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# *Perkinsus olseni* and other parasites and abnormal tissue structures in New Zealand Greenshell™ mussels (*Perna canaliculus*) across different seasons

**Farhana Muznebin[1,2] · Andrea C. Alfaro[1] · Stephen C. Webb[3]**

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**Abstract**
The New Zealand Greenshell™ mussel (*Perna canaliculus*) supports the largest aquaculture industry in the country. However, summer mortality events and potential disease outbreaks may threaten the growth of this industry. As an approach to gauging potential threats through the seasons, a detailed histopathological examination was conducted on 256 adult cultured mussels collected from a farm between April 2018 to September 2019, which covered the austral autumn, winter, spring and summer seasons. Histological sections followed by confirmatory in situ hybridization (ISH) resulted in the identification of *Perkinsus olseni* at an overall prevalence of 56%. Other parasites and pathogens were identified by histology: apicomplexan parasite X (APX) (78%), copepods (*Pseudomyicola spinosus* or *Lichomolgus uncus*) (1%), *Microsporidium rapuae* (1%), intracellular microcolonies of bacteria (IMCs) (2%) and bacilli and cocci bacteria (4%) in gills, mantle, gonads, digestive epithelium and digestive tubules. There was a significant association between *P. olseni* and APX infection in mussels. This is the first report on seasonal variations of *P. olseni* and APX in New Zealand Greenshell™ mussel. There was a significant association between seasons and the presence of *P. olseni* and APX in mussels. A significant positive association between the brown material accumulation and parasites (*P. olseni* and APX) and between haemocytosis and *P. olseni* infections were recorded. A significant association between presence of parasites and health condition (healthy and unhealthy) of mussels was observed. Moreover, a significant association between digestive tubule deterioration (large lumen, with a thin epithelial wall) and *P. olseni* infection was noted. Therefore, this study provides information regarding the infections of potential parasites and pathogens for the first time in *P. canaliculus*, their seasonal variations and host-parasite interactions within a commercial farm.

**Keywords** Apicomplexan X · Ceroid material · Haemocytosis · In situ hybridization · *Perkinsus olseni* · Semi-quantitative histological scale

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✉ Andrea C. Alfaro
andrea.alfaro@aut.ac.nz

Extended author information available on the last page of the article

**Abbreviations**

| | |
|---|---|
| H&E | Haematoxylin and eosin |
| ISH | In situ hybridization |
| APX | Apicomplexan parasite X |
| IMCs | Intracellular microcolonies of bacteria |
| PBS | Phosphate-buffered saline |
| DIG | Digoxigenin |

## Introduction

New Zealand Greenshell™ mussels (*Perna canaliculus*) are endemic to New Zealand and support the largest aquaculture industry in the country. Unlike other shellfish species, both cultured and wild Greenshell™ mussels have had relatively few disease issues (Castinel et al. 2019), but they are affected by several pathogens and parasites, including *Vibrio* spp. (Kesarcodi-Watson et al. 2009a, b), digestive epithelial virosis (Diggles et al. 2002), *Microsporidium rapuae* and *Bucephalus* spp. (Castinel et al. 2019; Webb 2008), *Tergestia agnostomi* (Jones 1975), rickettsiae and apicomplexan parasite X (Hine 2002a, b; Webb 2013) and *Perkinsus olseni* (OIE 2017; Webb and Duncan 2019). Among them, the main pathogens and parasites of interest are *P. olseni* and APX, affecting these mussels (OIE 2017; Suong 2018).

*Perkinsus olseni* is a significant parasite associated with inflammatory responses and mass mortalities in *Haliotis laevigata* (Goggin and Lester 1995). This parasite was reported for the first time in farmed *P. canaliculus* from the top of the South Island in New Zealand in 2014 (OIE 2017), then in 2018 (Webb and Duncan 2019). *Perkinsus* spp. (*P. olseni* and *P. marinus*) affect a variety of bivalve species around the world and are linked with severe mortalities (Ramilo et al. 2015; Villalba et al. 2011). Heavy *P. marinus* infection in oyster, *Crassostrea virginica* results in the massive accumulation of haemocytes in epithelia, connective tissue, muscle fibres and haemolymph spaces close to the parasite (Villalba et al. 2004). Moreover, the haemocytes phagocytose *P. marinus* cells and the phagocytosed parasites multiply inside the haemocytes of *C. virginica* causing them to rupture (Villalba et al. 2004). Therefore, *P. marinus* are involved with tissue damage and deformation of infected organs (La Peyre et al. 1995; Mackin 1951; Perkins 1996) and may eventually lead to organic abnormalities and death (Choi and Park 2010) in clam and oyster populations. Mortalities associated with *P. olseni* infection are especially acute when environmental circumstances (e.g. elevated temperatures, increased salinity, host density) are favourable for the proliferation, activity (e.g. cell division, metabolism, reproduction) and transmission of the parasite (Park and Choi 2001; Soudant et al. 2013; Villalba et al. 2004). Furthermore, studies reported the influence of temperature in the occurrence of annual patterns of perkinsosis (diseases caused by *P. olseni* and *P. marinus*) in the clam *Tapes decussatus* (Casas 2002). Although some studies have focused on the annual and seasonal patterns of *P. olseni* infections in clams (*Ruditapes philippinarum* and *T. decussatus*) (Casas 2002; Dang et al. 2010; Lassalle et al. 2007; Villalba et al. 2005), there is no information on infection by *P. olseni* and its seasonal variations in mussels (*P. canaliculus*).

Another potential parasite of New Zealand shellfish is apicomplexan X (APX), which is endemic to New Zealand and reported in different bivalves, such as *Ostrea chilensis* (Diggles et al. 2002; Hine 2002a), *P. canaliculus* (Suong 2018; Suong et al. 2019; Webb 2013), *Mytilus galloprovincialis* and *Modiolus areolatus* (Suong 2018; Suong et al. 2019). APX

is often seen in the haemolymph sinuses and supra branchial sinuses of flat oysters, *Ostrea chilensis* (Hine 2002a), but lesions are noticed in the digestive tract, gills and mantle with associated mortalities in acute APX infections (Hine 2002a; Webb and Duncan 2019). In addition, heavy infections of APX in flat oysters are related to the destruction of haemocytes and connective tissue (Hine 2002a), and the depletion of host glycogen (Hine 2002a; Suong 2018). Furthermore, APX zoites frequently cause severe infection in oysters during the peak spawning period (summer/autumn) (Hine 2002a). While seasonal changes of APX infection in New Zealand oyster *Ostrea chilensis* have been reported (Hine 2002a), such studies on the mussel *P. canaliculus* are lacking.

Observations of inflammatory responses, tissues damages or abnormal tissue morphologies in histology can be the signs of pathogenic and parasitic infection in shellfish. Inflammatory responses following pathogenic and parasitic infections, such as those caused by *Perkinsus* spp. are local defence reactions in host tissues (Cone 2001; Choi and Park 2010). The inflammatory response may be seen as abnormally elevated numbers of haemocytes in a tissue area (haemocytosis). This accumulation of haemocytes has been described as an attempt by the host to destroy, dilute or isolate the invading factors (Sparks 1972). Another immunological tissue response is the presence of brownish-yellow pigmentation (ceroid material or lipofuscin), frequently observed across tissues in many molluscan species (Webb and Duncan 2019) and generally occurs in the vicinity of parasites, such as APX (Webb and Duncan 2019). This brownish-yellow pigment is also associated with inflammation and contamination or necrotic lesions in *C. virginica* (Wood and Yasutake 1956; Zaroogian and Yevich 1993).

In addition to the occurrence of known pathogens and parasites, other conditions are also encountered in shellfish, including digestive gland and gill pathology caused by pathogens and parasites (*Perkinsus* spp.) (Lee et al. 2001; Choi and Park 2010). Digestive gland pathologies, such as abnormal tubule structures and lumen modifications, including thinning, sloughing and damage were seen in other bivalves- *Cardium edule*, *Crassotrea virginica* and *Mytilus galloprovincialis* (Morton 1970; Winstead 1995, 1998; Carella et al. 2015a) have also been observed in *P. canaliculus* (Webb and Duncan 2019). These digestive tubule abnormalities and morphological changes may occur during the digestive process or under stress conditions, such as changes in the food supply, starvation or exposure to pollutants and biotoxins (Smolowitz and Shumway 1997; Ellis et al. 1998; Rolton et al. 2019). The pathological changes in the digestive gland of the mussel (*Crenomytilus grayanus*) may be caused by chronic pollution and parasitic infestation (Usheva et al. 2006) and affect the absorptive capacity of the digestive system. Another condition where causality is usually uncertain is the pathology of the gills, such as epithelial erosion, loss of cilia (Webb and Duncan 2019) and alterations in the ciliary discs (the attachment connecting gill filaments laterally along their length) (Sunila and Lindstrom 1985) which are common in aquaculture species. Depending on the level of the erosion and the extent of the obstructed area, the erosion of the gill epithelial cells could disrupt respiratory function (Webb and Duncan 2019). In addition, the ciliary discs prevent gill filaments from separating in the mussels (de Oliveira David et al. 2008). Therefore, alterations in the ciliary discs of mussels will cause the gill filaments to separate and may lead to the disruption of the entire gill structure (de Oliveira David et al. 2008).

It is well known that seasonal temperature changes have a significant impact on the pathogen and parasite load in bivalves (Aagesen and Häse 2014; Malham et al. 2009; Viergutz et al. 2012). Some parasites show positive responses to increases in temperature, including the increased transmission of parasites between hosts (Mouritsen and Jensen 1997; Moore et al. 2000; Poulin 2006) and can modify host-parasite interactions (Malek and Byers

2018). Furthermore, a parasite such as *P. marinus* may expand its geographical range due to climate-induced rises in winter water temperatures (Ford and Chintala 2006; Ford and Smolowitz 2007). Although such increasing water temperatures have been reported to be associated with massive mortalities in *P. canaliculus* during summer (Dunphy et al. 2015), little is known about the relationship between seasonal temperature changes and parasitic and pathogenic infections in this species.

This study reports on the presence of parasites and health conditions in *Perna canaliculus* collected from Kaiaua mussel farms. In this study, a detailed histopathological examination of a targeted survey/sampling of farmed mussels (*P. canaliculus*) was undertaken to identify potential pathogens and parasites and their seasonal variations, as well as to study the immunological tissue responses to pathogens and parasites to gauging potential threats (summer mortality events and potential disease outbreaks) at the farms. The prevalence and abundance of parasites (e.g. trophozoites of *Perkinsus olseni*, zoites of APX), inflammatory tissue responses (haemocytosis, ceroid material) and abnormal tissue structures (especially in the gills and digestive tubules caused by pathogens and parasites) which are indicative of host health condition (Howard et al. 2004) were assessed with quantitative and semi-quantitative approaches.

## Materials and methods

### Sample collection

A total of 256 adult (2 years old) mussels (shell size ranging from 69.2 to 121 mm, mean ± SE of 94.2 ± 0.9 mm), from the same batch of seed were obtained from Kaiaua mussel farms (Whakatiwai, New Zealand: 37°02′51.2″S, 175°18′56.1″E) between April 2018 and September 2019 (20 samples each month randomly collected) and transported to the Auckland University of Technology (AUT) laboratory, Auckland, New Zealand. The monthly sampling covered the austral summer (average temperature 21 °C; months: December, January, February), autumn (average temperature 17 °C; months: March, April, May), winter (average temperature 12 °C; months: June, July, August) and spring (average temperature 14 °C; months: September, October, November).

### Histology

On arrival, specimens were processed on the same day. After measuring the weight and shell length, specimens were shucked and the soft parts sectioned to give a 2–5-mm-thick tissue slice (Howard et al. 2004) followed by standard histological processing (OIE 2016). The tissue slices were placed in histological cassettes, fixed in 4% formalin (1-part concentrated formalin solution plus 9 parts filtered seawater) for 48 h and then stored in 70% ethanol. Specimens were dehydrated in a series of ascending ethanol concentrations with two changes of xylene and then embedded in paraffin wax. Sections of 5 µm were obtained using a microtome. Slides with adhering tissue sections were dewaxed in xylene and then rehydrated through a descending series of ethanol concentrations followed by distilled water. Slides were stained with regressive Mayer's haematoxylin and eosin (H&E) stains, rinsed with deionized water and then taken through an ascending series of ethanol concentrations. After that, slides were rinsed in two changes of xylene. Then, DPX mountant was used to seal glass coverslips over the sections.

### In situ hybridization (ISH)

ISH was used to corroborate *Perkinsus* infections for *P. canaliculus* in which *Perkinsus* was encountered during the histological assessment. ISH analyses were conducted at the Animal Health Laboratory, Wallaceville, Upper Hutt, New Zealand. Sections of wax-embedded blocks corresponding to histological slides showing *Perkinsus* infections were used for ISH. Tissue sections of 5 µm in thickness, each on silane-prep™ slides (Sigma-Aldrich, France), were dewaxed in two changes of xylene for 5 min and 2 min and rehydrated in a descending alcohol series. Subsequently, the sections were rinsed in distilled water for 1 min and then in phosphate-buffered saline (PBS) for 1 min. The sections were treated with 100 µl proteinase K (concentration of 100 µg/ml) for 15 min at 37 °C, washed in 1% glycine in PBS for 5 min and briefly air-dried at room temperature before pre-hybridization. Sections were then incubated at 42 °C for 30 min with 100 µl of pre-hybridization buffer containing 10 µl of digoxigenin (DIG)-labelled probes (Eurogentec). A DIG-labelled ISH probe (10 µl) was prepared using the DIG PCR probe synthesis kit (Roche). A DIG-labelled probe to *Perkinsus olseni* was generated using the PCR primers Pols140F (5′ GAC CGC CTT AAC GGG CCG TGT T 3′) and PolsITS-600R (5′ GGR CTT GCG AGC ATC CAA AG 3′) (Moss et al. 2006). The DIG-labelled probe was used at a concentration of 5 ng/µl. A no-probe control (25 µl of hybridization buffer only) was included with each experiment. Target DNA and DIG-labelled probes were denatured at 95 °C for 5 min and the hybridization was carried out overnight at 42 °C. The next day, sections were washed in 2 × SSC at RT (2 × 5 min), in 0.75 × SSC at 42 °C (10 min) and in Solution 1 (100 ml maleic acid buffer, 205 g NaCl, pH 7.5) for 5 min at RT. Tissues were then blocked for 30 min at RT with 100 µl blocking solution 1. Specifically, the bound probe was detected using 100 µl anti DIG conjugate in blocking solution (2 h, RT). Washed with solution 1, 2 × 1 min and equilibrated with detection buffer (solution 2) for 2 min at RT. Slides were incubated for 1 h at RT in NBT/BCIP, a chromogenic substrate for alkaline phosphatase, diluted in solution 2 (100 µl) in the dark by covering a humid chamber with foil. The reaction was stopped by rinsing briefly in distilled water (10–15 dips) and briefly in 1 × PBS two times. Slides were counterstained for 1 min with Bismarck brown (0.5%), dehydrated with ethanol (96% ethanol 2 × 1 min and absolute ethanol 3 × 15 s) and cleared with xylene 3 × 15 s, mounted with a drop of Eukitt resin and cover slipped.

Controls for each *Perkinsus olseni* species-specific probe were tested in the same manner except that they received a hybridization buffer lacking probe during the hybridization step. Positive controls were tissue sections from the susceptible host (*P. canaliculus*) infected with *P. olseni* and negative controls were no-probe assays.

### Microscopic observations

Prepared histological (H&E) and ISH slides were examined under the microscope using 4 ×, 10 × and 40 × objectives. A 100 × objective (oil) was used to identify suspected pathogens and parasites as required. Morphology, size and location on the tissues were used to identify the parasites and their life stages (Bruno et al. 2006). Images were taken using a Leica DM2000 microscope with 40 × and 100 × objectives.

### Quantification of parasites

Each tissue was examined to record the number of parasites, which was then used to calculate the prevalence and gauge the abundance of parasites per individual or tissue, by visual examination of a single histological section (the entire section) for each case at 40 × and 100 × magnification. Prevalence and abundance of parasites were calculated according to Bush et al. (1997).

(i). Prevalence: prevalence is the number of hosts infected with one or more individuals of a particular parasite species divided by the number of hosts examined for that parasite species. It is commonly expressed as a percentage or proportion.

(ii). Abundance: the relative number of individuals of a particular parasite in/on a single host regardless of whether or not the host is infected. This is often expressed quantitatively for calculating the abundance of parasites and is calculated as the sum of all parasites in each examined host divided by the total number of hosts examined. In the present work, a semi-quantitative scale was used for determining the abundance of parasites, as not all the parasites could be counted in each host and one histological section per animal was used for histopathological evaluation. Abundance is therefore indicative and comparative rather than an absolute value. It differs from the intensity parameter in that intensity of "0" is not possible whereas an abundance of "0" is appropriate and therefore allows the inclusion of data from individuals with no detected infections.

### Quantitative and semi-quantitative evaluation of parasite infections, inflammatory tissue responses and abnormal tissue structures

The relative abundance of *P. olseni* was evaluated by a semi-quantitative grading scale modified from Ray's scale (Ray 1954) and adapted by da Silva et al. (2013). Ray's scale estimated the intensity of infection in cultured tissues and the scale of da Silva et al. estimated the intensity of infection with *Perkinsus* spp. from the tissues stained with Lugol's solution (diagnosed *Perkinsus* spp. by Ray's fluid thioglycollate medium). Thus, a modified scale was used in this study to evaluate *P. olseni* infection of histological sections (Table 1). Since the scale (0–4) modified from Ray's scale was not suitable for evaluating the abundance of APX, another semi-quantitative grading scale (0–5) modified by Hine

**Table 1** Whole animal grading for *P. olseni* infection (for each mussel, 10 random fields for each tissue were examined at 100 × magnification)

| Score | Grading scale | Description |
|---|---|---|
| 0 | Null infection | No trophozoites observed after extensive searching of all tissues for 5 min per slide |
| 1 | Very light infection | Up to 10 trophozoites (in total) observed after extensive searching of all tissues (5 min) |
| 2 | Light infection | 11–20 trophozoites observed per tissue |
| 3 | Moderate infection | > 20–40 trophozoites readily observed in all tissues, scattered throughout the slide |
| 4 | Heavy infection | > 40 trophozoites present in all tissues |

(2002b) was used in this study to assess the relative abundance of APX (Hine's scale classified APX-infected oysters into one to five grades of the intensity of infection). Although Hine's scale evaluated APX infection from histological sections, observed parasites (zoites) numbers were not included in that grading scale (1–5). In this modified grading scheme (0–5) for APX infection, observed parasites (zoites) number for each scale were mentioned (Table 2). In the current study, the semi-quantitative grading scales were based on grading from 0 (indicating no infection) to 4 (indicating a high level of infection) for *P. olseni* and from 0 (indicating no infection) to 5 (indicating a high level of infection) for APX.

The abundance of *P. olseni* and APX for different tissues, including gills, mantle, the connective tissue around digestive tubules, gonads and digestive epithelium was evaluated with a grading scale from 0 to 4 (Table 3). Hine's (2002b) scheme recorded the distribution of APX zoites in different areas (tissues) of the oyster (*Ostrea chilensis*) at different levels of intensity (grades 1–5) of infection. Observed parasites (zoites) numbers were not included in Hine's scale. However, in this modified grading scheme (0–4) for APX and (0–3) for *P. olseni* infection, observed parasites (trophozoites/zoites) number per tissue of mussels for each scale were mentioned (Table 3). Therefore, this modified scheme is more appropriate for evaluating tissue grading of *P. olseni* and APX infection in New Zealand Greenshell™ mussel (*Perna canaliculus*).

### Inflammatory responses

Inflammatory responses, such as haemocytosis and ceroid material in gills, mantle and the connective tissue around digestive tubules, gonads and digestive epithelium were evaluated semi-quantitatively on a scale of 1–3, where grade 1 = light (few, < 30 haemocytes per field at 40 × magnification), grade 2 = moderate (medium number, 31–200 of haemocytes per field at 40 × magnification) and grade 3 = heavy (high number, > 200–500 of haemocytes per field at 40 × magnification). All samples had some presence of haemocytes as this is the normal condition. Haemocyte proliferation (diffuse and focal haemocytosis) in haemolymph spaces and tissues were observed. In *Perna canaliculus*, haemocytosis can be a sign of infection by parasites (copepods or *Perkinsus*) (Webb and Duncan 2019) and heavy haemocytosis might be indicative of health problems.

A semi-quantitative scale of 1–3 was also applied to the amount of observed inflammatory response in the form of ceroid material, where grade 1 = light (low concentration of ceroid material), grade 2 = moderate (medium concentration of ceroid material) and grade 3 = heavy (high concentration of ceroid material) (Muznebin et al. 2021). Ceroid material is often recognized as intra and extracellular granules associated with haemocytes (granulocytes and hyalinocytes) and may indicate an immune response (Webb and Duncan 2019).

### Tissue conditions

Abnormal digestive tubule and gill morphologies were also assessed semi-quantitatively (Tables 4 and 5).

### Digestive tubule structures

A grading scale (1–4) was applied for evaluating digestive tubule structures (Table 4).

**Table 2** Whole animal grading for APX infection (for each mussel, 10 random fields for each tissue were examined at 100× magnification)

| Score | Grading scale | Description |
|---|---|---|
| 0 | Null infection | No parasites (zoites) observed after extensive searching of all tissues for 5 min per slide |
| 1 | Light infection | A few (< 5) parasites (zoites) present (in total), only observed after extensive searching of all tissues (5 min) |
| 2 | Light to moderate infection | > 5–20 parasites (zoites) observed per tissue |
| 3 | Moderate infection | > 20–40 parasites (zoites) readily observed in all tissues, scattered throughout the slide |
| 4 | Moderate to heavy | > 40–100 parasites (zoites) present in all tissues |
| 5 | Heavy infection | > 100 parasites (zoites) present and tissues congested |

**Table 3** Tissue grading for *P. olseni* (0–3) and APX (0–4) infection (for each mussel, 10 random fields for each tissue were examined at 100 × magnification)

| Score | Grading scale | Description |
|---|---|---|
| 0 | Null infection | Parasites (Trophozoites/zoites) absent in tissue |
| 1 | Low infection | 1–5 parasites (Trophozoites/zoites) present per tissue |
| 2 | Medium infection | > 5–20 parasites (Trophozoites/zoites) present per tissue |
| 3 | High infection | > 20–40 parasites (Trophozoites/zoites) present per tissue |
| 4 | Extremely high infection | > 40 parasites (Trophozoites/zoites) present per tissue |

**Table 4** Grading for digestive tubule structures (Fig. 1A–D)

| Score | Grading scale | Description |
|---|---|---|
| 1 | Very healthy | Normal, cruciform or Y-shaped lumen (D1) (Fig. 1A) |
| 2 | Healthy to fair | Normal lumen but sloughed epithelial cells inside lumen (D2) (Fig. 1B) |
| 3 | Poor | Small or no lumen (D3) (Fig. 1C) |
| 4 | Very poor | Large lumen, with a thin epithelial wall (D4) (Fig. 1D) |

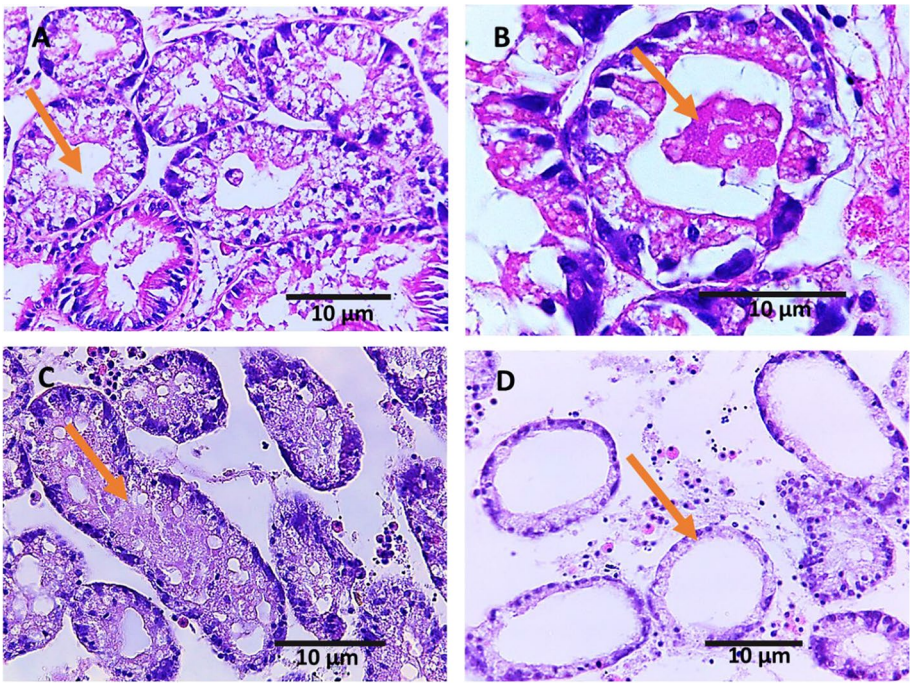


**Fig. 1** Digestive tubule structures (orange arrows) of *P. canaliculus*. **A** Normal or cruciform or Y-shaped lumen (D1). **B** Normal lumen but sloughed epithelial cells inside lumen (D2). **C** Small or no lumen (D3). **D** Large lumen, with a thin epithelial wall (D4). Scale bars = 10 µm (D1–D4 stand for the grading scale of digestive tubule pathology)

**Table 5** Grading for gill structures (Fig. 2A–D)

| Score | Grading scale | Description |
|---|---|---|
| 1 | Healthy | Normal structure with ciliated lateral cilia in the frontal zone, with ciliary discs in the abfrontal zone of gill filament and a medium number of haemocytes in the gill haemolymph space (G1) (Fig. 2A, B, C) |
| 2 | Fair | Ciliary discs appear in the abfrontal zone and destroyed/broken epithelium of gill filament and with few haemocytes in the gill haemolymph space (G2) (Fig. 2D) |
| 3 | Poor | Without ciliated lateral cilia, ciliary discs are atrophied/separated in the abfrontal zone of gill filament and very few haemocytes in the gill haemolymph space (G3) (Fig. 2E, F) |

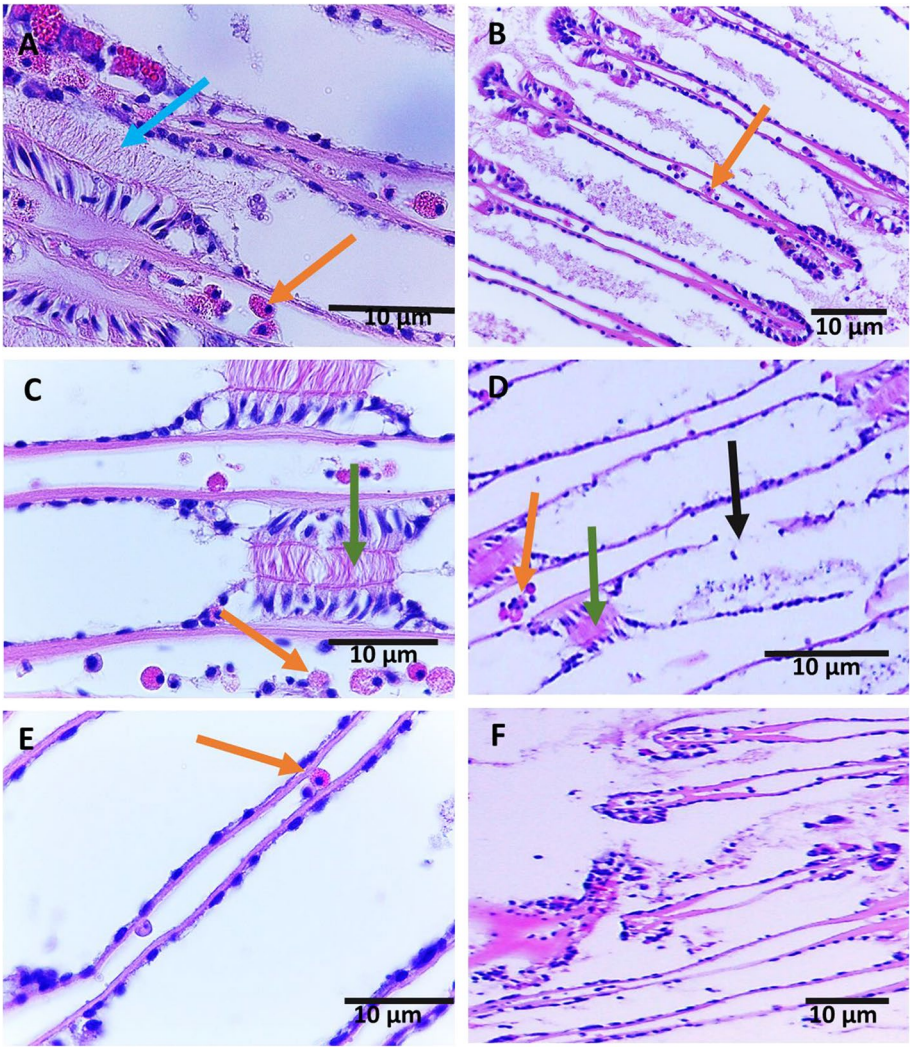


**Fig. 2** Gill structures of *P. canaliculus*. **A** and **B** Normal structure with ciliated lateral cilia (blue arrow) in the frontal zone of gill filament and with a medium number of haemocytes (orange arrows) in the gill haemolymph space (G1). **C** Normal structure with ciliary discs (green arrow) appear in the abfrontal zone of gill filament and with a medium number of haemocytes (orange arrow) in the gill haemolymph space (G1). **D** Ciliary discs (green arrow) appear in the abfrontal zone of gill filament and destroyed/broken epithelium (black arrow) of gill filament and few haemocytes in the gill haemolymph space (G2). **E** and **F** Without ciliated lateral cilia in the frontal zone of gill filament, ciliary discs are atrophied/separated in the abfrontal zone of gill filament and very few haemocytes (orange arrow) in the gill haemolymph space (G3). Scale bars = 10 µm (C1–C3 stand for the grading scale of gill pathology)

## Gill structures

A typical arrangement of mussel gills is composed of ascending and descending lamellae (Sunila and Lindström 1985). Each gill lamella consisted of filaments that have three different zones: frontal, intermediate and abfrontal (Gregory et al. 1996). The frontal zone

differs from other zones by the presence of ciliated cells. Filaments are laterally joined along their length at regular intervals by discrete ciliary discs or ciliary junctions. Ciliary discs are formed by condensed tufts of simple cilia, which are fixed from a base extended from the latero-abfrontal surfaces of the lamellae (Gregory et al. 1996; Akşit, and Mutaf 2014). Interfilamentar junction break up can occur either in a mechanical or chemical method (Sunila and Lindström 1985). The ciliary discs of an interfilamentar junction can be changed and separated by the exposure of copper or cadmium (Sunila and Lindström 1985). In this study, ciliary discs of *Perna canaliculus* can be atrophied or separated due to infection of parasites and might be indicative of health issues.

Gill structures were evaluated by a semi-quantitative grading scale (1–3), where grade 1 = healthy, grade 2 = fair and grade 3 = poor (Table 5).

### Statistical analyses

Statistical analyses were performed using Pearson's chi-square test with IBM® SPSS® Statistics software (version 23). Pearson's chi-square test statistics and the associated *p*-values were applied for the comparisons between seasons and parasites, the association between haemocytosis and parasites infection, the association between tissue conditions and parasites infection and the association between health conditions and parasite infections. Prevalence of parasites and season*year were analysed via two-way ANOVAs. Associations were statistically significant at $p < 0.05$.

## Results

### Epidemiological parameters of *P. olseni* infection

Histological sections followed by confirmatory in situ hybridization (ISH) resulted in the identification of *Perkinsus olseni* at an overall prevalence of 56%. *Perkinsus olseni*-infected mussels with prevalence 45% ($n = 112$) were in whole animal grade 1, whereas 0.4% ($n = 1$) were determined as grade 4.

Trophozoites of *P. olseni* were observed in the connective tissue surrounding the digestive tract epithelium, digestive tubules and gonads. They were also seen in the haemolymph space of gills and outside the gill epithelium, in space between digestive tubules, mantle and adductor muscle (Fig. 17). Most organs/tissues showed a low prevalence (2–25%) of *P. olseni*. In gills, 20% of the mussels were infected with *P. olseni* at level 1. In the mantle, 10% of mussels showed *P. olseni* infection at grade 1 and 1% were suffering from a severe infection at grade 3. In the connective tissue around digestive tubules, 30% and 9% of the mussels were infected with *P. olseni* at grades 1 and 2, respectively. Thirteen to fifteen percent of the mussels presented infection by *P. olseni* at grade 1 in the connective tissue surrounding digestive epithelium and gonads (Fig. 3).

The highest prevalence (80%-95%) of mussels with *P. olseni* infection was recorded in March 2019, May 2019 and July 2019, respectively (Table 6).

A high number (60–61%) of mussels showed *P. olseni* infection at grade 1 in October 2018, March 2019, May 2019, June 2019 and July 2019 (Fig. 4).

The highest prevalence (70%, $n = 53$) of mussels with *P. olseni* infection was recorded in autumn. Fifty-three of mussels presented with *P. olseni* infection in both spring and summer (Fig. 5).

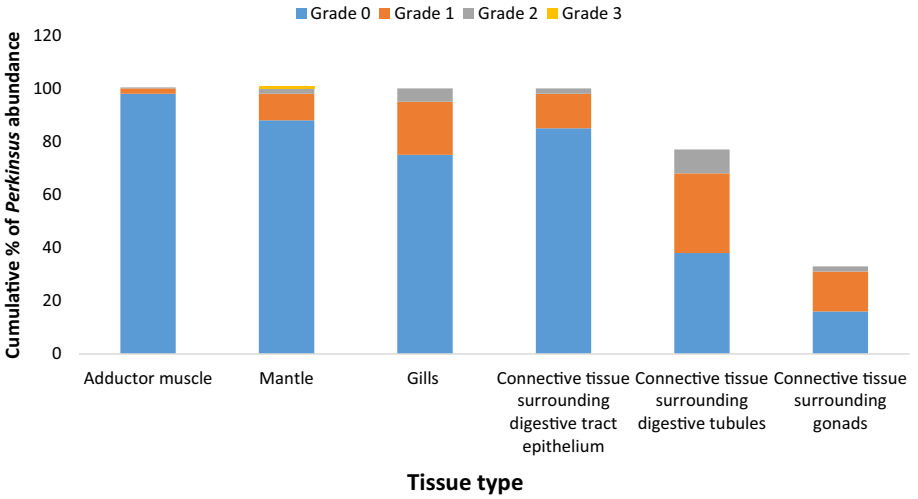


**Fig. 3** Cumulative percentage of *P. olseni* abundance grading (0–3) of mussels in different tissues, $N = 256$ (abundance here applied only to organs/tissues rather than the whole mussel)

Pearson's chi-square test resulted in a significant association between seasons (summer, winter, spring and autumn) and the presence of *P. olseni* in mussels ($p$-value = 0.029). The prevalence of *P. olseni* in summer, winter and spring are the same. However, the prevalence of *P. olseni* in autumn was significantly higher than in the other three seasons.

A significant ($p = 0.004$) number of *P. olseni* was detected in mussels in both years (2018 and 2019) and seasons (summer, winter, spring and autumn) (2-way ANOVAs) (Supplementary Table S1).

**Table 6** Prevalence of *P. olseni* associated with mussels in different months ($n$ = number of mussels infected with parasites)

| Months & years | Sample/mussels examined ($N$) | *Perkinsus olseni* prevalence (%) | *Perkinsus olseni* frequency ($n$) |
|---|---|---|---|
| Apr. 2018 | 18 | 50 | 9 |
| May 2018 | 20 | 45 | 9 |
| July 2018 | 20 | 15 | 3 |
| Oct. 2018 | 20 | 65 | 13 |
| Nov. 2018 | 20 | 60 | 12 |
| Jan. 2019 | 20 | 35 | 7 |
| Feb. 2019 | 20 | 70 | 14 |
| Mar. 2019 | 20 | 95 | 19 |
| May 2019 | 18 | 80 | 16 |
| June 2019 | 20 | 75 | 15 |
| July 2019 | 20 | 80 | 16 |
| Aug. 2019 | 20 | 30 | 6 |
| Sep. 2019 | 20 | 35 | 7 |

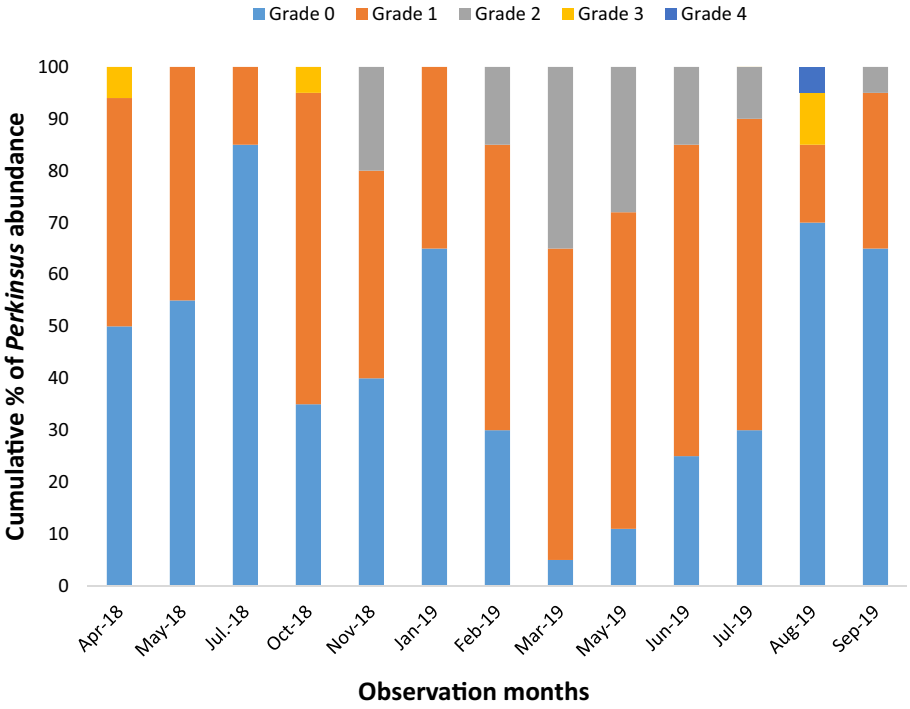


**Fig. 4** Cumulative percentage of *P. olseni* abundance grading (whole animal grading = 0–4) of mussels in different months

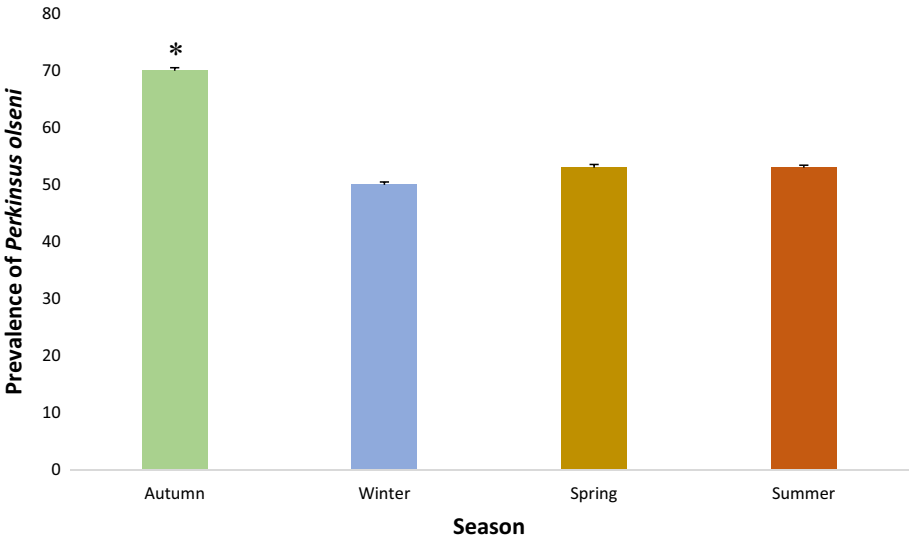


**Fig. 5** Mean prevalence (± SE) of *P. olseni* in summer, winter, spring and autumn [summer ($n = 40$), winter ($n = 80$), spring ($n = 60$), autumn ($n = 76$)]. [* indicates significance]

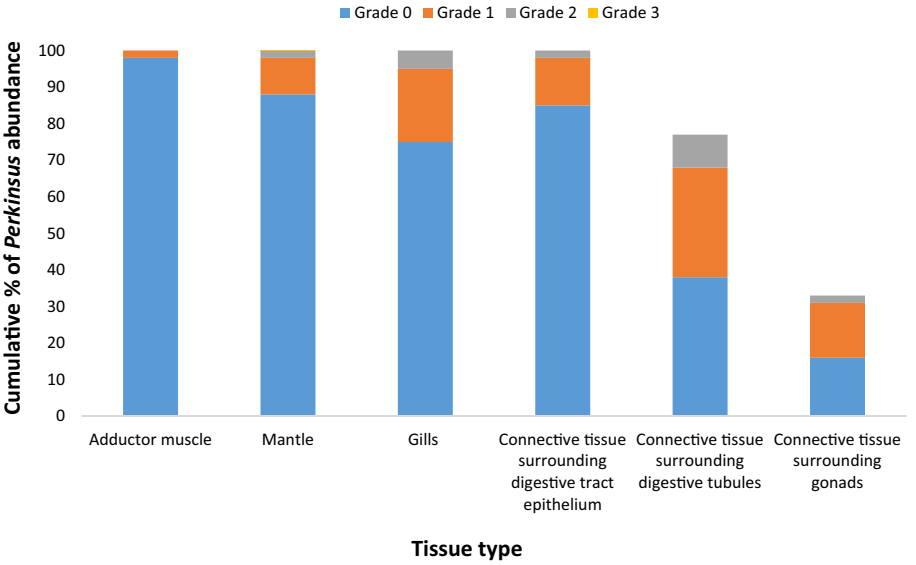


**Fig. 6** Cumulative percentage of *P. olseni* abundance grading (0–4) of mussels in different seasons (summer, winter, spring and autumn) [summer ($n=40$), winter ($n=80$), spring ($n=60$, autumn ($n=76$)]

In autumn, mussel samples presented a high abundance ($n=40$, 53%) of *P. olseni* with grade 1 (very light infection) and 16% of the mussels ($n=12$) presented *P. olseni* abundance with grade 2.8% ($n=6$) of the mussels showed a mild infection (grade 2) by *P. olseni* in winter, spring and summer. In winter, 4% ($n=3$) of mussels were infected with *P. olseni* at a medium level (grade 3). In contrast, grade 3 infection of *P. olseni* in mussel samples was not recorded during the summer. The severe infection of *P. olseni* (grade 4) in mussels was only noticed in winter (Fig. 6). There was no significant association between seasons (summer, winter, spring and autumn) and *P. olseni* abundance gradings (0–4) in mussels (Pearson's chi-square test, $p=0.233$).

## Epidemiological parameters of APX infection

Out of total 256 mussel analyzed, 78% ($n=199$) were APX positive. APX-infected mussels with prevalence 27% (this is prevalence of the infection at this grade) ($n=68$) were in whole animal grade 2 and only 2% ($n=4$) had severe infection with abundance at grade 5. Twenty-six percent of mussels ($n=67$) were at APX abundance grade 1 and 22% ($n=57$) had no observable APX infection (whole animal abundance grade 0).

Individual tissue grade 0 (absence of infection) with APX in the gills, mantle, adductor muscle, the connective tissue surrounding digestive tract epithelium, digestive tubules and gonads were observed from 45 to 79% mussels. For 31–37% of APX-infected mussels with abundance grade 1, infections were observed in the mantle, and the connective tissue surrounding digestive tract epithelium and gonads. On the other

hand, only 0.4% ($n=1$)–1% ($n=3$) of mussels presented a tissue grade 4 (extremely high) infection of APX in the mantle and the connective tissue around digestive tubules and digestive epithelium. Three percent of mussels was suffering from a high infection of APX with individual tissue grade 3 in both the mantle and the connective tissue around digestive tubules (Fig. 7).

The highest prevalence (100%) of mussels with APX infection was observed in June 2019, August 2019 and September 2019 (Table 7).

The highest prevalence (88%, $n=70$) of mussels with APX infection was noted in winter. On the other hand, only 55% of mussels had APX infection in summer, a lower percentage than in other seasons (Fig. 8).

There was a highly significant association between seasons (summer, winter, spring and autumn) and the presence of APX in mussels (Pearson's chi-square test, $p$-value $<0.001$). The prevalence of APX in winter and spring was almost the same. However, the prevalence of APX in autumn and summer was different from each other and also from the other two seasons.

A significant ($p=0.004$) number of APX was detected in mussels in both years (2018 and 2019) and seasons (summer, winter, spring and autumn) (2-way ANOVAs) (Supplementary Table S2).

A high number (45%) of mussels with heavy APX infection at whole animal grade 4 were recorded in June 2019. Fifty percent mussels were infected with APX abundance grade 3 in July 2019. The highest number of mussels (60%) with APX abundance whole animal grade 2 was found in September 2019. A higher number (55%) of mussels had no observable APX infection (grade 0) in February 2019. Fifty-five percent of mussels presented with APX abundance grade 1 in March 2019. Mussels with medium to heavy (grade 3–grade 5) infection of APX was recorded in May 2019, June 2019, July 2019 and August 2019 (Fig. 9).

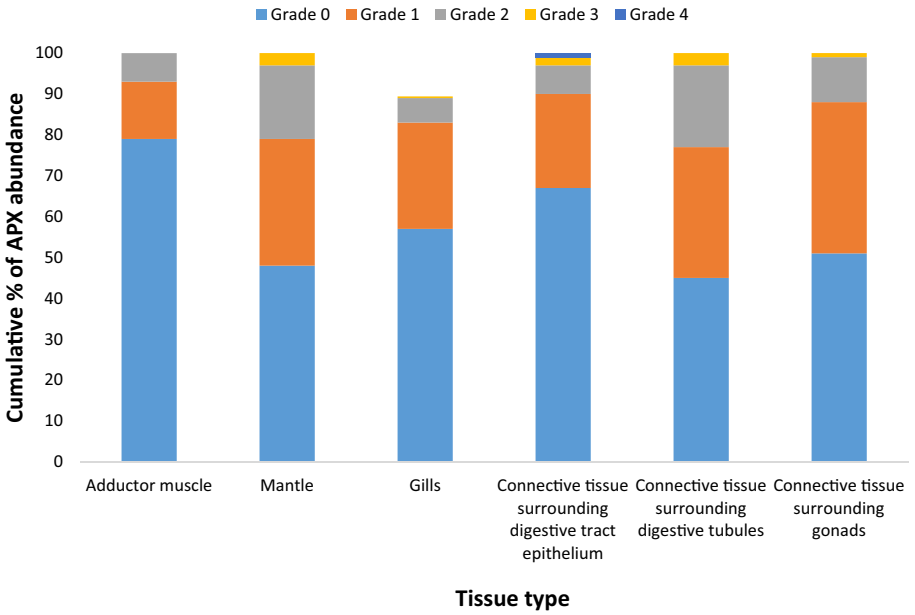


**Fig. 7** Cumulative percentage of APX abundance grading (0–4) of mussels in different tissues, $N=256$ (abundance here applied only to organs/tissues rather than the whole mussels)

**Table 7** Prevalence of APX associated with mussels in different months ($n$ = number of mussels infected with parasites)

| Months & years | Sample/mussels examined (N) | APX prevalence (%) | APX frequency ($n$) |
|---|---|---|---|
| Apr. 2018 | 18 | 72 | 13 |
| May 2018 | 20 | 55 | 11 |
| July 2018 | 20 | 60 | 12 |
| Oct. 2018 | 20 | 85 | 17 |
| Nov. 2018 | 20 | 70 | 14 |
| Jan. 2019 | 20 | 65 | 13 |
| Feb. 2019 | 20 | 45 | 9 |
| Mar. 2019 | 20 | 75 | 15 |
| May 2019 | 18 | 94 | 17 |
| June 2019 | 20 | 100 | 20 |
| July 2019 | 20 | 90 | 18 |
| Aug. 2019 | 20 | 100 | 20 |
| Sep. 2019 | 20 | 100 | 20 |

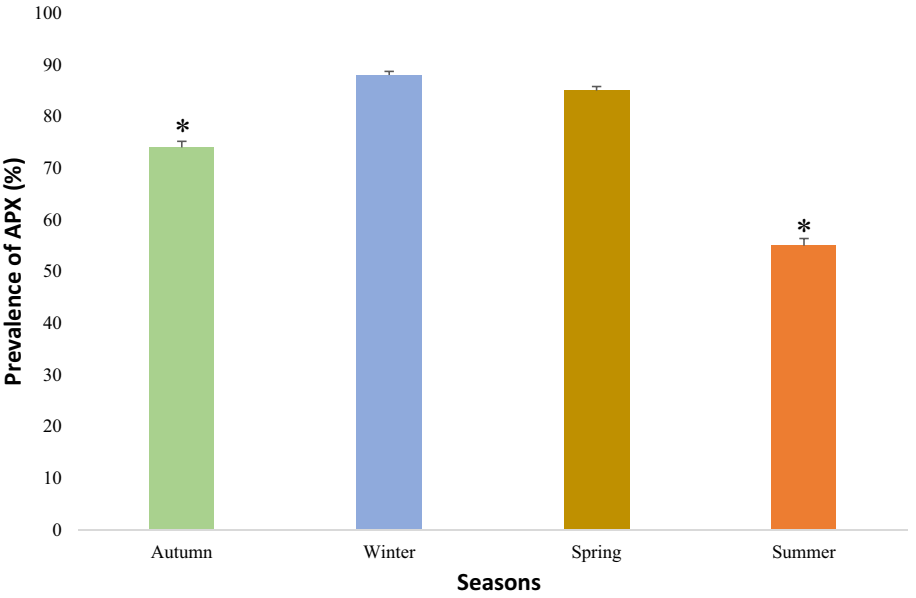


**Fig. 8** Mean prevalence (±SE) of APX in summer, winter, spring and autumn [summer ($n$ = 40), winter ($n$ = 80), spring ($n$ = 60), autumn ($n$ = 76)]. [* indicates significance]

In summer, 45% of the mussels ($n$ = 18) had no observable APX infection (grade 0). On the other hand, 40–43% of mussels presented with an APX abundance at grade 1 in autumn and grade 2 in spring. Four percent ($n$ = 3) and 1% ($n$ = 1) of the mussels were infected with a severe APX infection (abundance grade 5) in winter and autumn respectively. In the winter season, 15% of the mussels ($n$ = 12) showed a high level (grade 4) of APX infection (Fig. 10).

There was a highly significant association between seasons and APX abundance gradings of mussels (Pearson's chi-square test, $p$-value < 0.001).

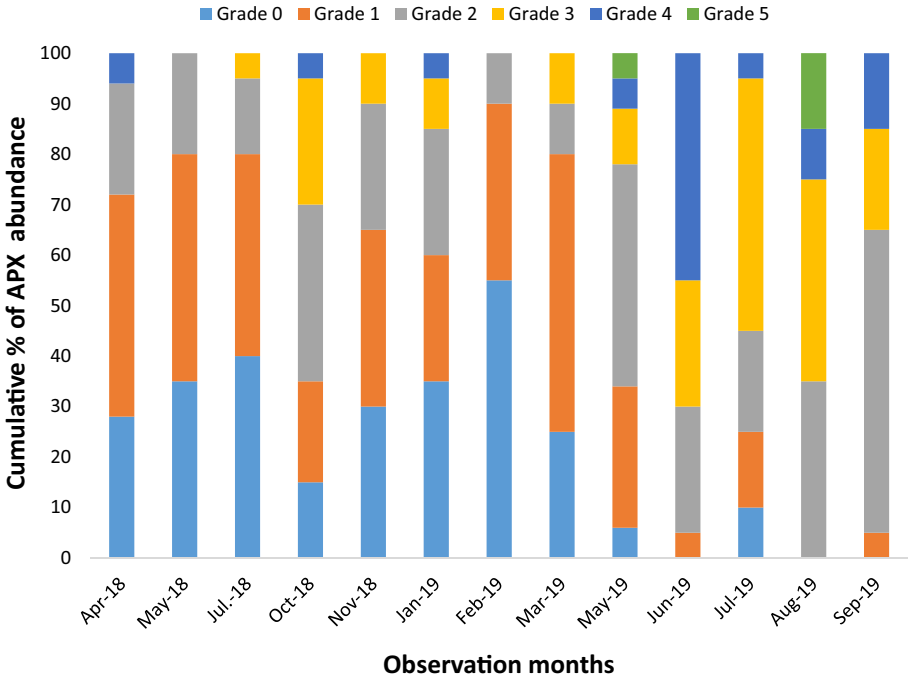


**Fig. 9** Cumulative percentage of APX abundance grading (whole animal grading = 0–5) of mussels in different months

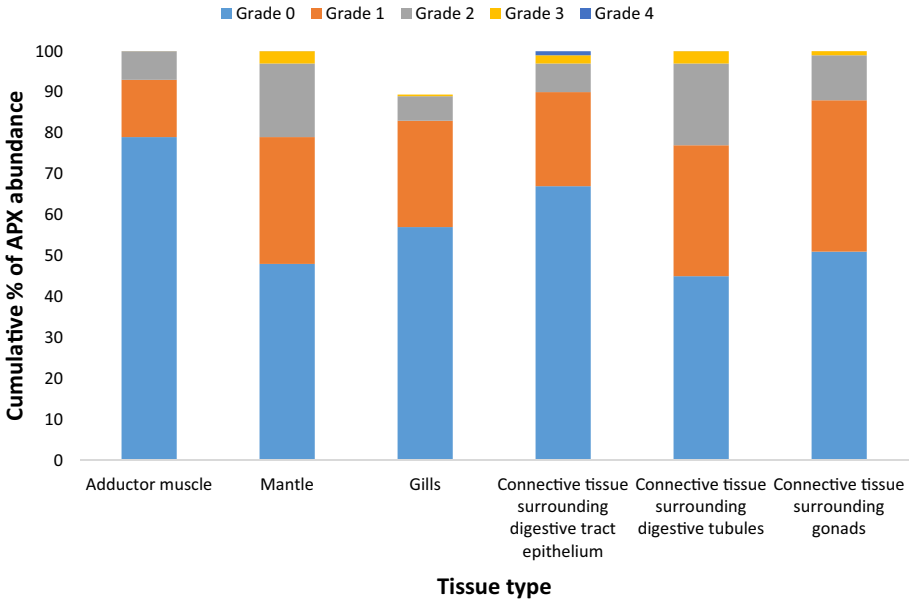


**Fig. 10** Cumulative percentage of APX abundance grading (0–5) of mussels in different seasons (summer, winter, spring and autumn) (summer ($n = 40$), winter ($n = 80$), spring ($n = 60$, autumn ($n = 76$))

### Co-infection of *P. olseni* and APX

There was a significant association between *P. olseni* and APX infection in mussels (Pearson's chi-square test, $p$-value < 0.017) Table 8.

Heavily infected mussels with *Perkinsus* did not tend to carry heavy APX burdens (Supplementary Table S3).

### Epidemiological parameters of other parasite infections

Four percent ($n = 11$) of the mussels analyzed were associated with bacteria (bacilli and cocci), 1–4% were infected with copepods ($n = 2$), *M. rapuae* ($n = 3$) and IMCs ($n = 4$) (Fig. 11).

The highest prevalence of infection for most of the parasite species was recorded during September 2019, except for copepods, which were only present in June 2019. In April 2018, 28% of mussels with bacterial association were observed (Table 9).

All the parasites showed the same prevalence (3%, $n = 2$) in the winter season. However, the highest prevalence (10%) of mussels with the bacterial association was recorded

**Table 8** Statistical analysis: chi-square test for the association between *P. olseni* and APX infection in mussels.

| Parasite | | APX | | Chi-square test for association (*P*-value) |
|---|---|---|---|---|
| | | No | Yes | |
| *Perkinsus olseni* | Absent | 32 | 78 | 0.017 |
| | Present | 25 | 121 | |

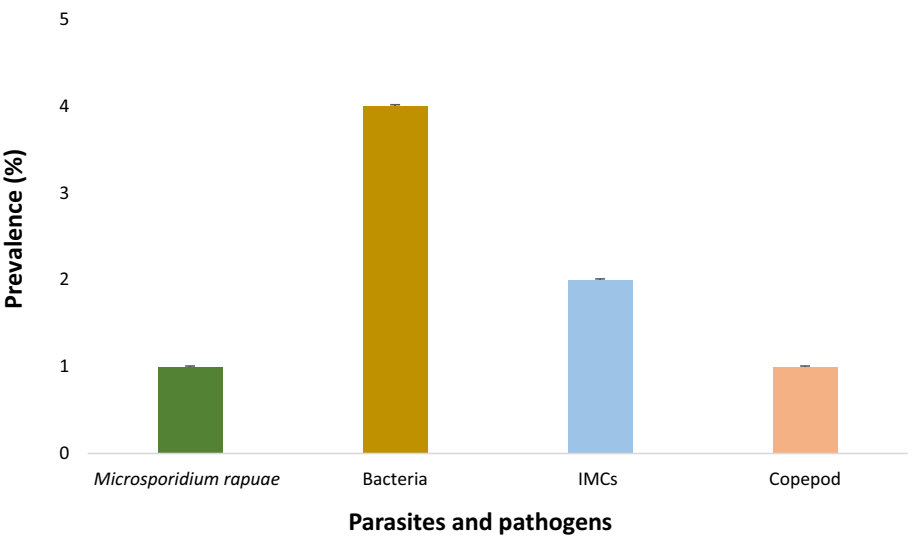


**Fig. 11** Mean prevalence (± SE) of parasites and microorganisms associated with mussels (per slide of the whole animal) ($N = 256$)

**Table 9** Prevalence of parasites associated with mussels in different months ($n$ = number of mussels infected with parasites)

| Months & years | Sample/ mussels examined ($N$) | Bacteria | | *M. rapuae* | | IMCs | | Copepod | |
|---|---|---|---|---|---|---|---|---|---|
| | | $n$ | % | $n$ | % | $n$ | % | $n$ | % |
| Apr. 2018 | 18 | 5 | 28 | 0 | 0 | 0 | 0 | 0 | 0 |
| May 2018 | 20 | 3 | 15 | 0 | 0 | 1 | 5 | 0 | 0 |
| July 2018 | 20 | 0 | 0 | 1 | 5 | 0 | 0 | 0 | 0 |
| Oct. 2018 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nov.2018 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Jan.2019 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Feb.2019 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mar. 2019 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| May 2019 | 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| June 2019 | 20 | 0 | 0 | 1 | 5 | 2 | 10 | 2 | 10 |
| July 2019 | 20 | 1 | 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| Aug. 2019 | 20 | 1 | 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sep. 2019 | 20 | 1 | 5 | 1 | 5 | 1 | 5 | 0 | 0 |

in autumn. There was a significant association between seasons (summer, winter, spring and autumn) and the presence of bacteria in mussels (Pearson's chi-square test, $p$-value < 0.014). There was no association between seasons (summer, winter, spring and autumn) and the presence of copepods, *M. rapuae* and IMCs in mussels.

## Immunological tissue responses (haemocytosis)

Haemocytosis was observed in the mantle and the connective tissue around digestive tubules, digestive epithelium and gonads (Fig. 12A–D). Haemocytosis was significantly correlated to the presence of *P. olseni* in the tissues (Pearson chi-square test, $p$-value = 0.03).

Overall percentages (15–18%) of haemocytosis were recorded in the mantle and in the connective tissue around digestive tubules and gonads, whereas only 2% were observed in gills and the connective tissue surrounding the digestive epithelium of mussels.

A medium level (grade 2) of haemocytosis was observed in the connective tissue surrounding digestive tubules of 68% mussels, whereas 21% of mussels showed haemocytosis at a high level (grade 3) in the connective tissue surrounding the digestive epithelium. Nine to ten percent of mussels showed heavy haemocytosis (grade 3) in the connective tissue surrounding digestive tubules, gills and mantle. Forty-five percent of mussels showed haemocytosis at a low level (grade 1) in the connective tissue surrounding gonads (Fig. 13).

## Immunological tissue responses (ceroid/brown material)

Ceroid/ brown material accumulation (light, moderate and heavy) was recorded in different tissues of *P. canaliculus* including mantle, gills and the connective tissue surrounding gonads and digestive tubules (Fig. 14A–D). A chi-square test indicated a significant association ($p$-value = 0.016) between the brown material accumulation and *Perkinsus* infection.

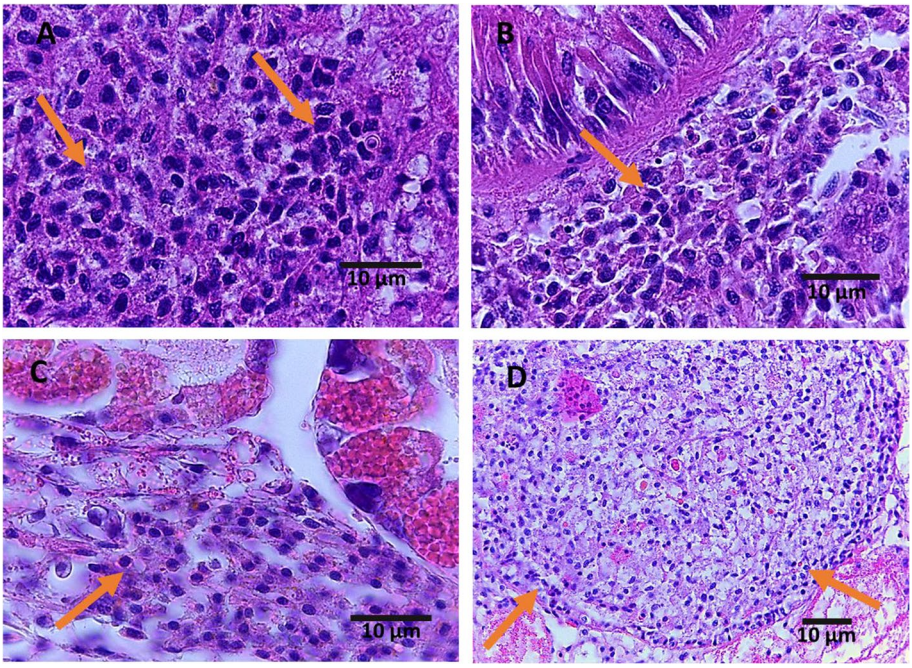


**Fig. 12** Haemocytosis (orange arrows) in the different tissues of *P. canaliculus*. **A** Mantle. **B** Connective tissue surrounding digestive tract epithelium. **C** Connective tissue surrounding digestive tubules. **D** Connective tissue surrounding gonads. Scale bars = 10 µm

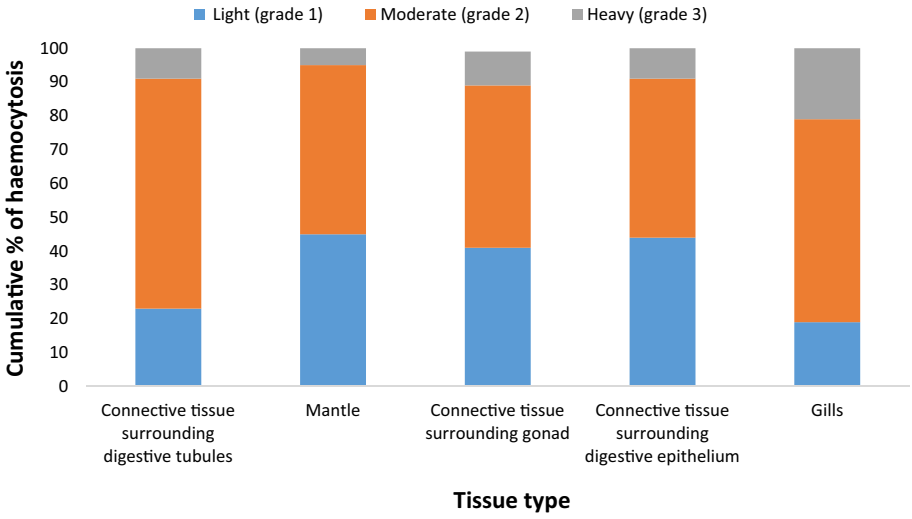


**Fig. 13** Cumulative percentage (occurrence rate) of haemocytosis grading (0–3) in different tissues of mussels, $N = 256$, % = occurrence rate (the term occurrence rate was used rather than prevalence to avoid confusion between organ/tissue values and prevalence)

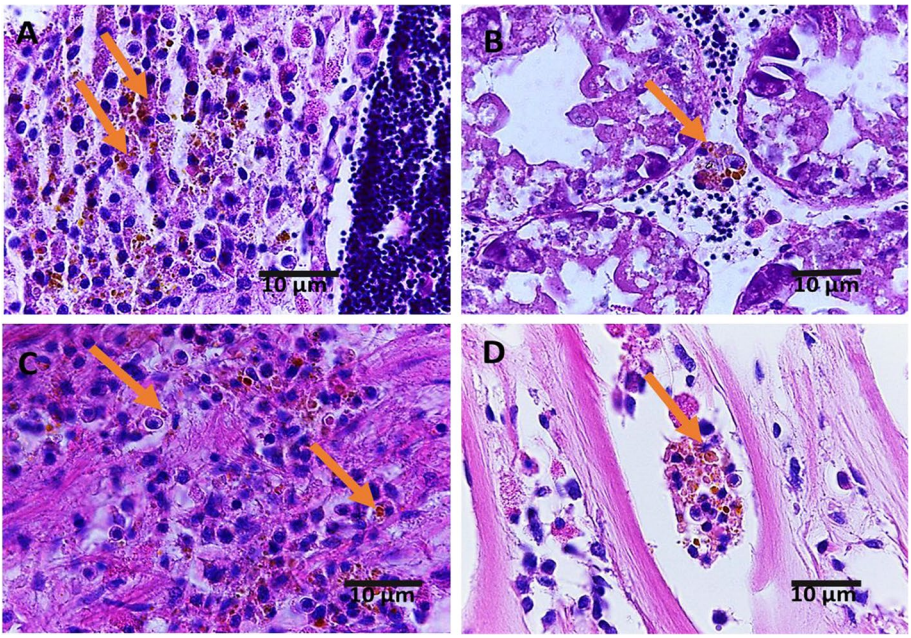


**Fig. 14** Ceroid/brown material accumulation (orange arrows) in *P. canaliculus*. **A** Connective tissue surrounding gonads. **B** Connective tissue surrounding digestive tubules. **C** Mantle. **D** Gills (blood space). Scale bars = 10 µm

A significant association ($p$-value = 0.014) was also observed between the brown material accumulation and APX infection (chi-square test). The presence of ceroid material was often noted in the tissues of mussels where APX was detected (Fig. 14A–D).

Seventy percent of the mussels analyzed showed ceroid accumulation at low levels (grade 1) in gills and the connective tissue surrounding the digestive epithelium. In the connective tissue surrounding digestive tubules, 66% and 10% of mussels showed ceroid material accumulation at a medium level (grade 2) and high level (grade 3), respectively (Fig. 15) (light/grade 1 = normal level).

## Digestive tubule structures

Seventy-nine percent (occurrence rate) of the mussels analyzed showed digestive tubule structures with the normal or cruciform or Y-shaped lumen (D1) ($n$ = 202). However, 10% (occurrence rate) of mussels ($n$ = 25) showed digestive tubule structure with normal lumen but sloughed epithelial cells inside lumen (D2). Eighteen percent (occurrence rate) of mussels ($n$ = 45) showed digestive tubule structures with the large lumen, with a thin epithelial wall (D4) and small or no lumen (D3). A chi-square test indicated a highly significant association ($p$-value = 0.001) between digestive tubule structures and *Perkinsus* infection. However, there was an insignificant association ($p$-value = 0.484) between digestive tubule structures and APX infection.

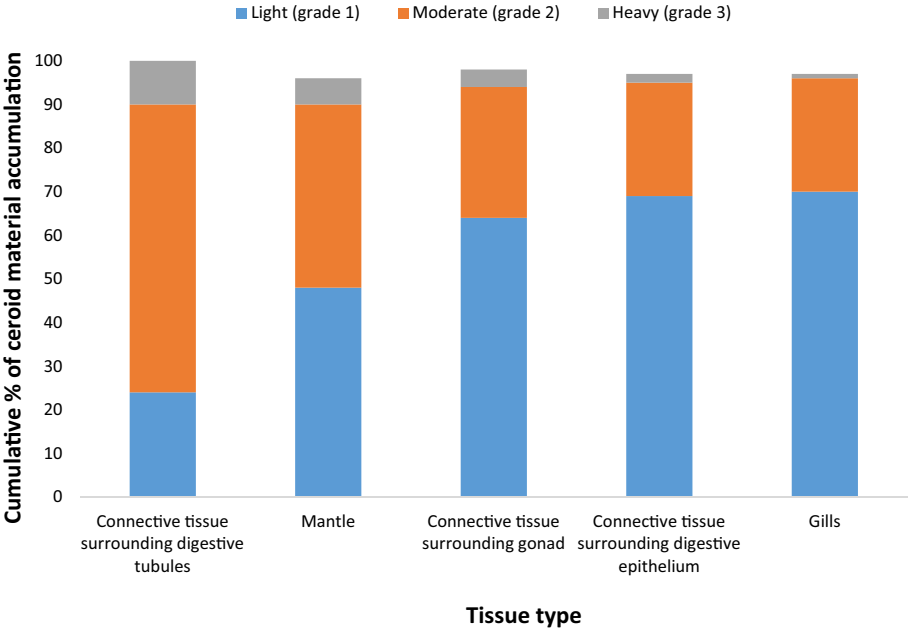


**Fig. 15** Cumulative percentage of ceroid material accumulation levels (0–3) in different tissues, $N = 256$, % = occurrence rate

## Gill structures

Out of 256 mussels, 190 samples (occurrence rate of 74%) analyzed showed gills with normal structure, with ciliated lateral cilia, ciliary discs and medium number of haemocytes in the gill haemolymph space (G1), whereas 10% (occurrence rate) of samples ($n = 26$) analyzed showed gills with ciliary discs, destroyed/broken epithelium of gill filament and few haemocytes in the gill blood space (G2). Twenty-four percent (occurrence rate) of samples ($n = 62$) analysed showed gills without ciliated lateral cilia, ciliary discs are atrophied/separated and with very few haemocytes in the gill blood space (G3).

A significant association between presence of parasites and health condition (healthy and unhealthy) of mussels was observed (Pearson Chi-square test, $p$-value = 0.030) Table 10.

**Table 10** Statistical analysis: chi-square test for the association between parasites and health condition of mussels

| Health condition | | Parasites | | Chi-square test for association ($P$-value) |
|---|---|---|---|---|
| | | No | Yes | |
| Healthy or unhealthy | Healthy | 12 | 124 | 0.030 |
| | Unhealthy | 21 | 99 | |

### Observation of pathogens and parasites

#### Infection with *P. olseni*

Trophozoites of *P. olseni* are spherical cells, 3–5 µm in diameter, with a large, eccentric vacuole occupying most cytoplasm and a peripheral nucleus, giving them a "signet ring" appearance (Fig. 17A–H). Phagocytosis of *P. olseni* by mussel haemocytes was frequently ($n=120$, detection rate 47%) observed (Figs. 16A, B and 17).

#### Specificity test of *Perkinsus olseni* by ISH

The digoxigenin-labelled probes (species-specific ISH assay) demonstrated hybridization to *P. olseni* cells in digestive epithelium, digestive tubules, gills, mantle, adductor muscle and gonads as blue staining (Fig. 18A, B) and all controls performed as expected. No hybridization of other parasite cells was observed. No probe labelling was detected within slides that had been incubated either in the absence of the probe or from the negative control tissue sections (Fig. not shown).

#### Infection with APX

APX zoites were observed in the connective tissue surrounding the digestive tract epithelium, digestive tubules and gonads. They were also seen in the haemolymph space of gills, mantle and adductor muscle (Fig. 19). The zoites are oval/elongated and elliptical, 5–8 µm long and 3–5 µm wide with a round nucleus located to the middle of their length (Fig. 19 and Fig. 20). Phagocytosis of APX by mussel haemocytes was also observed (Fig. 19D).

#### Infection with other parasites

Other parasites and pathogens were noted (Fig. 20A–F). *Microsporidium rapuae* (sporocyst) was observed in connective tissue near the digestive epithelium. Copepods were found inside the gut lumen. Identification of the copepods is difficult in the histological section, but they might be *Pseudomyicola spinosus* or *Lichomolgus uncus* as they have been reported by Hine (2002a). Intracellular microcolonies of bacteria (IMCs) were observed as colonies or cysts in muscle tissues and in the mantle (note: Rickettsia-like organisms (RLOs) are now referred to as IMCs (Cano et al. 2020)). Bacteria (bacilli and cocci) were found in the gills, mantle, connective tissues near gonads, digestive tubules and digestive epithelium of mussels.

## Discussion

#### Infection with *P. olseni*

*Perkinsus olseni* was tentatively identified by histology and confirmed by ISH in mussels (*P. canaliculus*) (prevalence 56%) collected from Coromandel, North Island, New Zealand. *P. olseni* was also identified by histology and Ray's fluid thioglycollate medium (RFTM) from *P. canaliculus* in the South Island, New Zealand (OIE 2017). In this study, 56% of mussels sampled were infected with *P. olseni*. A similar finding was recorded in clams (*Ruditapes philippinarum*) in Isahaya Bay, Japan by Choi (2002). A high prevalence

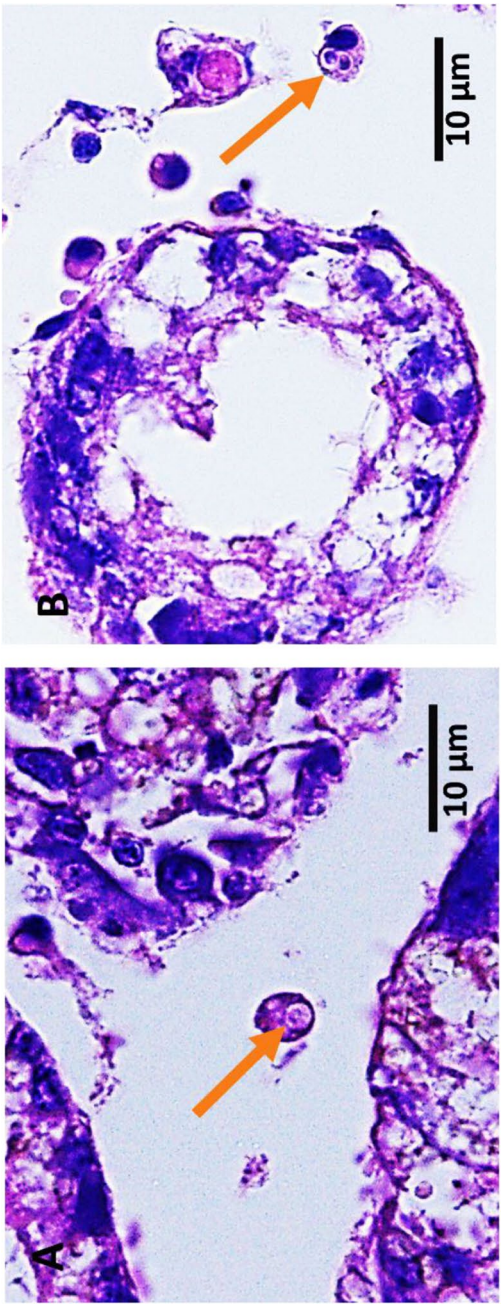


**Fig. 16** **A** and **B** Histology of Greenshell™ mussel (*P. canaliculus*) showing *P. olseni* cells phagocytosed by haemocytes in space between digestive tubules (orange arrows). Scale bar = 10 µm.

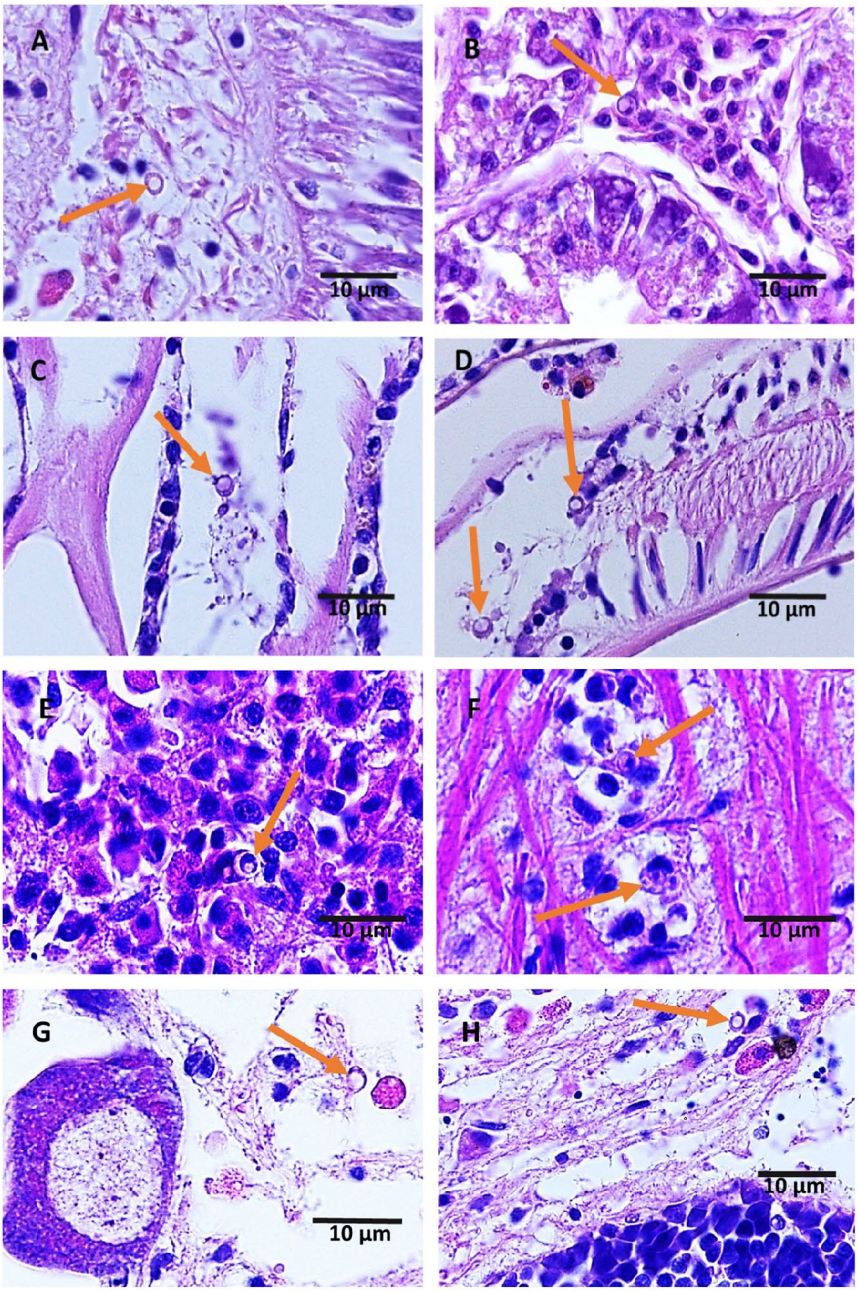


**Fig. 17** Trophozoites (orange arrows) of *P. olseni*. **A** Connective tissue surrounding digestive epithelium. **B** Connective tissue surrounding digestive tubules. **C** Gills (haemolymph space). **D** Gills (near the gill epithelium). **E** Mantle. **F** Adductor muscle. **G** Connective tissue surrounding female gonads. **H** Connective tissue surrounding male gonads. Scale bars = 10 µm

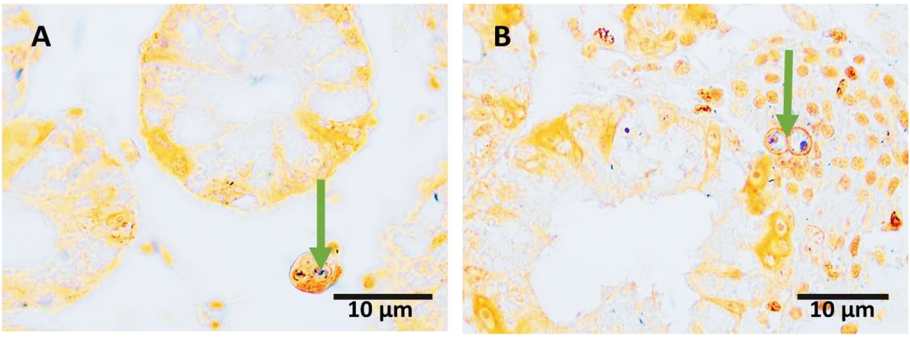


**Fig. 18** **A** and **B** ISH of New Zealand Greenshell™ mussels showing blue labelled *P. olseni* trophozoites near digestive tubules (green arrows). Scale bar = 10 µm

(97–100%) of *P. olseni* in clams (*R. philippinarum*) was observed in Ariake Bay, Kyushu, Japan and Komsoe Bay, Korea by Park et al. (1999, 2008). Combined, these findings indicate that high infection rates of *P. olseni* are recorded in bivalves, including *P. canaliculus*.

In this study, trophozoites of *P. olseni* were recorded in connective tissues surrounding digestive tract epithelium, digestive tubules and gonads, and also in gills, mantle and adductor muscle. *Perkinsus* trophozoites were also observed in the same tissues of Manila clams (*Ruditapes philippinarum*) by Choi (2002) and Park and Choi (2001). In the present study, trophozoites of *P. olseni* were mainly recorded in the connective tissue surrounding digestive tubules and gills, but fewer were noticed in the adductor muscle. Choi (2002) also reported similar findings in Manila clams.

Seasonal variations of *P. olseni* infection in *P. canaliculus* were demonstrated in this study. However, Yang et al. (2012) observed no clear seasonality in *P. olseni* prevalence in adult Manila clam (*R. philippinarum*) in Gomsoe Bay, Korea but reported the proliferation of *P. olseni* in clam tissues during winter. Park et al. (1999) noted extremely high levels of *P. olseni* in clams in late summer in Gomsoe Bay, Korea. Uddin et al. (2010) also observed a seasonal difference in the prevalence (38–97%) annually, and the highest prevalence was in fall/autumn in Manila clams in Incheon Bay, Korea, when most clams are in the post-spawning stage. In contrast, Villalba et al. (2005) found a high prevalence of *P. olseni* infection in the clam *Tapes decussatus* during spring and summer when surface water temperatures increased. According to Yang et al. (2012), food accessibility is relatively low during late fall/autumn to winter, and during summer and early fall/autumn, spawning activities exhaust clams, which may impair their defence capacity. Therefore, the combined impacts of food shortage and poor physiological condition of clams will accelerate the spread of *P. olseni* in fall/autumn and winter (Yang et al. 2012). Further research may focus attention to find out the link between *P. olseni* and seasonal variations, and the food supply and physiological conditions of the mussels.

*Perkinsus olseni* trophozoites were detected in the haemocytosis/haemocytic infiltration of mussel tissues and phagocytosis of *P. olseni* by the haemocytes was frequently seen (detection rate 47%). In addition, a significant association was recorded between the inflammatory tissue responses (haemocytosis and brown material accumulation) and *Perkinsus* infection in this study. According to Choi and Park (2010), heavy infection with *P. olseni* often involves tissue inflammation and results in mass mortalities in clam and oyster populations. Villalba et al. (2004) noted that severe infection with *Perkinsus* spp. in oysters (*Crassostrea virginica*) induces enormous haemocytic infiltration of tissues where

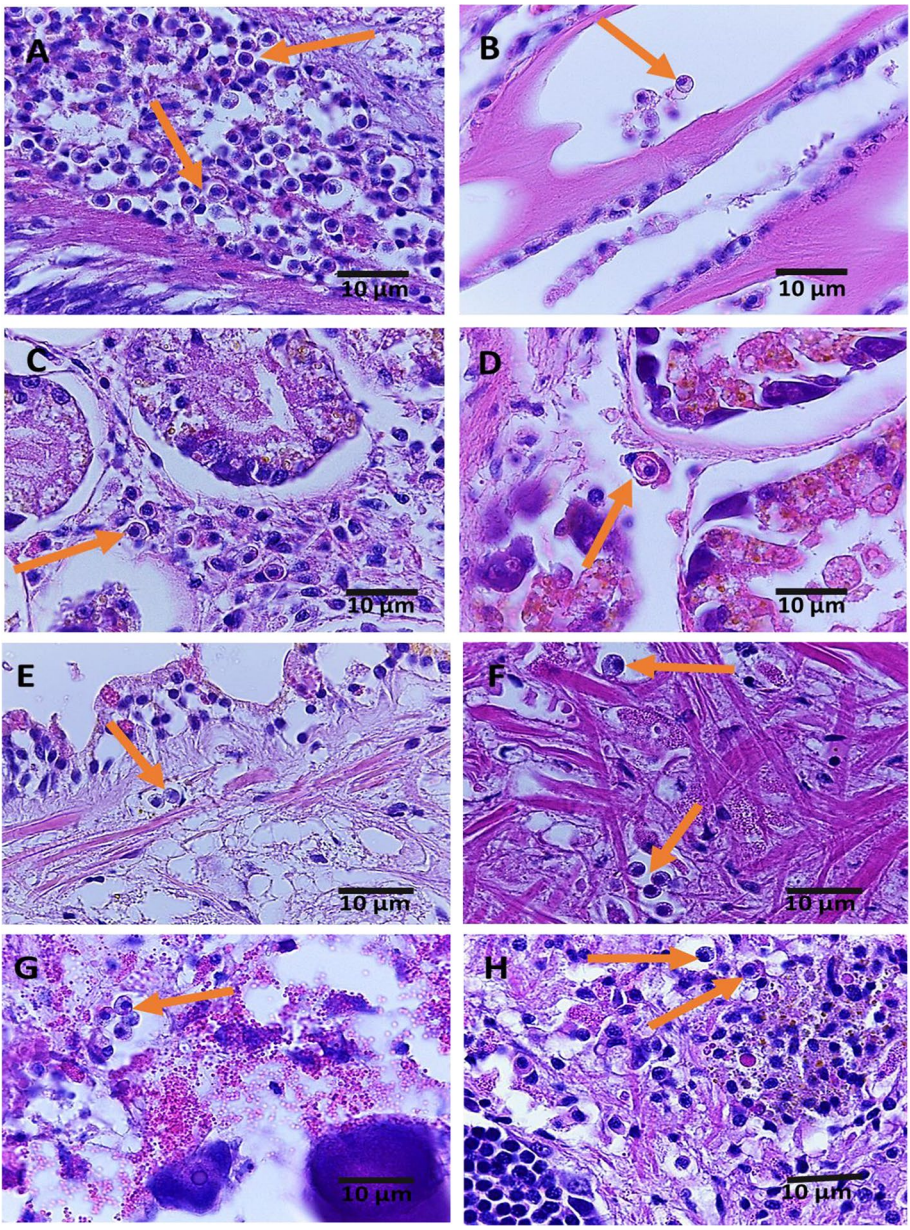


**Fig. 19** APX (orange arrows) in different tissues of *P. canaliculus*. **A** Connective tissue surrounding digestive epithelium. **B** Gills (haemolymph space). **C** Connective tissue surrounding digestive tubules. **D** Phagocytosis by haemocytes near digestive tubules. **E** Mantle. **F** Adductor muscle. **G** Connective tissue surrounding female gonads. **H** Connective tissue surrounding male gonads. Scale bars = 10 µm

the parasite is identified, and the haemocytes actively phagocytose *Perkinsus* cells. In addition, *Perkinsus* parasites multiply within the haemocytes and involve in their rupture (Villalba et al. 2004). Therefore, these findings indicate that *P. olseni* infections are linked with haemocytosis and brown material accumulation which may debilitate the mussels.

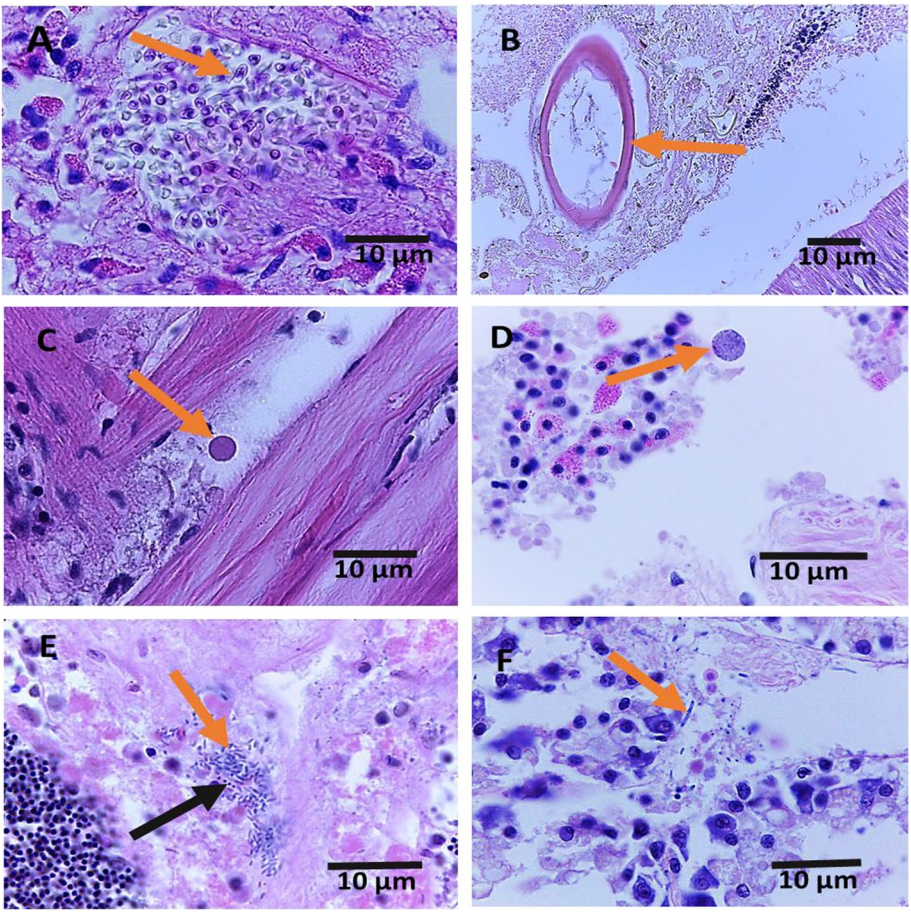


**Fig. 20** Parasites (orange arrows) and microorganisms (orange arrows and black arrow) in tissues of *P. canaliculus*. **A** *M. rapuae* sporocyst in connective tissue near digestive epithelium. **B** Part of copepod (*P. spinosus* or *L. uncus*) exoskeleton inside the gut. **C** and **D** IMCs in muscle and near the mantle respectively. **E** Bacteria (bacilli = orange arrow and cocci = black arrow) in the connective tissues near male gonads. **F** Rod-shaped bacteria (bacilli) in the connective tissues surrounding digestive tubules. Scale bars = 10 µm

## Infection with APX

APX zoites with a high prevalence rate (78%) corresponding to the mean detection in whole mussels were found in different tissues of *P. canaliculus* collected from Coromandel, New Zealand. Suong (2018) also observed a high prevalence (60%) of APX in cultured *P. canaliculus* from Coromandel, New Zealand, in 2016. Other researchers have detected APX in *P. canaliculus* (Diggles et al. 2002; Suong et al. 2019; Webb 2013), Mediterranean mussels, *Mytilus galloprovincialis* (Suong et al. 2019) and hairy mussels, *Modiolus areolatus* (Suong et al. 2018). In the current study, light to moderate numbers of parasites were observed in most tissues, but a high number of APX was recorded in the mantle and the connective tissue around digestive tubules and digestive epithelium of *P. canaliculus*. In contrast, Suong (2018) identified a few APX-like zoites in the connective tissues in various mussel species including *P. canaliculus*. According to Suong (2018), all the mussel

samples had only very light infections, and only a few APX-like zoites were scattered or gathered into small groups (3–5 zoites), mainly in the connective tissue of the digestive gland and mantle tissue. In this study, higher prevalence of APX infection in mussels was found in the winter season. In addition, a highly significant association between seasons and the presence of APX in mussels was noted. A significant association was observed between the ceroid/brown material accumulation and APX infection and the presence of ceroid/brown material was often noted close to APX. Consistent with this study, the presence of brown cells and the accumulation of haemocytes around APX-like cells have been recorded in the tissues of several mussel species, including *P. canaliculus*, *M. galloprovincialis* and *M. areolatus* (Suong 2018). Webb and Duncan (2019) also found areas of brown pigment in bivalve tissues, mostly in the mantle, gonad and digestive gland interstitial tissues, and brown pigmentation appears in the vicinity of parasites such as APX (Webb and Duncan 2019). According to Suong (2018), APX may be related to the morbidity and mortality of mussels under certain stress conditions. Moreover, biotic factors (e.g. host density, common pathogens and host developmental stages) and abiotic factors (e.g. temperature, pH and salinity) can cause APX to propagate and result in harmful outbreaks in New Zealand aquaculture industry (Suong 2018). These observations suggest that environmental factors affect the spreading of APX infection and, in severe cases, APX may be associated with disease outbreaks.

### Other parasites and pathogens

Other parasites and pathogens were observed, including *Microsporidium rapuae* in the connective tissue near the digestive epithelium, copepods (*Pseudomyicola spinosus* or *Lichomolgus uncus*) inside the gut, IMCs in muscles and mantle, and bacteria (rods/bacilli and cocci) in the gills, mantle, connective tissues near gonads, and in the digestive tubules and digestive epithelium. A few mussels (low infection prevalence) were infected with these parasites and pathogens, and there were no noticeable pathological effects. *Microsporidium rapuae* was also identified in connective tissue near the digestive tract in *P. canaliculus* and *Ostrea chilensis* by Jones (1981) and Hine and Jones (1994). According to Webb (2013), *M. rapuae* appears to have no pathological impact on *P. canaliculus* and *O. chilensis.*

Webb (1999) and Webb and Duncan (2019) reported that copepods are frequently seen attached to gills or embedded in digestive tissues in bivalves such as South African *Perna perna*, *Aulacomya ater*, *Choromytilus meridionalis*, *M. galloprovincialis*, *P. canaliculus*, *Crassostrea gigas* and *O. chilensis*, and copepods are surrounded by heavy haemocytosis in the tissues of bivalves. According to Caceres-Martinez and Vasquez-Yeomans (1997), copepods are not host-specific and are found worldwide, and in light infestation, copepods are harmless to their host, but, in heavy infestation, they may lead to gill erosion and haemocyte infiltration. Although the impact of copepods on the host may be negligible, the resultant haemocytosis can conceal other haemocytosis-activating infections, such as perkinsosis whose trophozoites are small and inconspicuous compared with the haemocyte profusion associated with them (Webb and Duncan 2019).

IMCs/RLOs were also reported in the cytoplasm of gills, digestive and mantle epithelial cells of mussels, oysters and other bivalves without significant pathological effects by Webb and Duncan (2019). Other pathogens such as bacteria (*Splendidus clade*, *Harveyi clade*, *Vibrio aestuarianus*, *V. tubiashii*, *V. coralliilyticus*, *V. tapetis*) was identified in marine bivalves as important aquaculture pathogens by. According to Travers et al. (2015), bivalves are adversely affected by many bacteria and are associated with mortality events

of hatcheries and natural beds. In the present study, there was a significant association between seasons (summer, winter, spring and autumn) and the presence of bacteria in mussels. According to Nguyen and Alfaro (2020), in the summer season, aquaculture species may experience heat stress and pathogen loads which tends to overwhelm their immune systems and cause summer mortality events. Pathogens, parasites and pests (PPP) are a long-lasting threat for the aquaculture sector, and the increase of production and expansion of trade (Stentiford et al. 2012). Global cultivation of many bivalve species have been significantly threatened by the substantial mortality outbreaks (Nguyen and Alfaro 2020). There is a critical need to investigate this health threat to safeguard the future of the industry. According to Assefa and Abunna (2018), implementation of best management practices (e.g. for site and system selection, stocking densities, species rotations, broodstock, and feed quality, filtration and parasite monitoring and removal, and surveillance) has been the key ways of minimizing diseases (Assefa and Abunna 2018). It is vital to conduct surveillance frequently in order to decrease the risk of the spread of pathogens (Oidtmann et al. 2011). Thus, strict quarantine measures, sanitation of equipment, disinfection of eggs, traffic control, water treatments, maintaining feed quality and removal of dead mussels should be properly implemented during the introduction of new stock as well as implementing them for decreasing pathogens and to circumvent transferring pathogens from one stock to another (Assefa and Abunna 2018). Therefore, it is important to monitor parasites and remove the infected animals throughout the year to minimize diseases and to reduce the risk of spread of pathogens and parasites.

## Gill pathology and digestive tubule pathology

Gill pathology (e.g. loss of cilia, destroyed gill filament, atrophied/separated ciliary discs in the abfrontal zone of gill filament and very few haemocytes in the gill blood space) was observed in *P. canaliculus.* Erosion of the gill epithelium and loss of cilia were observed in *C. gigas*, by Webb and Duncan (2019), who indicated that damage to the gill epithelial cells may lead to significant disturbance of respiratory functions in affected bivalves. Moreover, gill tissues may be affected by severe infection with *Perkinsus* which may result in deteriorating filtering activity and efficiency (Choi and Park 2010).

The pathology of digestive tubules was noted in this study. Digestive tubule structural abnormalities, such as thinning and sloughing (Carella et al. 2015a; Morton 1970; Winstead 1995, 1998), have been recorded in *P. canaliculus*, *C. gigas* and *M. galloprovincialis* by Webb and Duncan (2019). Carella et al. (2015b) also assessed damage to the digestive epithelia in connection with lumen modifications in mussels exposed to the harmful dinoflagellate *Ostreopsis cf. ovata*. These digestive tubule structural anomalies have been related to stresses such as starvation and xenotoxins (Rolton et al. 2019). In the present study, a significant association between digestive tubule structure (large lumen, with a thin epithelial wall) and *Perkinsus* infection was recorded. According to Lee et al. (2001), the infestation of the *Perkinsus* parasites in digestive tubules may cause digestive tubule atrophy and detrimental effects during food digestion. According to Morton (1970), the intracellular digestive procedure in the digestive diverticula is linked with the development, absorption, breakdown and reformative phases of the digestive tubules. Morton (1970) noted that in digestive cycle (tidal cycle), the variations of the digestive tubules structure (due to different physiological factors) are associated with feeding and digestion of cockle (*Cardium edule*). Thus, it is likely that the digestive tubule structural abnormalities observed in this study may have resulted from pathogen/parasite infections, natural cycle

and/or biotic factors that lead to disruptions in digestive tubule functions, such as food digestion and nutrition absorption.

## Conclusions

This is the first report on seasonal variations of *P. olseni* and APX in New Zealand Greenshell™ mussels (*P. canaliculus*). The presence of *P. olseni* and APX in mussels are significantly related to the seasons (summer, winter, spring and autumn). A high prevalence of *P. olseni* and APX was found in different tissues of mussels and there was a significant association between *P. olseni* and APX infection in mussels. However, a few moribund mussels were associated with bacteria (bacilli and cocci) and infected with copepods, *M. rapuae* and IMCs, and appears to have no pathological effect on *P. canaliculus* as indicated by histological condition. The abundance of *P. olseni* and APX in *P. canaliculus* was evaluated semi-quantitatively using two separate modified grading scales, which are suitable to assess *P. olseni* and APX infection of the histological section. In addition, tissue gradings for *P. olseni* and APX infection, inflammatory tissue responses (haemocytosis, ceroid material) and abnormal tissue structures (gill pathology and digestive tubule pathology) were evaluated with semi-quantitative scales. An association between haemocytosis and *P. olseni* infection was noted. A significant association between the brown material accumulation and parasites (*P. olseni* and APX) infection was recorded. The pathologies of digestive tubules and gills were observed in this study and *P. olseni* infection is related to digestive tubule deterioration (large lumen, with a thin epithelial wall). A significant association between presence of parasites and health condition (healthy and unhealthy) of mussels was observed. Therefore, the findings in this study provide information regarding the infections of potential parasites and pathogens and their abnormal tissue structures in *P. canaliculus* across different seasons.

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**Author contribution** Farhana Muznebin: conceptualization, investigation, methodology, formal analysis, data curation, roles/writing—original draft; Andrea C. Alfaro: conceptualization, writing—review and editing, resources, funding acquisition; Stephen C. Webb: conceptualization, methodology, writing—review and editing.

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**Data availability** Data available upon request.

**Code availability** Not applicable.

## Declarations

**Ethics approval** Not applicable.

**Competing Interests** The authors declare no competing interests.



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## Authors and Affiliations

**Farhana Muznebin[1,2] · Andrea C. Alfaro[1] · Stephen C. Webb[3]**

[1] Aquaculture Biotechnology Research Group, School of Science, Auckland University of Technology, Auckland, New Zealand

[2] Department of Zoology, Faculty of Life and Earth Science, Jagannath University, Dhaka, Bangladesh

[3] Cawthron Institute, Nelson, New Zealand