Showcasing metabolomics in aquaculture: a review

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Abstract

Aquaculture production is currently challenged to meet the growing demands for seafood protein throughout the world. To achieve this growth in an efficient, safe and sustainable manner, novel tools and applications will need to be incorporated at each step of the production line. A variety of 'omics' (e.g., transcriptomics, proteomics, metabolomics) applications have already begun to emerge in aquaculture research with extreme success. A promising new 'omics' approach is metabolomics, which aims to use metabolite profiles to identify biomarkers indicative of physiological responses of living samples (e.g., whole organism, tissues, cells) to environmental or culture conditions. One of the benefits of this approach is that it uses a broad scan of biological conditions to identify often unexpected problem or risk areas to focus management attention. In this contribution, we have selected relevant research examples to showcase the applications of metabolomics in aquaculture in four major areas: hatchery production, nutrition and diet, disease and immunology, and food safety and quality. The novelty of this approach is highlighted by the fact that we have cited the majority of published papers in this field, and these are all recent (within the last decade) contributions.

Key words: Aquaculture, Biotechnology, Metabolism, Metabolomics, Omics

Global aquaculture

We live in a world of increasing environmental impacts and diminishing food resources. In addition, our growing population places more and more demands on food supplies, including animal-based protein. Undoubtedly, seafood supplies and demands have increased exponentially in recent years, and are expected to continue to grow in the years to come. This growth is mainly focused on aquaculture, since global wild-caught fish resources have declined due to overexploitation, and are unlikely to recover in the near future. Thus, over 50% of fish and seaweed food supplies currently come from aquaculture, amounting to about 90 million tonnes and worth US$144 billion in 2012 (FAO 2014). It is estimated that production will need to almost double by 2050, if we are to meet the growing demands (Waite et al. 2014). There have been many suggested scenarios as to how this growth could be achieved (Gjedrem et al. 2012; Merino et al. 2012; Bhattacharyya et al. 2015; Jobling 2015), but it is clear that future production will need to improve in efficiency, safety, sustainability and environmental performance (Edwards 2015; Jones et al. 2015). Regardless, technological innovation will be required to enhance reproduction and conditioning, larval rearing, disease diagnostics and immunology, nutrition and feed formulation, cultivation systems performance, and food safety and quality (Browdy et al. 2012). Recent biotechnological advances have produced improvements in all of these areas, and new tools and approaches proliferate in the literature daily. One of the most promising biotechnological areas is that of 'omics' approaches. With rapidly expanding capabilities and diversity of analytical platforms and computational analysis, 'omics' applications are likely to revolutionize aquaculture research and development in the near future.

'Omics' approaches

During the last century, biological research has benefited from the knowledge generated around the genome and a myriad of applications. A strong emphasis on the idea that genes direct all processes within a cell, and thus the organism, was based on the assumption that proteins, via mRNA, and then metabolites are synthesized in a hierarchical manner when genes are activated. However, this idea has now been superseded by the knowledge that many metabolic processes are controlled by complex feedback mechanisms and regulatory networks involving post-translational protein modifications and metabolite fluxes, which are de-coupled from gene expression (Saroglia & Liu 2012; Chubukov et al. 2013; Feussner & Polle 2015). At the heart of this new thinking are a range of newly created fields under the 'omics' banner, which promise to revolutionize biotechnological research across all life-based investigations. 'Omics' is a term which refers to the collective technologies used to explore the structures, functions, relationships and dynamics of various biomolecules within the cells of organisms (Coughlin 2014). Recent advances in technology now enable us to use non-targeted 'omics'-based approaches to identify the global set of gene products (transcripts, proteins and metabolites) within a biological sample, rather than single products at a time. The exploratory nature of these approaches means that novel hypotheses are often generated rather than validated. At the same time, one of the advantages of 'omics' is that often unexpected information is revealed, leading to high innovation and discovery in a very efficient manner.

New 'omics' fields are continually being generated, but the bulk of research from genotype to phenotype falls within genomics, transcriptomics, proteomics and metabolomics (Fig. 1). Genomics aims to sequence, assemble and analyze the structure and function of a given genome (Primrose & Twyman 2003). The DNA sequence provides information about what could potentially happen in the organism if it was to express particular genes. Transcriptomics focuses on sequencing the RNA transcripts that are produced by the genome and identifies which genes are actually being expressed under specific biological circumstances (McGettigan 2013). This knowledge provides information about what appears to be happening in the organism at a given time. Proteomics is the study of proteins as a way to elucidate the structure, function and regulation of biological systems (Mishra 2010). This includes proteins which are constructed by direct instruction from genes, but also encompasses the structure and functions of proteins which occur due to post-translational protein modifications (Olsen & Mann). Since enzymes are involved in the operation and regulation of metabolic pathways in every living organism, their expressions reflect accurate responses to cellular and environmental conditions. This information provides understanding about what makes cellular processes happen. Metabolomics (the study of small molecules) represents a better picture of an organism's phenotype, since metabolites are most sensitive to environmental changes and provide information about what is actually happening on a metabolic and physiological level (Patti et al. 2012).

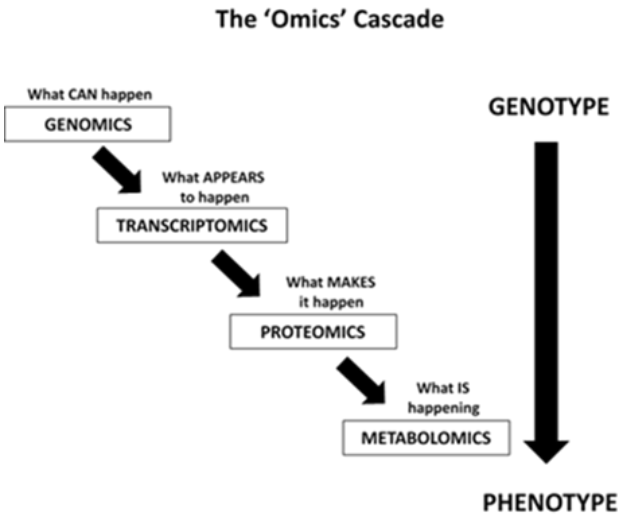

Figure 1: Diagram of 'omics' cascade defining genomics, transcriptomics, proteomics and metabolomics, and depicting their position along the genotype to phenotype continuum.

The rapid expansion in capabilities and applications within these fields owes a great deal to recent advances in analytical platforms (e.g., next generation sequencing, mass spectrometry, nuclear magnetic resonance) and bioinformatics, which allow us to obtain and mine huge amounts of data previously unattainable (Bantscheff et al. 2012; Berger et al. 2013; Mutz et al. 2013). These changes are reflected in the widening scope of biological studies from simple biochemical analyses of individual genes, proteins or metabolites to measurements of complex component mixtures within biological systems. This type of research is possible with the use of new, sophisticated, multi-platform technology that allows high-throughput analysis. The constant generation of new algorithms for software and search engines ensures maximum data production (Misra & van der Hooft 2015). These data can then be compared to gene and protein sequences and chemical fingerprints of metabolites stored in the public domain, and also to proprietary databanks that continue to grow exponentially (Longnecker et al. 2015; Perez-Riverol et al. 2015).

What is metabolomics?

Metabolomics is the study of chemical processes involving metabolites. Metabolites comprise all compounds in a biological matrix that are typically smaller than 1 kDa in size

(Beyoğlu & Idle 2013), and includes small peptides, oligonucleotides, sugars, organic acids, ketones, aldehydes, amino acids, lipids, steroids, alkaloids, and xenobiotics. Metabolite profiles can be obtained and investigated to find chemical signatures that reflect specific cell activities, such as a metabolic response to an environmental stimulus or stress (reviewed by Lankadurai et al. 2013). This field does not use DNA, RNA or proteins, but only metabolites that are produced as a result of genetic coding specific to a given cell or organism, or as a response to environmental perturbation In simple terms, it is possible to say that metabolomics is a 'physiological snapshot of a living cell'. This approach can then be used to identify physiological differences between cells, tissues, organs or organisms that have been exposed to different environmental conditions (or culturing conditions). Thus, analytical techniques are used to search within the metabolomes (the total inventory of metabolites) of biological samples (reviewed by Young & Alfaro 2016). Then, statistical examination of the metabolite profiles is performed to look for differences among samples (Young & Alfaro 2016).

From such differences we can identify metabolite signatures that serve as biomarkers for specific conditions and identify the activities of particular metabolic pathways (e.g., glycolysis, TCA cycle, lipid degradation, amino acid biosynthesis). Within experimental trials, metabolomics can be used to identify effects of water temperature (e.g. Dunphy et al. 2015), oxygen levels (e.g. Lardon et al. 2013), dietary manipulations (e.g. Cheng et al. 2015), culture conditions (e.g. Young et al. 2015a), and pathogen or toxin exposures (e.g. Peng et al. 2015; Chong et al. 2016), among others.

Advantages of metabolomics

While other 'omics' approaches have their own unique benefits, there are a number of advantages to conducting metabolomics-based research. With metabolites representing the end products of cellular regulatory processes, and being incredibly sensitive towards exogenous influences, their profiles can be regarded as the ultimate response of biological systems to genetic or environmental change (Fiehn 2002). Metabolomics also can provide a wealth of highly sensitive information that cannot be gained through the analysis of gene and protein expressions. For example, not all genes that are expressed are translated into functional products (Fernie & Stitt 2012), and there can be significant divergences in the temporal scales of translation, protein turnover and metabolite formation (Feussner & Polle 2015). These aspects may hinder identification of particular physiological processes in response to environmental perturbations at given times. However, it should be noted that omics-based approaches are by no means exclusive of one another. For example, when used in conjunction with other omics-based approaches, metabolomics has proven extremely useful for identifying gene function (Haitao 2012), and discovering new loci/genes for selective breeding trait improvements (Fernie & Schauer 2009).

Compared to proteomic and transcriptomic analyses, metabolomics generally involves less sample preparation, has much shorter turnaround times from sample collection to data interpretation, and a typical experiment can be conducted for a fraction of the cost. In addition, for many organisms there are far fewer types/classes of metabolites that exist compared to genes or proteins, and metabolite data processing is generally less complex (Wang et al. 2006; Lu & King 2009). Metabolomics can be performed using non-invasive body fluids/solid, such as plasma and faeces, which may be of particular value for studies involving fish. In addition, a number of analytical techniques can be used without destruction of samples (Young & Alfaro 2016). This is extremely useful when biological material is limited and/or when multiple analyses are to be conducted on a single sample with the aim of data integration.

Another distinguishing characteristic beneficial for aquaculture researchers is that the majority of metabolite structures are not species-specific, unlike many nucleic acid and amino acid sequences of genes and proteins. Consequently, the analytical assay does not need to be redeveloped for every animal model. Furthermore, metabolomics can be applied easily to the study of non-model organisms without prior knowledge of their genome. This makes the approach particularly well-suited to research involving new and emerging aquaculture species, but also is applicable to organisms with long culture histories. Due to these advantages and the informative power that metabolomics techniques can deliver, it is of no surprise that this approach is becoming increasingly used in various research arenas, including biomedical science (Jones 2014), veterinary science (Ryan 2015), plant science (Obata et al. 2012), ecology (Kuhlisch & Pohnert 2015), microbiology (Xu et al. 2014), nutritional science (Gibbons et al. 2015), food science (Rubert et al. 2015), agriculture (Nadella et al. 2012), and, most recently, within various fields of aquaculture research.

Scope

As can be expected, there is a wealth of information related to 'omics' research in the literature, especially for genomics and transcriptomics. However, newer 'omics' fields, such as metabolomics are represented by fewer publications, especially with regards to aquaculture applications. Indeed, there are comprehensive reviews for genomics (Quinn et al. 2012; Huete-Pérez & Quezada 2013), transcriptomics (Saroglia & Liu 2012; Qian et al. 2014) and proteomics (Rodrigues et al. 2012; Zhou et al. 2012; Carrera et al. 2013; Peng 2013) in aquaculture and seafood-related research. However, as yet there is no such review for metabolomics. To date, there are over150 papers published that use metabolomics to address a range of issues with relevance to aquaculture (Fig.

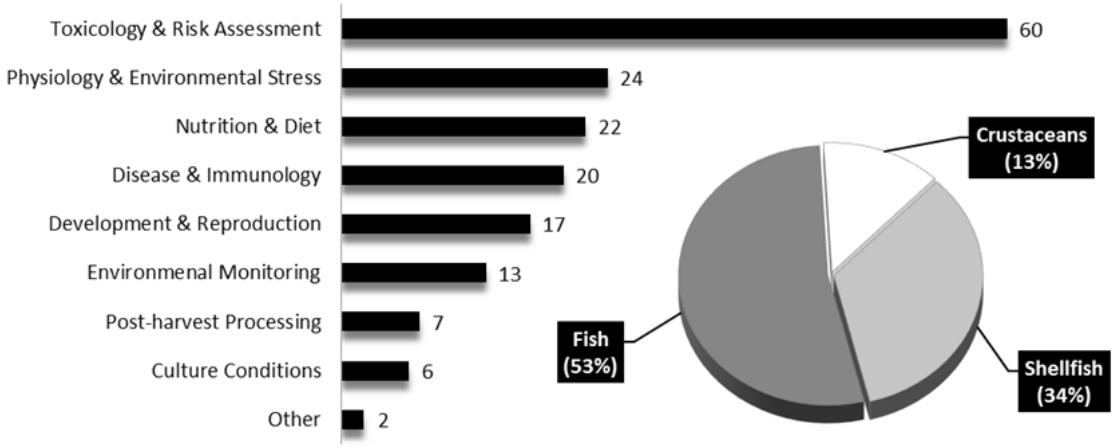

Figure 2: Trends in metabolomics-based research with applications to the aquaculture sector. The bar chart displays the number of publications from 2000-2014 within various categories. The pie chart displays the proportion of those publications, which involve relevant aquaculture organisms.

Metabolomics applications in aquaculture

Hatchery production

Successful larviculture of fish and marine invertebrates presents one of the foremost challenges in the development of full life-cycle rearing practices. Advances in hatchery technology are often incremental and directed by technical managers. Unfortunately, technical developments are poorly captured in the primary scientific literature and often not shared due to concerns over losing commercial advantage (Allan & Burnell 2013). While significant improvements in hatchery production technology have been made over the past two decades, considerable opportunities exist for optimizing the quality of broodstock, feeding regimes and culture systems (Hamre et al. 2013).

For many species, production yields are greatly hampered by high (>90%) mortality rates during larval rearing (Salze et al. 2011; Purcell et al. 2012; Sørensen et al. 2014). In some cases, this appears to be due to inadequate culture practices for new species and a lack of knowledge regarding their fundamental requirements (Calado et al. 2009; Manley et al. 2014). In other cases, reduced successes have been attributed to environmental (Barton et al. 2015), genetic (Islam et al. 2015), nutritional (O'Connor et al. 2012; Kousoulaki et al. 2015) or disease-related (Blindheim et al. 2015; Richards et al. 2015; Solomieu et al. 2015) factors. However, unexpected batch crashes and low production yields are routinely observed in some hatcheries and causation is often not identified (Hardy-Smith & Humphrey 2010; Hamasaki et al. 2011; Beal 2014). Metabolomics could be employed within various frameworks to help solve some of these issues by providing mechanistic and functional biochemical information, and to support development of remedial strategies for poor hatchery performance.

There are few reports on the application of metabolomics-based approaches to investigate the larval biology of aquatic organisms. However, recent inroads have been made with research on developmental biology using Zebrafish (Danio rerio) as a model organism (Papan & Chen 2009). The Zebrafish larval metabolome is highly dynamic through embryogenesis and can be used to successfully predict development stage, identify provisional requirements for energy acquisition and growth, and reveal coordinated flux in gene and protein expressions (Soanes et al. 2011; Huang et al. 2013; Raterink et al. 2013). While the zebrafish is not a commercially cultured species, such research provides an exemplary application and highlights the potential for studying the early life-stages of other organisms.

Thus far, the only two published studies of specific applications of metabolomics in hatchery production of larvae is that of Young et al. (2015a,b), who investigated intraspecific growth variations in mussel larvae and the effects of handling stress and culture conditions. As an exemplar, Young et al. (2015b) used a metabolomics approach to investigate the physiological condition of mussel (Perna canaliculus) larvae during hatchery production to assess larval quality. Gas chromatography mass spectrometry (GC-MS) was used to obtain metabolite profiles from fast-growing and slow-growing larval samples collected over time from the same cultures. Good candidate biomarkers for the separation of these two larval groups were identified with a variety of univariate and multivariate feature selection methods, including volcano plot analysis, supervised projection to latent squares discriminant analysis (PLS-DA), significant analysis of metabolites (SAM) and empirical bayesian analysis of metabolites (EBAM). These methods independently and consistently identified metabolite–metabolite ratios involving levels of succinic acid, glycine, alanine, pyroglutamic acid and myristic acid as biomarkers of mussel larval quality. A closer look at the function of these metabolites suggests the

involvement of various biochemical pathways in larval performance. These pathways include energy metabolism, osmotic regulation, immune function and cell-cell communication. This study illustrates not only how metabolomics can be used to assess mollusc larval quality, but signals the potential to generate predictive models of larval performance and provides mechanistic insights of biological pathways for further investigation. In the future, a great benefit to the industry would be the development of an easy-to-use tool kit to evaluate the physiological state of larvae throughout the rearing process.

Our limited knowledge of exogenous and endogenous regulation of early development in different marine invertebrate and fish species restricts rapid advancements in larviculture practices. It is crucial that we mature our understanding of the factors that influence developmental timing, energy acquisition and allocation, nutritional requirements and preferences, and immunological response mechanisms. With the imminent likelihood of an expansion in the diversity of farmed marine species, it becomes especially urgent that we develop solutions to address the problem of larval nutrition and disease. Use of metabolomics could also be applied to other areas of hatchery production to help close the loop on full life-cycle culture for many species. For example, routine production of high-quality gametes for successful fertilisation and on-growing could be achieved through better broodstock management and understanding of maternal provisioning, paternal effects and factors associated with high fecundity. While not yet realised within the aquaculture industry, metabolomics has benefitted other areas of developmental biology with respect to investigating reproductive disorders (Courant et al. 2013); identifying biomarkers for assessment of sperm fertility (Kumar et al. 2015); assessing oocyte quality and predicting embryo viability (Bertoldo et al. 2013; Cortezzi et al. 2013); and identifying the coordination of metabolic traits during selective breeding for stress resistance and longevity (Malmendal et al. 2013). Metabolomics will undoubtedly be a useful tool in the progression of future hatchery technologies.

Nutrition and diet

Nutritional research in aquaculture aims to improve the health of cultured species through diet, and increase growth trajectories and production yields. Providing the appropriate nutrition quality and quantity is likely to have broad ramifications into other cultivation aspects, such as individual performance improvement, disease prevention, enhancement of broodstock and gamete quality, development of sustainable and high-quality feed alternatives, and mitigation of environmental impacts, among others. Optimal nutritional requirements for many new and emerging aquaculture species are unknown. Thus, there is an urgent need to determine peak dietary conditions for these organisms to support sector expansion and diversification. Even for many well-established species, the complex interactions between nutrition, health and environment are poorly understood. Furthermore, efforts to maximize production yields are inhibited by limited knowledge of larval dietary preferences and nutritional requirements for a number of species. Thus, considerable scope exists to boost full life-cycle culture productivities. Metabolomics is uniquely suited to assess metabolic responses to nutritional deficiencies or excesses, and can provide in-depth mechanistic insights to assist development of optimised feeding regimes.

Currently, nutritional metabolomics research in aquaculture is an emerging field. Thus far, metabolomics-based approaches have proved useful for assessing: positive and negative effects of food deprivation in mussels, trout and salmon (Tuffnail et al. 2009; Kullgren et al. 2010; Baumgarner & Cooper 2012; Thunathong et al. 2012; Cipriano et al. 2015; Sheedy et al. 2015); interactions between diet, environment and disease in abalone and seabream (Rosenblum et al. 2005; Silva et al. 2014); effects of nutrient supplementation in seabream, salmon and carp (Cajka et al. 2013; Robles et al. 2013; Anderson et al. 2014; Wagner et al. 2014); effects of dietary protein substitution and utilization in salmon and carp (Bankefors et al. 2011; Jin et al. 2015); effects of feeding charr with contaminated and decontaminated fishmeal to sustainably expand resource use (Cheng et al. 2015); effects of using reduced fishmeal-based feed alternatives in cobia and charr (Schock et al. 2012; Abro et al. 2014); effects of replacing fish oil with vegetable oil in sea bass feeds to decrease reliance on wild-caught stocks (Castro et al. 2015); effects of newly introduced plant-derived contaminants in salmon feeds (Søfteland et al. 2014); and development of non-invasive dietary inspection techniques in various finfish species (Asakura et al. 2014).

To illustrate the use of metabolomics to investigate dietary performance we highlight a study by Abro et al. (2014) who tested the use of a protein-rich zygomycetes fungus (Rhizopus oryzae) as a substitute for the traditional fish meal protein in Arctic charr (Salvelinus alpinus) diets. The authors produced metabolite profiles of fish fed a commercial diet of unknown composition, a diet with mostly fish meal protein, and a diet with mostly zygomycetes protein. Analysis of metabolite profiles from liver samples indicated that the zygomycetes protein diet did not differ from the fish meal protein diet, and suggests similar physiological responses to these diets. However, significant metabolite differences were observed between fish fed the commercial diet and fish fed each of the other two protein-based diets, with the former being an inferior diet. The study used liver samples (quenched in liquid nitrogen) to extract metabolite signatures since this organ is actively involved in metabolism of absorbed nutrients, such as proteins. The combination of a nuclear magnetic

resonance (1H-NMR) analytical platform and statistical analyses, including orthogonal projection to latent squares discriminate analysis (OPLS-DA), provided a powerful pathway to identify differences and similarities among the metabolite profiles to test for diet effects. In addition, the study highlights the possibility of using alternative protein sources, which may prove to be more sustainable for fish cultivation practices in the future.

In another study of fish diets, Robles et al. (2013) used the short-chain fatty acid butyrate as a diet supplement to increase body weight and enhance intestinal tract activity in sea bream (Sparus aurata). Using a high performance liquid chromatography mass spectroscopy (HPLC-MS) platform, the authors measured over 80 metabolites from fish intestine samples before and after feeding with the butyrate supplement diet. Initial samples were taken after a 12-h starvation period to obtain a basal metabolite profile with a non-active intestine. Three hours after the initial feeding, intestine samples were collected from fish that were fed the butyrate supplemented diet and fish fed diets without the supplement. Growth measurements of the remaining fish within each treatment were taken after 8 weeks. Results showed significant improvements with butyrate diets, including weight gains, increases in several essential amino acids and nucleotide derivatives, and potential increases in cell energy provisions through glucose and amino acid oxidation pathways. Based on these results, it appears that butyrate may be a good natural supplement to enhance fish growth and metabolic activity.

As an emerging tool in nutrition research, metabolomics offers a unique potential to unravel the complex intertwining mechanisms involved in nutrient utilisation, reproduction, growth and disease progression. Through the catabolic breakdown of macromolecules in foods and direct incorporation of smaller components, the metabolome is the receiving depot for the raw materials required by cells to synthesise new products. These materials are essential for the formation and repair of body tissues, and the production of energy to support and maintain life. As signalling molecules and enzymatic co-factors, metabolites are involved in the synthesis, degradation and modification of proteins, which regulate gene expression and metabolic pathways. It is the intricate combination of these multifaceted processes which are required for biological systems to maintain homeostasis. Metabolomics can provide important mechanistic insights to identify how regulation of homeostatic control is disturbed in the early phases of diet-related diseases. This knowledge could be used to identify new metabolic biomarkers for health and nutritional status and to develop strategies for the dietary prevention and intervention of diseases. Future nutrition research in aquaculture will undoubtedly be radically advanced through application of metabolomic approaches.

Disease and immunology

The successful management of health and diseases is a major challenge for aquaculturists. Health is characterized by the optimal functioning of biological systems. Within this framework, disease can simply be defined as the lack of health. However, disease is commonly associated with interactions between host organisms and pathogens (bacterial, viral or parasitic). Disease outbreaks can arise due to transmission from wild-stocks, accidental transfer of diseased animals between farms, use of pathogen-infected feeds, poor water quality, lack of sanitary barriers, failure to identify and isolate unhealthy organisms, and impaired animal welfare as a result of overstocking and inadequate nutrition, among others. It is estimated that up to 20% of potential aquaculture production in China, which is responsible for over two thirds of the world's production, is lost due to the occurrences of diseases (Li et al. 2011). In some cases, mass mortalities as a result of severe disease epidemics have decimated certain sectors, leading to complete collapse of the industry and having huge socioeconomic impacts (e.g., herpes virus in oysters, white spot syndrome virus in shrimp) (Sánchez-Martínez et al. 2007; Peeler et al. 2012).

It was previously thought that diseases had single causes with single diagnostic targets. However, considerable research has established tight links with intricate metabolic imbalances. Subtle changes in entire metabolic pathways can be responsible for the onset and development of particular health conditions. Imbalances caused by genetic, nutritional or environmental factors can lead to suppression of immune function, which in turn can cause organisms to be more susceptible to pathogen exposure. Host organisms may maintain healthy lives in the continuous presence of pathogens, and only when they experience stressful conditions will the equilibrium shift, favouring the dominance of the pathogen. Alternatively, the encounter of a healthy organism with a highly virulent pathogen can quickly overwhelm the immune system, leading to major instability in metabolic homeostasis and ultimately resulting in death. Due to the involvement of metabolic networks in the initiation and proliferation of diseases, metabolomics can provide unique insights into the effects of pathogen exposure and the mechanisms of resistance. Furthermore, metabolomics can be applied as a valuable tool to determine the efficacy of disease treatments and management.

Practical applications of metabolomics in aquaculture research to investigate host-pathogen interactions are developing rapidly. In general, these findings are revealing that pathogen exposure tends to cause severe disturbances to host energy metabolism, osmotic regulation, oxidative stress, cell-signalling pathways and respiratory mechanisms. A suite of recent metabolomics-based investigations on the immunological responses of bivalves to infections with various bacterial

pathogens have also revealed that the molecular response-mechanisms are differentially regulated depending on bacterial strain (Ji et al. 2013; Wu et al. 2013; Liu et al. 2013a, 2014a,b), the host species and pedigree (Liu et al. 2013b; Wu et al. 2013a), the sex of the host (Ellis et al. 2014; Liu et al. 2014a), and between different tissues (Ji et al. 2013; Wu et al. 2013a; Liu et al. 2014b). These results highlight and reinforce the need for different disease management strategies to be developed for particular conditions. Metabolomics also has been used to investigate the interactions of nutrition, temperature, withering syndrome disease and disease treatments in abalone (Rosenblum et al. 2005, 2006); to evaluate the effect of superintensive aquaculture systems on the health of shrimp (Schock et al. 2013); to identify biomarkers involved in the host responses of shrimp to white spot syndrome virus (Liu et al. 2015); to characterise metabolic demands of hepatic tumors in flatfish (Southam et al. 2008); to discover metabolite biomarkers associated with enhanced host defence against bacterial infection in carp (Guo et al. 2014); to reveal the biochemical mechanisms underlying the efficacy of bacterial vaccines in fish (Guo et al. 2015); to identify simple strategies for potentiating the sensitivity of aquatic pathogens to antibiotics (Su et al. 2015); to identify simple treatments for increasing disease resistance of tilapia against bacterial pathogens (Peng et al. 2015) and determine the mechanism of action (Ma et al. 2015); to identify metabolic responses of crabs to vibrosis (Schock et al. 2010); to enhance the health of cobia through dietary manipulation (Schock et al. 2012); and to develop nutritional and non-nutritional treatments for enhancing survival of diseased tilapia (Cheng et al. 2014; Zhao et al. 2015).

A good example of metabolomics applications to assess health parameters in cultured marine invertebrates is a study by Schock et al. (2013). In this case, the authors used NMR-based metabolomics to monitor health factors along the production line of shrimp (Litopenaeus vannamei) from nursery to harvest. Shrimp were grown in a superintensive aquaculture system with minimal water exchanges and a biofloc system to promote growth of beneficial bacterial communities. The aim of this superintensive system was to increase production (year-round high density farming) while reducing disease susceptibility, and to enhance water quality (via reduction of waste products). Weekly shrimp samples were collected throughout the nursery (over about 2 months) and growout raceways (for about 4 months). A robust quality control procedure was performed to evaluate experimental biases in extraction process and timing with the use of standard reference material, extractions of different tissues from the same animal, and various pooled and blank replicates as controls. Such protocols for verification of sound data collection procedures are essential for metabolomics projects, and reporting on these results should be common practice in the literature, although they are thus far seldom encountered. Comparison of NMR spectra of biological samples from nursery and raceway phases resulted in important differences that could be interpreted as stressful conditions to shrimp. Specifically, the compounds iosine and trehalose were found to be good biomarkers for stress in this species. From an industry perspective, these physiologically stressful conditions were attributed to three main events – a total ammonia nitrogen spike in the nursery, a period of reduced feeding due to surface scum build-up in raceways, and the transition of stock from the nursery to raceways. Clearly, this metabolomics-based study identified specific areas where facility managers could focus to improve production efficiency. Such results are likely to have required considerably more time and financial investment had they been illuminated with traditional experimental approaches.

Another exemplary study is provided by Guo et al. (2014). In their investigation, crucian carp (Carassius auratus) were assessed for their ability to survive infections caused by the bacterium Edwardsiella tarda. Using a GC-MS platform, the authors compared metabolite profiles from infected fish that either survived or died, and their respective controls (not infected). The results not only identified biomarkers that could be used to signal infection in the fish, but also biochemical pathways likely to be involved in immune responses to infections by E. tarda. The bioinformatics analysis involved the use of the web-based MetaboAnalyst 2.0 pipeline for metabolomics data analysis and the pathway library of zebrafish (surrogate for carp) to identify biochemical pathways that were indicative of infections. The identified biomarkers for this infection include increased levels of palmitic acid and decreases in the amount of D-mannose. Relevant pathways included elevated unsaturated fatty acid biosynthesis and decreases in fructose and mannose metabolism. The broader implications of these results are that metabolomics may be an effective approach to identify early signs of pathogens and infections that could be mitigated at the onset of a crisis. In addition, the improved understanding of biochemical pathways involved in immunological responses may be extremely valuable when selecting optimal conditions for growth of cultured species. It is important to re-iterate that the strength of the metabolomics approach is that it provides a wide scan of physiological activities and identifies areas where problems may be encountered. Thus, this is a powerful approach to inform more detailed studies of production issues such as health threats.

The deleterious effects of pathogens and viruses are enhanced by exposure of aquatic organisms to immunotoxic contaminants, which supress the immune system and hasten the onset of disease (Morley 2010; Girón-Pérez 2010). Poor water quality is a major problem for the aquaculture industry in many regions. Rapid industrialisation and urbanisation has resulted in various organic and synthetic pollutants being introduced into

aquatic ecosystems which pose serious threats to the health of cultured organisms (Li et al. 2011). The number of new and emerging contaminants being released into the environment is increasing, and their biological fate and effects are often unknown. Using fish and shellfish, metabolomics is progressively being used to characterize mechanisms of toxicity and to develop novel methods to assess environmental contamination. For example, metabolomics-based studies have proven useful for: identifying physiological responses of various molluscs to agricultural run-off, heavy metals and endocrine disruptors (Wu et al. 2013b; Hanana et al. 2014; Ji et al. 2014; Leonard et al. 2014; Zhou et al. 2014; Campillo et al. 2015; Ji et al. 2015a,b; Ji et al. 2016; Song et al. 2016); characterising the consequence of pesticide exposure in carp (Kokushi et al. 2015); assessing the effects of petrochemical contamination at industrialised sites harbouring caged mussels (Fasulo et al. 2012; Cappello et al. 2013, 2015); detecting freshwater locations which have multi-contaminant sediment loadings (Watanabe et al. 2015); identifying new and highly sensitive bioindicator species of clams for monitoring environmental contamination (Ji et al. 2015c); and developing non-invasive methods for pollution assessment by profiling metabolites in the excreted mucus of fish skin (Ekman et al. 2015). The results of these studies clearly demonstrate promising applications. It is expected that metabolomics will be used extensively in the future to monitor the health of sentinel species, deliver forensic capabilities for evaluating pollution exposure, enhance the sensitivities of ecotoxicological assessments, and to provide authorities with information to set new regulatory guidelines. The exposure of aquatic organisms to environmental contaminants and disease-related factors during their cultivation has major implications for biosecurity, market opportunity, product quality, product value, and most importantly food safety.

Post-harvest quality control

One of the biggest challenges for the aquaculture industry is to achieve and maintain a high quality product from farm to market, and into the hands of consumers. One person's view of quality may be different from another's and numerous factors are normally involved in its classification. Nutritional value is an objective aspect based on the presence of essential amino acids, highly digestible proteins, vitamins, minerals and a high content of polyunsaturated fatty acids (e.g., omega-3 fatty acids). Meat quality is also defined by other compositional features, such as the lean to fat ratio and water content (Brewer 2012). Consumers' perceptions of quality is perhaps one of the most important aspects of aquaculture, and involves palatability factors, which can be highly subjective. These include visual appearance, smell, firmness, juiciness, tenderness and flavour (Nollet & Toldrá 2010). Product quality is a multifaceted term and there is still much to learn about how the various indices are perceived and interact.

In order to achieve a quality product prior to harvesting, it is critical that culture conditions are optimized and well-managed to enhance animal welfare and improve nutritional and compositional traits. On the other hand, post-harvest processes, such as transport, storage and packaging have major impacts on product freshness and food safety. Due to the involvement of metabolites in flavour and aroma profiles, nutritional value and degradation processes, metabolomics provides an exciting opportunity for food quality assessment and development of new strategies for quality preservation and enhancement. Examples of metabolomics-based analysis in food quality, post-harvest processing and storage are growing rapidly (Rubert et al. 2015; Trimingo et al. 2015; Kim et al. 2016). For instance, metabolomics has thus far proved useful for: identifying degradation of specific nutrients, flavours and aromas in cold-stored fish (Castejón et al. 2010; Leduc et al. 2012; Shumilina et al. 2015); examining interactions between culture conditions, post-harvest quality and storage (Savorani et al. 2010; Picone et al. 2011); development of rapid assessment techniques and discovery of biomarkers to determine fish meat freshness and quality during extended freezing (Duflos et al. 2010; Leduc et al. 2012; Heude et al. 2014); identifying metabolic factors during semi-anhydrous living-preservation of scallops to improve meat quality and value after transport (Chen et al. 2015); and investigating compositional changes in salmon during and after irradiation processing to reduce foodborne infection, monitor storage effects and enhance product safety (Villa et al. 2013; Castejón et al. 2016).

A rapid and straightforward method to assess fish freshness and quality was used by Heude et al. (2014), based on indicative metabolite quantification using high resolution magic angle spinning (1H HR-MAS) NMR spectroscopy. The procedure was aimed at quantifying two well-known indicators of fish freshness and quality (K value and trimethylamine nitrogen [TMA-N] content) in fish samples that had been stored at 0°C. The K value is a measure of autolytic process (spontaneous disintegration of cells or tissues that occurs after death), and TMA-N content is indicative of bacterial spoilage. These parameters are based on measurements of metabolite concentrations, which were recorded in fish from four commercially important species (sea bream, sea bass, trout, and red mullet). Both the NMR approach and traditional methods were compared, and produced similar results. However, NMR had the advantage that it could be used directly on unprocessed fish without prior extraction procedures with an average time of 40 min per sample. Conversely, traditional analytical methods require extensive and complex extraction procedures that are time-consuming. Another advantage of this new approach is that small sample sizes (about 10-15 mg) are needed for metabolite

analyses, and these amounts can be collected with a biopsy, thus, affording considerable benefits to the producer.

The same platform (1H HR-MAS NMR) was used to test the effects of irradiation treatment on cold-smoked Atlantic salmon (Salmo salar) as a means to prevent bacterial and parasite infections (Villa et al. 2013). The authors used white salmon muscle samples from live fish from three irradiation treatments (0, 1, and 4 kGy), and non-irradiated controls, to obtain NMR spectra that were statistically analyzed (principal components analysis [PCA], and they utilized analysis of variance [ANOVA]) to identify differences in degradation of fish samples during storage. In this study, several compounds, including creatine, trimethylamine oxide and the sum of phosphorylcholine and glycerophosphorylcholine were found to be diagnostic of irradiation treatment. A huge advantage of this approach is that this is a non-destructive analysis to evaluate food processing and physiological changes (e.g., bioactive compounds) due to fish storage prior to market delivery.

Traceability is another important aspect of food quality control. Regulatory authorities may ask aquaculture producers to identify the origin of their product and stipulate the culture methods to prevent fraudulent activities and ensure food safety. Responsible farmers are concerned with protecting their market share from products from unknown sources that may be of inferior quality. Methods for food authentication and traceability have been advanced through application of metabolomic-based approaches (reviewed by Castro-Puyana & Herrero 2013; Cubero-Leon et al. 2014). For example, metabolomics has been useful for discriminating between wild and farmed sea bream reared in different culture locations and conditions, which are of various qualities but are marketed as the same high-quality product (Melis et al. 2014). Similar techniques were also successfully used to classify wild-caught and cultured salmon and seabass and expose fraudulent labelling of seafood products (Aursand et al. 2007, 2009; Mannina et al. 2012). In Sardinia, Italy, dried and salted mullet row is processed using special methods which result in a unique high-value product. Metabolomics has been shown to be extremely valuable for distinguishing this locally-produced roe from other sources to protect Sardinia's market (Locci et al. 2011).

Indeed, the study by Locci et al. (2011) perfectly illustrates the use of metabolomics in product traceability. In this case, 1H-NMR was used to obtain metabolite signatures that could differentiate salted and dried mullet (Mugil cephalus) roe samples from Sardinia (Italy) and those produced in other geographical regions. The study found that the different storage and manufacturing procedures from different mullet sources could be identified by several metabolites (e.g., free amino acids, organic acids) found in the aqueous phase extracted from samples. This traceability technique is crucially important to Sardinian communities with long traditions of processing a high quality roe from this species, called 'bottarga'. The uniqueness of this study is that it shows that this approach can be used not only to identify the provenance of the fish samples (previously possible only with DNA and protein analyses), but also allows fingerprinting of the manufacturing and processing style of this traditional Italian artisanal fishery product. In today's global food markets, this rapid and straightforward metabolomics approach can be used effectively to provide authentication and traceability for the industry and consumers.

Future applications and directions

As we have discussed, improving nutrition to enhance larval production, animal health and meat quality will be important for the future of aquaculture. In order to advance research in feed formulation and to speed discovery of alternative feed sources for improved sustainability, an in depth understanding of nutrient requirement and utilisation is needed. Metabolomics is uniquely suited to this task. Individual molecules within test diets can be chemically labelled and traced during the digestive breakdown, uptake and redistribution and energy conversion processes. Incorporation of comprehensive metabolomics-based tracer studies in nutritional research is an area which should be explored. Other nutrition-based challenges which require attention include enhancing low carbohydrate utilisation in fish, replacement or elimination of antibiotic and chemical use in feeds, improving flesh quality in some species, and lengthening the shelf life of fish and shellfish meat (Tincy et al. 2014).

Successful management of diseases is also a major challenge. Although development of vaccines to remediate many diseases have been indispensable for the successful expansion of the fish sector (Ringø et al. 2014), there are no effective treatments as yet for other organisms (e.g., viral infection in shrimp [Seibert & Pinto 2012]). Incorporation of metabolomics-based approaches to better understand the reasons for disease susceptibility, and to develop methods for enhancing immunodiagnostics and host resistance, is an area which requires further exploration. New advances in invertebrate immunology are radically challenging the concepts of innate and adaptive immunity, and the line between them is starting to blur (Sun et al. 2014; Wang et al. 2015). Furthermore, large inter- and intraspecific variations in immune function and responses to pathogen exposure exist. Metabolomics has recently been shown to be extremely useful for investigating mechanisms of immunity and the physiological impacts of pathogen exposure in mammals (Dorrestein et al. 2014; Noto et al. 2014; Gray et al. 2015), and could easily be applied more widely to focus on cultured aquatic species.

Other relevant areas in which metabolomics could be applied in the future include climate change research, cryopreservation and selective breeding. The threats of

global warming and ocean acidification are increasingly becoming a major concern for aquaculture producers and stakeholders (Reid & Jackson 2014). Changes in sea surface temperatures and water pH can enhance the frequency of harmful algal blooms, alter primary productivity, escalate incidences of marine infectious diseases, and reduce growth rates and survival of cultured organisms (Burge et al. 2014; Gilbert et al. 2014; Richards et al. 2015). Metabolomics has recently been used successfully to investigate the effects of ocean acidification on mussels (Ellis et al. 2014) and oysters (Wei et al. 2015a,b), thermal stress on mussels (Ellis et al. 2014; Dunphy et al. 2015) and sea cucumbers (Shao et al. 2015), and the interactions between red-tide forming dinoflagellates and other phytoplankton species (Poulson-Ellestad et al. 2014). We anticipate there will be substantial growth in the application of metabolomics to this research area. Cryopreservation of gametes and embryos provides a means to preserve genetic material for future use. While there have been many successes in this field for some species and/or certain types of biological material, a number of challenges remain. For example, cryopreservation of fish and shellfish sperm has been achieved; however, cryopreservation of oocytes is still highly problematic (Herráez et al. 2011; Wang et al. 2014; Hassan et al. 2015). In molluscs, cryopreservation of fertilised embryos can be accomplished, but survival rates are often extremely low (Wang et al. 2011; Parades et al. 2012) and the reasons for poor success are unknown. Metabolomics could be used to better understand the physiological mechanisms involved in cellular damage during the cooling and warming processes (Abuja et al. 2015), and help develop remedial strategies for cryoinjury (Koštál et al. 2011). Selective breeding in aquaculture has a long history based on morphological and, more recently, genetic traits. Recent applications of metabolomics in agriculture have resulted in the identification of new phenotypes for selective breeding of crops and animals to enhance production, disease resistance and product quality (Buitenhuis et al. 2013; Kushalappa & Gunnaiah 2013; Pushpa et al. 2014; Sundekilde et al. 2014). This provides an exciting new avenue of research, which could be transferred to the aquaculture sector.

The future of biotechnology in aquaculture will undoubtedly benefit from building upon the knowledge which is starting to arrive from comprehensive exploratory metabolomics-based studies. Due to its infancy, we are still well within the discovery phase of research, and hence the full extent to which metabolomics will be applied is yet to be realised. We suggest three avenues in which metabolomics should be applied in the future to enhance production, increase efficiency and help supply the global demand for aquaculture-produced food.

- ***Knowledge discovery:*** Use of highly sensitive and precise analytical platforms/technologies within specialised laboratories should be enhanced in order to continue the expansion of knowledge in aquatic biology and the discovery of new applications. This high-level research requires broad expertise and access to state-of-the art equipment, and it is likely to be performed in collaboration with academic institutions.

- ***Development of biochemical assays:*** By building upon the knowledge gained from knowledge discovery, the identity of single or multiple biomarkers which reflect the physiological condition (nutritional status, stress, health, disease) of cultured organisms can be used to develop simple assays for monitoring organism status and for forensic purposes. These assays will ideally be accurate, cheap, easy to use, require low user skills, be applicable in field and hatchery environments, and deliver immediate results.

- ***Incorporation of alternative metabolomics-based platforms:*** Presently, metabolite fingerprinting/profiling is performed in well-equipped laboratories. However, new technologies are continually emerging. Use of lower resolution platforms (e.g., infrared spectroscopy) with proven application, sensitivity and robustness are being made accessible for field measurements at a fraction of the costs of current systems. With the development and validation of multivariate predictive classification models, metabolomics-based technology will be brought from the hands of scientists and into the hands of farm workers. This will also pave the way to provide and support a unique opportunity for method development and knowledge discovery to be performed in-house with minimal training and expenditure.

Conclusions

Metabolomics is a powerful, newly emerging field that has huge application potential in aquaculture. Recent advances in analytical capabilities and bioinformatics have made it possible to acquire and analyze large amounts of metabolite data more effectively and efficiently. These procedures are now being applied successfully to identify biomarkers to detect a range of cultivation problems and issues in areas such as larval production, nutrition, health and food safety and quality.

Since this is a recently evolving approach, few examples are available to illustrate the relevance of metabolomics in aquaculture. However, these recent publications clearly highlight the benefits of expanding this evolving field. We envisage that metabolomics will play an increasingly more important role in all aspects of aquaculture research. Indeed, metabolomics is well-placed to provide the biotechnological advances needed to meet our expected aquaculture growth in the coming years.

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