organisms, 126*(3), 229-238.

Zhang, J., Kong, X., Zhou, C., Li, L., Nie, G., & Li, X. (2014). Toll-like receptor recognition of bacteria in fish: Ligand specificity and signal pathways. *Fish & shellfish immunology, 41*(2), 380-388.

Zhang, J. M., & An, J. (2007). Cytokines, Inflammation and Pain. *International anesthesiology clinics, 45*(2), 27-37.

Zhang, X. T., Zhang, G. R., Shi, Z. C., Yuan, Y. J., Zheng, H., Lin, L., Wei, K. J., & Ji, W. (2017). Expression analysis of nine Toll-like receptors in yellow catfish (*Pelteobagrus fulvidraco*) responding to *Aeromonas hydrophila* challenge. *Fish & shellfish immunology, 63*, 384-393.

Zhang, Y. B., & Gui, J. F. (2012). Molecular regulation of interferon antiviral response in fish. *Developmental & Comparative Immunology, 38*(2), 193-202.

Zhong, Z., Wheeler, M. D., Li, X., Froh, M., Schemmer, P., Yin, M., Bunzendaul, H., Bradford, B., & Lemasters, J. J. (2003). L-Glycine: a novel antiinflammatory, immunomodulatory, and cytoprotective agent. *Current Opinion in Clinical Nutrition & Metabolic Care, 6*(2), 229-240.

Zhou, L. F., Zhao, B. W., Guan, N. N., Wang, W. M., & Gao, Z. X. (2017). Plasma metabolomics profiling for fish maturation in blunt snout bream. *Metabolomics, 13*(4), 40.

Zhou, Z. X., Zhang, B. C., & Sun, L. (2014). Poly(I:C) induces antiviral immune responses in Japanese flounder (*Paralichthys olivaceus*) that require TLR3 and MDA5 and is negatively regulated by Myd88. *PLoS One, 9*(11), e112918-e112918.

Zhu, L. Y., Nie, L., Zhu, G., Xiang, L. X., & Shao, J. Z. (2013). Advances in research of fish immune-relevant genes: A comparative overview of innate and adaptive immunity in teleosts. *Developmental & Comparative Immunology, 39*(1), 39-62.

References

Zhu, W., Zhang, Y., Zhang, J., Yuan, G., Liu, X., Ai, T., & Su, J. (2019). Astragalus polysaccharides, chitosan and poly(I:C) obviously enhance inactivated *Edwardsiella ictaluri* vaccine potency in yellow catfish *Pelteobagrus fulvidraco*. *Fish & shellfish immunology, 87*, 379-385.

Zhu, Y., Wu, J., Leng, X., Du, H., Wu, J., He, S., Luo, J., Liang, X., Liu, H., & Wei, Q. (2020). Metabolomics and gene expressions revealed the metabolic changes of lipid and amino acids and the related energetic mechanism in response to ovary development of Chinese sturgeon (*Acipenser sinensis*). *PLoS One, 15*(6), e0235043.

Zou, J., Carrington, A., Collet, B., Dijkstra, J. M., Yoshiura, Y., Bols, N., & Secombes, C. J. (2005). Identification and bioactivities of IFN-γ in rainbow trout *Oncorhynchus mykiss*: the first Th1-type cytokine characterized functionally in fish. *The Journal of Immunology, 175*(4), 2484.

Zou, J., Grabowski, P. S., Cunningham, C., & Secombes, C. J. (1999). Molecular cloning of interleukin 1β from Rainbow trout *Oncorhynchus mykiss* reveals no evidence of an ICE cut site. *Cytokine, 11*(8), 552-560.

Zou, J., Wang, T., Hirono, I., Aoki, T., Inagawa, H., Honda, T., Soma, G. I., Ototake, M., Nakanishi, T., & Ellis, A. E. (2002). Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*. *Developmental & Comparative Immunology, 26*(2), 161-172.