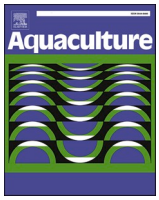
Aquaculture 607 (2025) 742683

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Reduced farming density of New Zealand flat oyster *Ostrea chilensis* is associated with decreased infection by *Bonamia ostreae* and other parasites

Zoë Hilton [a,1,*], Anne Rolton [a,1,*], Farhana Muznebin [b], Stephen C. Webb [a], Andrew Fidler [c], Andrew Elliot [d], Javier Atalah [a], Andrea C. Alfaro [b], Kate S. Hutson [a,e]

[a] Cawthron Institute, Nelson, New Zealand
[b] Auckland University of Technology, Auckland, New Zealand
[c] AquaGeneNZ Ltd., Nelson, New Zealand
[d] Kono Ltd, Nelson, New Zealand
[e] Centre for Sustainable Tropical Fisheries and Aquaculture, College of Science and Engineering, James Cook University, Australia

ARTICLE INFO

Keywords:
Bonamiosis
Protist
Shellfish mortality
Disease progression
Histopathology
Haplosporidean

ABSTRACT

The haplosporidian parasites *Bonamia ostreae* and *Bonamia exitiosa* are notifiable to the World Organisation for Animal Health. The first detection of *B. ostreae* in the southern hemisphere in 2015 was associated with high mortality in some stocks of farmed flat oysters, *Ostrea chilensis,* in the Marlborough Sounds, Aotearoa New Zealand (NZ), whereas *B. exitiosa* is considered endemic and commonplace. Subsequently, national restrictions were placed on flat oyster movements, and ultimately, in 2017, all flat oyster farms in NZ were de-populated and farming stopped. The farm where *B. ostreae* was first detected, adapted their practices following the detection and held oysters at both normal stocking densities and lowered stocking densities for 2+ years (2015 to 2017). Although this study was opportunistic and could not determine causality, and other factors may also have influenced parasite prevalence and intensity, flat oysters cultured at reduced densities had significantly lower prevalence and intensity of *B. ostreae* infections compared with those cultured at a higher (normal pre-2015) density on the same farm (22 % vs 95 % prevalence, and 0.18 vs 1.31 mean intensity grade on a scale of 1–3). There was also significantly lower prevalence of infection of other common parasites including apicomplexan X (APX; 3 % vs 45 %) and the trematode *Alcicornis longicornutus* (3 % vs 23 %) at lower density. We also hypothesise that some degree of resistance to *B. ostreae*, and tolerance to bonamiosis existed in this population of NZ flat oysters, as in the high-density group that experienced very high levels of mortality, around a quarter of the 5 % of survivors were healthy and lacked any detectable *Bonamia* infection, with some PCR negative for *Bonamia*. Regardless of density, there was a marked reduction in prevalence of *B. exitiosa* in 2017 (just 1/98 oysters) compared to a previous study of the same farm in 2015. Finally, we also provide novel insights into the progression of *B. ostreae* infection in NZ flat oysters. Infections were initially detected in the mid-gut, followed by the digestive gland, mantle, gill and finally gonad, and although APX prevalence was not correlated with *Bonamia,* both APX-infected animals and those infected with *A. longicornutus* had reduced gonad size and density compared to uninfected animals. These data suggest that reducing stocking density may significantly reduce parasite infection in this species. Changes in husbandry, along with production of disease-free juveniles and potential selective breeding, may enable re-establishment of this important aquaculture industry, with potential benefits for the long-term protection of fisheries and biodiversity in NZ.

1. Introduction

Haplosporidian micro-cell parasites of the genus *Bonamia* have had severe impacts on both farmed and wild populations of various flat oyster species throughout the world, including in Europe, North America, and Australasia (Arzul and Carnegie, 2015; Carnegie and Engelsma, 2014; Engelsma et al., 2014; Lane et al., 2016). These impacts have been most notable when the *Bonamia* species have been spread to naïve oyster

* Corresponding authors.
E-mail addresses: zoe.hilton@cawthron.org.nz (Z. Hilton), anne.vignier@cawthron.org.nz (A. Rolton).
[1] These authors are co-first authors of this manuscript and contributed equally to this work.

https://doi.org/10.1016/j.aquaculture.2025.742683
Received 30 June 2024; Received in revised form 19 April 2025; Accepted 5 May 2025
Available online 8 May 2025

hosts, a process that has repeatedly occurred over the last 50 years throughout the world (Carnegie et al., 2016; Doonan et al., 1994; Engelsma et al., 2014; Lane et al., 2016). *Bonamia* spp. microcells infect oyster haemocytes (blood cells) and can be transmitted directly host to host, inducing a disease called bonamiosis that often results in mortality (Engelsma et al., 2014). In naïve populations, these mortalities can be extremely high (Engelsma et al., 2014). Two *Bonamia* species, *B. ostreae* and *B. exitiosa,* are notifiable organisms to the World Organisation of Animal Health (WOAH; formerly OIE) (OIE, 2018a; OIE, 2018b) and many flat-oyster producing countries have national and regional control measures in place to prevent the anthropogenic spread of *Bonamia* (e.g. Hellberg and Hopkins, 2006).

Ostrea chilensis is a flat oyster native to Aotearoa New Zealand (NZ) and Chile (Foighil et al., 1999). In both countries it is highly valued for commercial and recreational fishing, and aquaculture, and in NZ it is considered a 'taonga' or treasured species. Taonga species are those which are highly valued both as a food resource for customary fishing (the traditional right of indigenous people to fish in a certain area for cultural [non-commercial] purposes), and as an integral part of indigenous cultural identity (Georgiades, 2015). In NZ, *B. exitiosa* has been observed in *O. chilensis* populations since the 1960s (Hine et al., 2001). In the Foveaux Strait in southern NZ (Fig. 1), a nationally iconic, historic, and locally important commercial dredge fishery operates (Cranfield et al., 2005; Cranfield et al., 1999; Doonan et al., 1994; Michael et al., 2016). Large-scale mortalities between 1985 and 1992 in the Foveaux Strait fishery attributed to *B. exitiosa*, caused estimated losses of 91 % of the mid 1970s biomass (Hine et al., 2001). Due to the ongoing impact of *B. exitiosa* on the wild flat oyster fishery in NZ, there has been both experimental and observational work carried out for many decades examining the epidemiology and pathology of *B. exitiosa* affecting *O. chilensis* in NZ (Cranfield et al., 2005; Diggles and Hine, 2002; Hine, 1991a; Hine et al., 2002). This includes ongoing regular surveys of the important fished wild populations of Foveaux Strait for *B. exitiosa* prevalence, to aid fisheries management (Michael et al., 2016).

Fig. 1. Map of New Zealand's three largest islands, indicating significant areas for aquaculture and commercial fishing of the native NZ flat oyster *Ostrea chilensis* up to 2017, as well as the location of the study site (Port Underwood, Marlborough Sounds).

The majority of the published scientific literature on epidemiology and pathology of any *Bonamia* spp. concerns the host-parasite relationship between the European native flat oyster *Ostrea edulis* and *B. ostreae* (see reviews by Arzul and Carnegie, 2015; Engelsma et al., 2014). *B. ostreae* has had a serious impact on European flat oyster stocks since the early 1980s after being introduced in the late 1970s from North America (Elston et al., 1986) and spreading to many different locations within Europe such as parts of Ireland, England, Scotland, Netherlands, France, Spain, and Denmark (Mialhe et al., 1988; Sas et al., 2020). Previously observed only in the northern hemisphere, *B. ostreae* was detected for the first time in the southern hemisphere in 2015 in NZ farmed flat oyster stocks within the nation's most important area for shellfish aquaculture, the Marlborough Sounds (Fig. 1; Lane et al., 2016). Its detection was associated with unusually high levels of mortality (in some cases over 95 %) on these oyster farms (A. Elliot and B. Hearn pers. comm; Lane et al., 2016), despite *B. exitiosa* being already present in these areas. According to retrospective PCR testing of available archived samples, *B. ostreae* was not detected in NZ prior to 2014, and it is thus believed to be a recent incursion (Lane, 2018; Lane and Jones, 2020). By contrast, *B. exitiosa,* originally thought to be distributed only in the Southern Hemisphere, has been shown to now be extensively and globally distributed, infecting a number of different oyster hosts throughout the world, including *O. edulis* in Europe (Arzul et al., 2012; Carrasco et al., 2012; Hill et al., 2014). Co-infections with both *B. ostreae* and *B. exitiosa* have been observed in the same *O. edulis* host (Abollo et al., 2008; Ramilo et al., 2014), and similarly, co-infections have now been observed in farmed *O. chilensis* in NZ (Lane and Jones, 2020; Lane et al., 2016).

Until 2017, *O. chilensis* was cultured on suspended longlines or in baskets or cages, in two of NZ's most significant aquaculture production areas: the Marlborough Sounds, and Big Glory Bay, on Stewart Island (Fig. 1), either as monoculture crops, or co-cultured with NZ's largest shellfish export species, the green-lipped or Greenshell™ mussel, *Perna canaliculus*. Despite *B. exitiosa* being present in these farms, flat oyster aquaculture production had been progressing for at least 30 years and was on a positive growth trajectory (Capson and Guinotte, 2014). Several small hatcheries had also been set up in Marlborough and Stewart Island, for producing spat for on-growing. When *B. ostreae* was detected in 2015 (Fig. 2), it was identified only in the Marlborough Sounds. Some farms in the area suffered very high mortalities, which

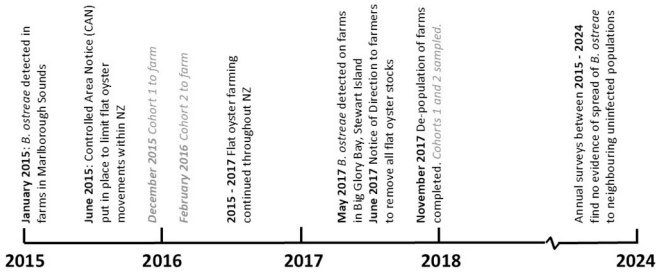


Fig. 2. Timeline of first detections of *B. ostreae* in NZ flat oyster *Ostrea chilensis* and response measures taken between 2015 and 2017 (black text), as well as dates of transfer of the animals sampled in this study (cohorts 1 and 2) to the on-growing sites on the farm, and timing of their sampling (grey text). Subsequent annual surveys administered by the New Zealand Government's Ministry for Primary Industries (MPI) indicated that *B. ostreae* had not spread from infected to neighbouring uninfected populations between 2017 and 2023 (MPI, 2023).

prompted farmers to change their farming practices according to recommendations from Europe (Georgiades, 2015) by increasing inter-line spacing, reducing stocking densities, and other husbandry practises (e.g. reduced frequency of grading or disturbance) to be able to successfully farm in the presence of this new disease (A. Elliot, B. Hearn, pers. comm).

After the detection of *B. ostreae* in NZ in early 2015, a 'controlled area notice' was put in place to control the movements of shellfish and associated equipment within different areas of NZ, reducing the likelihood of anthropogenic spread of *B. ostreae* within NZ waters (Fig. 2.). However, in mid-2017, *B. ostreae* was detected via targeted surveillance on farms in Big Glory Bay, approximately 800 km/500 mi south of the initial site of detection (Fig. 1; *MPI*, 2017). Although no unusual mortality was reported, this detection was of great concern, because Big Glory Bay is directly adjacent to the last remaining, commercially important, wild flat oyster dredge fishery in NZ, in the Foveaux Strait (Fig. 1). Subsequently, the NZ government's Ministry for Primary Industries gave a 'notice of direction' where all flat oyster aquaculture operations in both Marlborough Sounds and Big Glory Bay were closed, and all farmed flat oyster stocks were ordered to be destroyed due to concerns that infected farm stocks posed an unacceptable level of risk of accidental anthropogenic spread of *B. ostreae* to the wild fishery (Fig. 2). The removal of farms occurred during June – November 2017. Since then, there has been ongoing surveillance for *B. ostreae* in the uninfected wild populations adjacent to known infected sites, and to date, there has been no *B. ostreae* detected in the surrounding uninfected wild populations of Foveaux Strait, or Tasman and Cloudy Bays (MPI, 2023).

Prior to the closure of the flat oyster farms in 2017, farmers reported that oysters held at much lower densities than were previously routinely used up to 2015 had experienced lower mortality during 2016 and 2017. It was hypothesised that the reduced stocking densities may have led to lower prevalence and/or intensity of *Bonamia* infection. The aim of this study was to determine whether different farm grow-out methods were associated with reduced infection from *B. ostreae* and other parasites. We also examined oysters with a wide range of infection intensities so that inferences could be made regarding the progression of *B. ostreae* infection in this novel host-pathogen relationship in NZ. We examined *O. chilensis* from a single infected farm located in Port Underwood, Marlborough Sounds during the removal and destruction of all farmed flat oysters in November 2017. The Marlborough Sounds was the first and only location in NZ known to be highly infected with *B. ostreae* and the animals sampled for this study came from the same farm that was examined by Lane and collaborators in 2015 (Lane, 2018; Lane and Jones, 2020; Lane et al., 2016). Two different cohorts, originating from the same broodstocks, on the same farm, but transferred to the on-growing sites two months apart, had been on-grown in separate sites within this infected farm. The first cohort was deployed for on-growing in December of 2015 (early summer), and the second transferred in February 2016 (late summer). Thus, the cohorts were 2–3 months apart in age and had settled on the grow-out substrate at different densities, and then were grown in the same farm as they were collected on, but at different sites within this farm for the next 21–23 months until they were both sampled in November of 2017 (Fig. 2). The cohort in the later deployment (Feb 2016) was settled at lower density (50–80 animals per linear metre vs up to 200/m) on the droppers (vertical ropes/longlines), and also grown with increased spacing between droppers (800–1000 mm spacing vs 400 mm standard pre-2015 spacing). This was based on recommendations from Europe to reduce densities in order to help reduce the impact of bonamiosis from *B. ostreae* (Georgiades, 2015). Animals from low and high mortality sites were sampled with the aim of being able to examine the widest range of infection intensities possible, to enable inferences to be made about the progression of *B. ostreae* infection in NZ *O. chilensis*.

2. Materials and methods

2.1. Oyster origins, size and farming methods

The flat oysters sampled for this study were aged 22–26 months old; shell size 50 to 85 mm, mean = 68 ± 0.9 SE mm) and were collected from an oyster farm in Port Underwood, in the Marlborough Sounds, NZ on 27th November 2017. The farm contained many tens of thousands of oysters. The sampled individuals were from 2 separate grow-out 'cohorts' on-grown in separate locations within the farm area. These oysters had been collected as spat by settling directly on to rope on the same farm, (i.e. they were from the same parental broodstock pool). The cohorts were produced 2 years prior, between October 2015 and February 2016 by putting multiple groups of 8–12 adult broodstock oysters into large mesh bags at the beginning of the breeding season and surrounding them with settlement substrate rope. As *O. chilensis* brood for the entire larval phase, and the vast majority of spat settle immediately upon release from the parent oyster, the released juveniles (spat) settled on the ropes surrounding the parents, and these were subsequently removed and hung out on the same farm for on-growing as vertical ropes ('droppers') suspended from a horizontal 'backbone' rope, in a manner identical to typical NZ mussel longline culture. New rope was then placed in the same mesh bag of broodstock and in this way, successive cohorts were produced. Depending on the amount of time the settlement substrate was left in the bags, settlement density could be somewhat controlled.

2.1.1. Cohort 1: early grow-out at higher density

The first cohort was collected onto the settlement rope between Oct – Dec of 2015 and on-grown on droppers from Dec 2015 using 'normal' (standard pre-2015) husbandry methods for that farm: animals on longlines at a density of up to 200 per (vertical) linear metre (Fig. 3a), and the droppers spaced at 400 mm apart along the backbone. These oysters were therefore 24–26 months old at time of sampling in November 2017. Prior to sampling, they had undergone very high mortality (estimated to be >95 % pers. obs.) putatively due to the *Bonamia ostreae* epizootic ongoing since 2015. At the time of sampling, very few oysters on the droppers remained alive; most were empty shells. The droppers were searched extensively, and the sampled oysters consisted of the few remaining survivors.

2.1.2. Cohort 2: later grow-out at lower density

The second cohort was collected onto fresh settlement substrate that was placed into the same bags with the same parental pool as cohort 1 above, between Jan – Feb of 2016 and on-grown in a different location but on the same farm from Feb 2016 using modified husbandry methods: They had settled at lower densities of approximately 50–80 animals per linear metre (Fig. 3b) and were also farmed with increased inter-dropper spacing (800–1000 mm spacing vs 400 mm standard pre-2015 spacing). They were 22–23 months old at time of sampling in November 2017. The cumulative mortality levels had been low at the time of sampling and most of the oysters appeared to be alive, with few empty shells visible (pers. obs.).

In total, 98 individuals were sampled from the two cohorts (62 from cohort 1 and 36 from cohort 2). Shell size ranged from 50 to 85 mm, with an overall mean ± SE of 68 ± 0.9 mm. Size covaried with age, and although the size ranges for each cohort were almost identical, mean size differed significantly ($t = -4.26$, $p < 0.001$). Cohort 1, aged 24–26 months old mean 69.3 mm ± 8.4 S.D. ($n = 62$); Cohort 2, aged 22–23 months mean 62.4 mm ± 7.1 ($n = 36$; Fig. 4).

2.2. Oyster sample collection and processing

The oysters were transported live on ice to the NZ Ministry for Primary Industries' Animal Health Laboratory in Upper Hutt where they were held on ice in polystyrene bins for between 2 and 8 days before

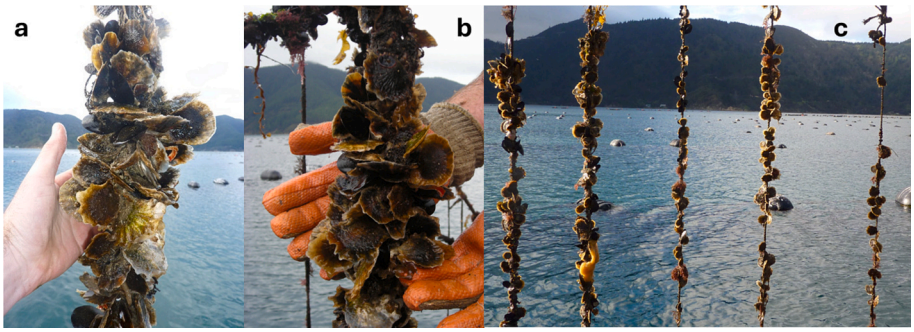


Fig. 3. a – b) Examples of high-density (up to 200 individuals per linear m) 12-month-old oysters on grow-out droppers. This was a common density used pre-2015. c) Example of lower-density settlement on droppers trialled after 2015; 50–80 individuals per linear m. Photos courtesy of A. Elliott.

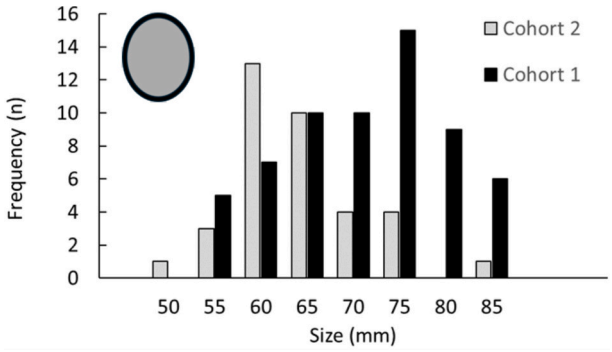


Fig. 4. Size (mm) frequency of flat oysters, *Ostrea chilensis*, obtained from the two different cohorts. Cohort 1 aged 24–26 months (n = 62; black) and Cohort 2 aged 22–23 months (n = 36; grey). Inset circles indicate graphically the relative difference in mean sizes between the cohorts. For context, the 'legal size' in NZ for fishing of flat oysters is >58 mm.

dissection. This duration of holding prior to tissue sampling was not likely to have any effect on the observed *Bonamia* status of the animals, as bonamiosis is a slowly-developing disease, with infections often observed to take many months to become visible via smears or histological methods after an initial exposure (Culloty et al., 1999; Grizel et al., 1988; Hervio et al., 1995; Montes, 1991).

Between 10 and 50 oysters were sampled each day. Prior to dissection each day, the animals' viability was assessed, by checking if they were actively closed, or if gaping, still actively closing when touched. On any day, any individual that was gaping and unable to close, was presumed dead or highly moribund and not sampled. Oysters were measured for shell length (mm) and whole weight (g), then were shucked and sectioned for subsequent histological and molecular analyses.

A 5 mm thick tissue slice was taken across the body, aiming to contain all major organs, and placed in an individual histological cassette which was immediately fixed in 4 % formalin in seawater for 48 h and then stored in 70 % ethanol. The fixed tissues were sent to Taranaki Medlab (New Plymouth, NZ) where they were dehydrated in a series of ascending ethanol concentrations, two changes of xylene, and then embedded in paraffin wax. Tissue sections of 5 μm were cut by microtome, mounted on glass slides before staining with Harris' haematoxylin and eosin for examination by light microscopy as described below (Howard et al., 2004 section 2.3). Subsequently, three to five 0.2 μm sections were cut off each wax block using a microtome and DNA was extracted from these sections to identify parasites via the PCR as described below (section 2.4).

2.3. Microscopic examination

General oyster health/vitality and gonadal assessments, as well as *Bonamia* infection intensity and pathological features in different oyster tissues were determined microscopically. Prepared slides were examined using an Olympus BX53 microscope equipped with an Olympus DP27 camera and CellSense software and a Leica DM2000 microscope.

2.3.1. Health assessment

Due to unavoidable logistical constraints, after removal from the farms, the live oysters were held in the laboratory out of water and on ice in polystyrene bins for between 2 and 8 days prior to dissection. Therefore, to provide an indication of their relative health status or 'vitality' *at the time of dissection* (independent of 'disease status'), oysters were categorised as either "live and apparently healthy", "live and unhealthy", or "moribund" (numerically coded as 1, 2 and 3 respectively) based on the integrity of the gill and digestive tubule structure and the presence of bacteria. 'Live and apparently healthy' oysters displayed typical gill structure with clear, defined, uniform filaments, cilia and relatively clear blood sinuses and the digestive tubules were thick with a cruciform lumen. In 'live but apparently unhealthy' oysters, the structure of the gills lacked cilia, there were an elevated number of haemocytes in the blood sinuses causing distention and bacteria (bacilli and/or cocci) were observed in tissues including the gill, and around the digestive gland. This was clearly discernible from *Bonamia* spp. infection, where although there were hemocytes in the blood sinuses, there was often also haemocytosis, cilia were still present on the gills and no bacteria were present. In 'moribund' oysters, the structure of the gills was destroyed, the underlying skeletal rods exposed, the digestive tubules were thin and without a cruciform lumen and bacteria (bacilli and/or cocci) were present in tissues as above.

2.3.2. Gonad assessment

The state of the gonads was assessed using a semi-automated method of digital image analysis. Slides were scanned using a VENTANA iScan Coreo whole slide brightfield slide scanner (Roche Diagnostics). Using 'Image J' version 1.48 software, the acquired digital images of the whole animal tissue sections were analysed to determine the percent gonadal area (% of total body cross-sectional area taken up by the gonad), percent gamete density (a relative measure of density of the gonad tissue), and the proportion of male and female gametes (Fig. 5).

For all analyses, measured areas were converted to pixel numbers. Percent gonadal area was calculated by first tracing around the whole body, excluding the gills, and then tracing around the entire gonad. The area (number of pixels) taken up by the gonad was divided by the area of the whole body and multiplied by 100, according to Morales-Alamo and Mann (1989) and Kang et al. (2003). The percent gonadal area thus represents relative reproductive effort (Todd and Havenhand, 1983; Normand et al., 2009).

Percent gamete density was calculated by tracing around the entire

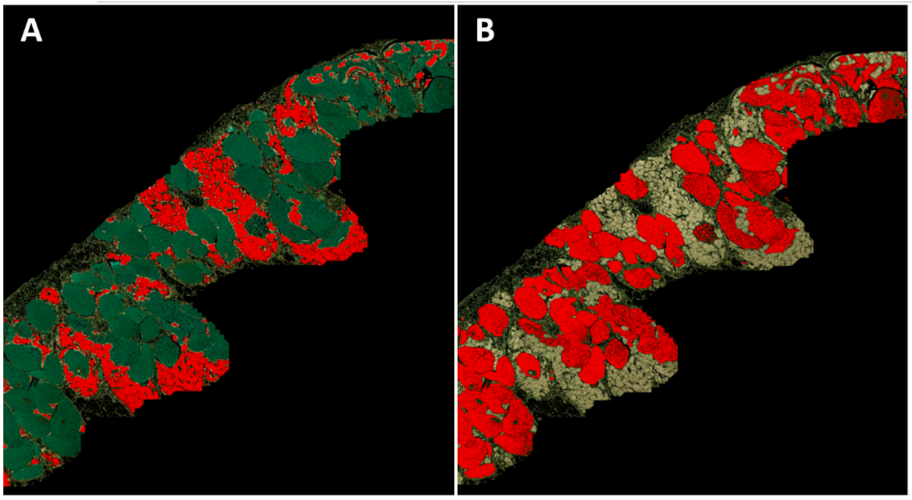


Fig. 5. A portion of the same section of gonad of adult *Ostrea chilensis* showing the differential selection (in red) of developing and mature A: sperm or B: oocytes using colour thresholds.

gonad, inverting the image and, then using set colour thresholds which differentially selected the gametic tissue (developing and mature, Fig. 5). Gamete density was the total area of the gonad divided by the total area of the gametic tissue multiplied by 100. To determine the proportion of the gamete that was male or female, either sperm or oocytes (developing and mature) were differentially selected using the same set colour thresholds (Fig. 5), and divided by the total area of both the sperm and oocytes and multiplied by 100.

2.3.3. *Bonamia* assessments

The presence of *Bonamia* microcells was recorded in five different tissues: gill; mantle; the digestive tubules and surrounding connective tissue; midgut (stomach and intestine) and gonad. From this, an overall *Bonamia* grade was given for each individual tissue type and then a mean grade was calculated for the whole animal as the mean of the tissue grades. The grades used were based on a subjective semi-quantitative grading scale, modified from previous scales of Hine (1991a) and Diggles et al. (2003) developed for *B. exitiosa* infection in *O. chilensis* and as described below. In addition, the number of *Bonamia* microcells and the proportion which were intracellular vs extracellular were also directly quantified for each tissue type.

2.3.3.1. Bonamia infection intensity grade: tissue and whole animal. After initial examination of the slides so that a large range of *Bonamia* infection intensities were observed (range-finding), the relative abundance of *Bonamia* microcells (i.e., infection intensity) in each tissue for all 98 oysters was given a grade on a scale of 0 to 3, where 0 = Completely absent (after extensive searching of each tissue-type for 5 min), 1 = Low, 2 = Medium and 3 = Heavy (See Fig. 10for examples). *Bonamia* infections were often 'patchy', especially at low infection intensities but the tissue grade was given according to the overall abundance of microcells observed in each tissue-type. For example, if there were no microcells in most of the tissue but, in one small area there were relatively few, or a moderate number of microcells, the overall infection intensity was 'Low' or 'Medium' respectively. High intensity infections were not 'patchy' in the same way as low or medium infection intensities - the infection was much more widespread. Each of the 5 tissues was individually graded this way, for each oyster, and then an overall grade was calculated for the whole animal as the mean of the 5 tissue grades. Thus, an animal where no infection was observed in any tissue type after extensive searching of all tissues for 5 mins each, received five tissue grades of 0 and an overall grade of 0; an animal where a low level of infection was observed in just one tissue type would receive a tissue grade of 1 for that tissue and a whole-animal grade of 0.2; an animal where a low level of infection was observed across all tissues would receive a tissue grade of 1 for all tissues and a whole-animal grade of 1; and an animal which had a high abundance of parasites in every tissue examined received a grade 3 for all tissues and an overall grade of 3. Thus, both individual tissue grades and overall grades range from 0 to 3, but the whole-animal grade, rather than just describing 'absent', 'low', 'medium' and 'high', described a combined measure of intensity and distribution, and ranged from 0 to 3 in 16 increments of 0.2 and could thus be treated as a continuous variable for statistical analyses by ordinal approximation of a continuous variable (section 2.5). The distribution and makeup of these grades are displayed in Figs. 8 and 9.

2.3.3.2. Proportion of hemocytes infected per tissue. The relative proportion of hemocytes infected with *Bonamia* microcells was also given a scale of 0 to 3, where 0 = no infected hemocytes (after extensive searching of the tissue for 5 min), 1 = less than half of the hemocytes infected, 2 = equal proportions of infected and non-infected hemocytes and 3 = more than 50 % hemocytes infected.

2.3.3.3. Intra and extracellular Bonamia assessments. Following examination of the slide, within each tissue of every animal, an area displaying the heaviest *Bonamia* infection intensity was selected to determine *Bonamia* microcell abundance within haemocytes, and whether the microcells were observed intracellularly (within haemocytes), extracellularly, or both. This information was used to infer disease progression and is displayed in Fig. 9.

The relative proportion of intracellular and extracellular *Bonamia* microcells was given a scale of 0 to 3, where 0 = completely absent (after extensive searching of the tissue for 5 min), 1 = mostly intracellular, 2 = equal proportions of intra- and extracellular microcells and 3 = mostly extracellular microcells.

Following identification of a heavily infected area, at 100 x magnification, the number of *Bonamia* microcells observed within infected haemocytes (intracellular) over 5 min were counted and are presented as the maximum number of microcells that could be observed within a haemocyte in each tissue. Additionally, the number of extracellular *Bonamia* microcells was quantified by counting the number of extracellular microcells observed in a single representative field of view for each tissue type in each sample. If extracellular microcells were too numerous to count in the entire field of view, the field was divided into equal segments, counted and multiplied by the number of segments.

2.3.4. Histopathological assessment

Histopathological features observed in the gill, mantle, digestive

tubules and surrounding connective tissue, midgut (stomach and intestine) and gonad were recorded as '0' for absent or '1' when present. The presence of brown pigments (i.e. ceroid), occluded blood vessels, diapedesis and haemocytosis, including ulcers and a loss of structure were recorded in several tissues, as was the presence of other parasites, copepods, and bacteria. The infection intensity (abundance) of the bucephalid parasite *Alcicornis longicornutus* was quantified using the same subjective semi-quantitative grading scale from 0 to 3 (0 = absent, 1 = low, 2 = medium and 3 = heavy) as described for *Bonamia* above.

2.4. Determination of parasite prevalence and Bonamia species identification using the PCR on DNA isolated from formalin-fixed paraffin embedded (FFPE) sections

2.4.1. Isolation of DNA from FFPE sections

Sections were cut from each of the 98 FFPE blocks that were used for the histological slide preparation using a microtome (0.2 μm; 3–5 replicate *sec*tions per sample). Great care was taken to clean equipment and change blades between each sample to ensure no cross-contamination of the samples. Given that the section used for DNA extraction was directly adjacent to the section used for microscopic examination, this ensured all tissue types were included for each sample for DNA extraction.

Genomic DNA (gDNA) was isolated from each sample using ZymoResearch, Quick-DNA™ FFPE MiniPrep Kit. Paraffin wax was dissolved first in 1.0 ml of xylene (55 °C, 2–3 min) followed by brief centrifugation and discarding of the xylene, and subsequently in 0.3 ml of the propriety paraffin wax solvent provided with the kit at 55 °C for 2–3 min. Samples were then digested overnight in proteinase K at 55 °C. The gDNA samples obtained were then used as templates for the PCR. Concentrations of the gDNA preparations were estimated on an Implen NanoPhotometer® (Implen, Bavaria, Germany). It is well established that the level of DNA fragmentation is highly variable in formalin fixed tissue samples as discussed in Fidler and Webb (2019). Thus, the first stage in the detection procedure was simply to confirm that gDNA isolated from the FFPE samples was suitable for use as a PCR template. To assess this the gDNA samples were used as templates in PCRs using two primer pairs amplifying a region from the host (*Ostrea chilensis*) 18S rRNA genes. Design of these primers is described in Fidler and Webb (2019). The two primer pairs used were as follows (AMQC: Aquaculture Mollusca Quality Control)l (i) AMQC18SFor1 (5′ – TAACGGGGAATCAGGGTTCGATTCC – 3′; coordinates 346–370 of accession number **KX977494**) paired with AMQC18SRev1 (5′- GAGCTGGAATTACCGCGGCTGCTGG −3′; coordinates 578–554 of accession number **KX977494**) generating an amplicon of 233 bp and (ii) AMQC18SFor1 paired with AMQC18SRev2 (5′- CGACGGTATCTGATCGTCTTCGAACC -3′; coordinates 1007–982 of accession number **KX977494**) generating an amplicon of 662 bp. Reaction conditions were (i) template gDNA (50–200 ng) (ii) forward and reverse primers at final concentrations of 0.5 μM, (iii) 1× MyTaq™ HS Mix (Bioline Cat. No. BIO-25046), (iv) ultrapure water (Invitrogen, Cat. No. 10977–015) added to a final reaction volume of 20.0 μl and (v) a thermocycling protocol: of 95 °C / 1 min. 1 time; 95 °C / 15 s., 58 °C / 30 s., 72 °C / X sec., 35 times; 72 °C / 5 min. 1 time; 15 °C / hold (X = 30 for AMQC18SFor1 / AMQC18SRev1, x = 60 for AMQC18SFor1 / AMQC18SRev2). Amplification products were electrophoresed through 1.5 (*w/v*) % agarose gels before staining in ethidium bromide (0.5 μg/ml) and photographing under UV trans-illumination. Molecular weight standards were pBR322 / *Bsu*R1 (Thermo Scientific Cat. No. SM0271).

The primer pair AMQC18SFor1 / AMQC18SRev1 (233 bp) generated easily detectable amplicons from all the 98 gDNA templates tested. The primer pair AMQC18SFor1 / AMQC18SRev2 (662 bp) generated detectable amplicons from almost all the template gDNAs tested although a number were faint. It was therefore concluded, with a high level of confidence, that the gDNAs isolated from the FFPE samples were suitable for generating amplicons of <300 bp.

2.4.2. Screening for Bonamia exitiosa, B. ostreae, and apicomplexan X (APX) using the PCR on isolated gDNA

The 98 FFPE gDNA samples were firstly screened for *B. exitiosa* gDNA by the PCR using the primers of Ramilo et al. (2014): forward primer BEXIT-F (5′- GCGCGTTCTTAGAAGCTTTG −3′; coordinates 1707–1726 of GenBank accession number **DQ312295**), reverse primer BEXIT-R (5′-AAGATTGATGTCGGCATGTCT −3′; coordinates 1931–1951 of **DQ312295**) to generate an amplicon of 246 bp. Reaction conditions are detailed in supplementary file 1.

The samples were next screened for *Bonamia ostreae* gDNA by the PCR using the primers of Ramilo et al. (2014): forward primer BOSTRE-F (5′- TTACGTCCCTGCCCTTTGTA −3′; coordinates 1622–1641 of GenBank accession number **AF262995**), reverse primer BOSTRE-R (5′-TCGCGGTTGAATTTTATCGT −3′; coordinates 1810–1829 of **AF262995**) to generate an amplicon of 208 bp. Reaction conditions are detailed in supplementary file 1.

The primers previously reported for amplification of APX 18S rRNA sequences (Suong et al., 2018) are not suitable for use with gDNA isolated from FFPE samples because of the length of the corresponding amplicon (723 bp) being beyond the limit considered viable for such gDNA. Therefore, alternative primer pairs were needed. Suitable regions, considered to minimise the chances of PCR primers annealing to non-target sequences, were identified by alignment of the 18S rRNA sequences: APX (GenBank acc. no.: **KX774501**, **KX774502**); *Ostrea chilensis* (**KX977494**); *Crassostrea gigas* (**AB064942**); *B. ostreae* (**AF262995**) and *B. exitiosa* (**JF495410**) following (Suong et al., 2018). It was decided that the forward primer to be used would be APX-For (5′-TCTTTGAGTGAGAATCCGGTTTG −3′; coordinates 645–667 of **KX774502**) from Suong et al. (2018) to be paired with two alternative reverse primers: (i) APXRevFFPE-2: 5′- TCTAAGAATTTCACCTCTGACAG −3′ (coordinates 881–903 of **KX774502**) generating an amplicon of 259 bp and (ii) APXRevFFPE-3: 5′- CTGACAGTACAAATACGAATG −3′ (coordinates 867–887 of **KX774502**) generating an amplicon of 243 bp. Reaction conditions are detailed in supplementary file 1. Molecular weight standards were pBR322 / BsuR1 (Thermo Scientific Cat. No. SM0271). The positive control was gDNA isolated from an *Ostrea chilensis* specimen (OC54) known to be infected with APX.

2.5. Statistical analyses

All analyses were performed in R V 4.1.0 Software (R Core Team, 2024). Differences in mean size of oysters between cohorts was tested for all 98 samples using a Student's *t*-test with significance set at $\alpha < 0.05$ for this and all subsequent tests mentioned below.

The histological examination identified that most animals dissected on day 8 (days out of water, on ice) were moribund and the accurate assessment of *Bonamia* infection intensity in those individuals was not possible. Thus, a subset of samples which excluded oysters sampled on the final day (day 8), was used for the linear model analyses that included *Bonamia* intensity outlined below ($n = 78$).

Data on parasite infection prevalence and infection intensity were firstly analysed using a Spearman's rank correlation analysis to examine correlations between all variables, accommodating a mix of ordinal and continuous variables. All of the different variables describing *Bonamia* infection intensity were highly correlated with each other (Fig. 6; Supp. File. 2). For example, the correlations between the tissue grades and the whole animal grade ranged from $r = 0.96$–0.97 (all $p < 0.0001$; Supp. File 2). Thus, a reduced set of variables was used to carry out subsequent models identifying significant effects. The models used the whole animal grade (mean of the five different tissue grades) as a proxy for *Bonamia* infection intensity.

Due to the binary (presence/absence) nature of the *prevalence* data, logistic regression (using the glm function from R's base stats package) was used to test the effect of cohort, the prevalence of other parasites and the % gonadal area and % gamete density on the prevalence of *B. ostreae*, APX and *Alcicornis longicornutus*. Logistic regression allows to

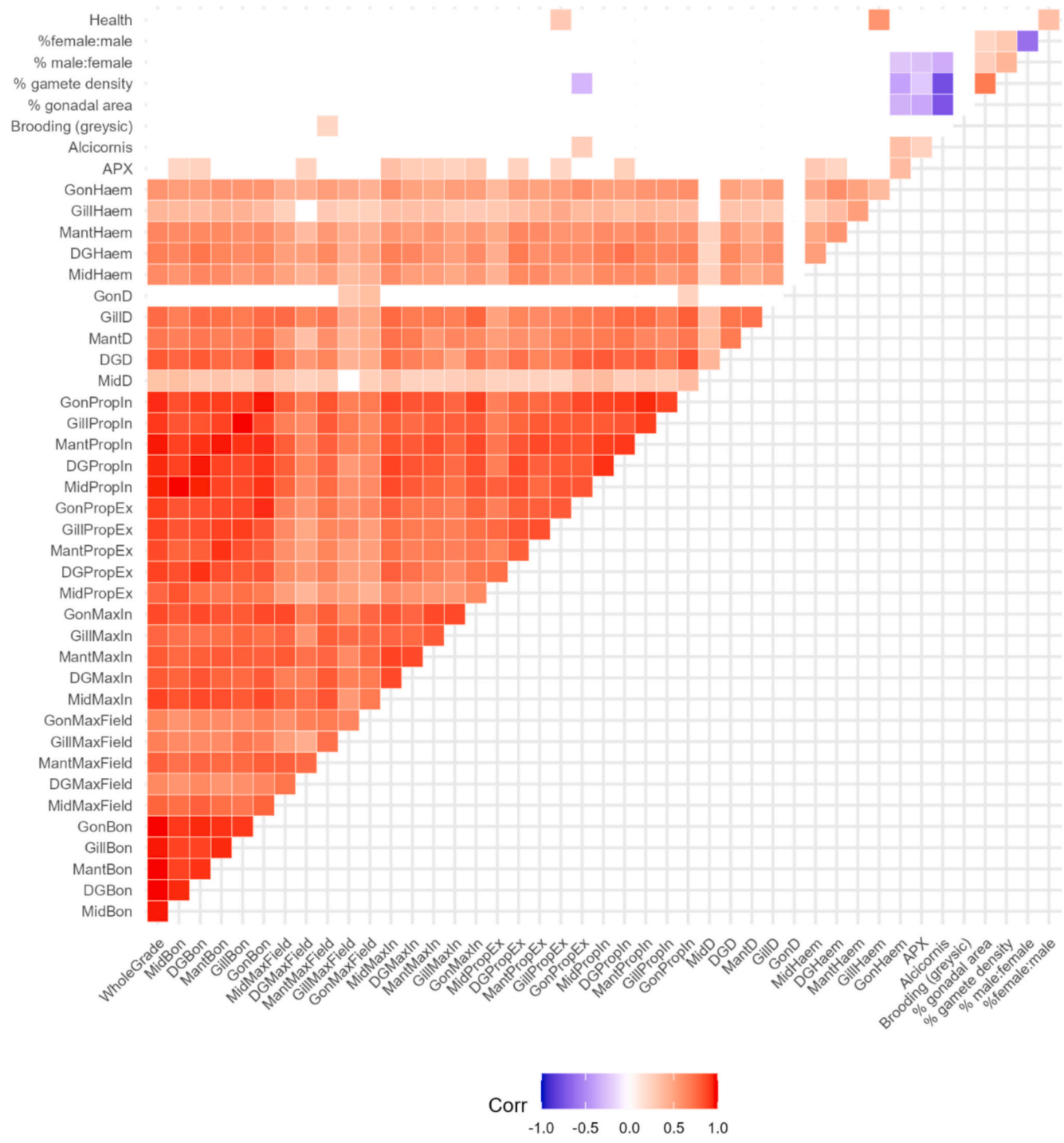


Fig. 6. Correlation analysis of all factors examined via histology and PCR including the parasites *Bonamia ostreae,* Apicomplexan X (APX), and the Bucephalid *Alcicornis longicornutus* (Alcicornis), in Ostrea *chilensis* obtained from an infected farm in Port Underwood, Marlborough Sounds, NZ in November 2017. Coloured squares indicate a statistically significant correlation (blue = negative, and red = positive). To decipher the various factors, 'Health' denotes apparent 'health status' graded as 'healthy' 'unhealthy' or 'moribund'; for each abbreviated tissue type (Mant = mantle, Gon = gonad, Mid = midgut, DG = digestive glad, Gill = gill) MaxField = maximum number of *Bonamia* microcells counted in a microscope field of view, MaxIn = maximum number of intracellular *Bonamia* microcells counted in a haemocyte in that tissue type, PropIn = relative proportion of *Bonamia* microcells that are intracellular, PropEx = relative proportion of *Bonamia* microcells that are extracellular, Bon = *Bonamia* 'intensity' grade for that tissue type, WholeGrade = overall mean *Bonamia* intensity grade for that animal (mean of the tissue grades), Haem = haemocytosis grade for that tissue type, D = diapedesis (presence/absence), and gonad descriptions are percent (%) gonad area, percent (%) gonad density and proportion of male:female or female:male gametes, as described in methods (section 2.3.2). Thus, for example 'MantHaem' denotes mantle haemocytosis grade, 'GonHaem' denotes gonad haemocytosis grade, 'GillBon' denotes *Bonamia* intensity grade for the gill tissue, and 'MidD' denotes Midgut diapedesis observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

test for several factor simultaneously and their potential interactions (e.g. cohort-dependent effects of the prevalence of other parasites). In addition, it allows to control for the effects of continuous covariates (e.g. percent gonadal area). Three separate logistic regression models were fitted for each species, with cohort and the prevalence of the other two parasites as fixed effects with two levels each. The interaction between cohort and the prevalence of other parasites was also included to test for potential cohort-dependent effects. Percentage gonadal area and % gamete density were fitted as continuous variables to control for their potential effect on parasite prevalence or vice versa. The regression coefficients are presented as odds ratios (exponentiated coefficients) that are interpreted as the ratio of the probability of an infection event or the probability of no infection.

As mentioned above, for the *Bonamia* infection *intensity* data, a linear model was run on a subset of variables chosen using results of the correlation analysis to test the effect of cohort, the prevalence of APX and % gonadal area on *Bonamia* infection *intensity* using the overall whole-animal *Bonamia* grade (mean of 5 tissue grades) as a proxy for *Bonamia* infection intensity; see section 2.3.3.1. Cohort and APX were fitted as fixed effects with two levels each, and percentage gonadal area and percent gamete density were fitted as continuous variables.

All models were selected using a stepwise procedure based on the Akaike Information Criterion (AIC) values. The final models were validated by inspecting simulated residuals and checking that model assumptions of over-dispersion and homogeneity of variance were met.

3. Results

3.1. Health assessments

The overall apparent health status of flat oysters at the time of dissection ('healthy', 'unhealthy' or 'moribund') was not correlated with *Bonamia* or other parasite infection prevalence or intensity (Fig. 6; Supp. File 2). There was a weak but statistically significant correlation between 'health status' and gill haemocytosis ($r = 0.58$; $p < 0.001$), similarly between 'health status' and the relative proportion of female: male gametes ($r = 0.35$; $p = 0.002$), and between 'health status' and the relative proportion of extracellular *B. ostreae* microcells in the gills ($r = 0.29$; $p = 0.01$; Fig. 6; Supp. File 2). Overall, 48 % of sampled oysters were observed to be 'healthy' (regardless of *Bonamia* or other parasite infection status), 26 % were observed to be 'unhealthy', and 26 % were regarded as 'moribund'.

The proportion of moribund individuals increased markedly over time as the oysters were held out of water. Histological analysis identified that most animals sampled on day 8 were moribund (Fig. 7), and that the accurate assessment of *Bonamia* infection intensity was not possible in these individuals. However, these animals were still used for molecular assessments of parasite presence/absence, but only 3 of the 17 animals sampled on day 8 were *Bonamia* positive. Any animals that were too moribund to accurately quantify *Bonamia* infection intensity were excluded from any statistical analyses involving *Bonamia* intensity. This included not only all animals that were sampled on day 8, but also two individuals sampled on previous days in which not all tissues were present for histological assessment.

3.2. Bonamia detection

The haplosporidian parasite *Bonamia ostreae* was detected by PCR in 68 % of samples overall, with 95 % of the oysters in cohort 1 being PCR positive for *B. ostreae*, compared with just 22 % of oysters in cohort 2. Overall, 81 % of PCR positive infections also had a *Bonamia* infection observable via histology (55 % of animals overall had an infection observable via histology: 79 % and 14 % of cohorts 1 and 2, respectively). The congeneric *B. exitiosa* was detected by PCR in just one of the 98 animals sampled: an apparently healthy individual from cohort 1 in which a light (grade 1) infection was observed in all tissues except for the gonad. Due to only a single infection of *B. exitiosa* being detected, and it also being a co-infection with *B. ostreae*, the following results describe only observations of *B. ostreae*.

3.3. B. ostreae assessments

When *B. ostreae* infection intensity was examined in known infected (PCR positive) oysters, the distribution was relatively uniform across the observed range of whole-animal grades (0–3) but with a left skewed u-shaped or inverted bell-curve distribution, indicating that around two thirds of oysters (68 %) had either a low or histologically un-detectable level of infection (grade < 2), and only around one third (32 %) had an overall grade of moderate to heavy (≥ 2; Fig. 8).

The 59 infected (PCR positive) oysters in cohort 1 demonstrated a wide range of infection intensities ranging from no infection observable via histology in any tissue, to highly abundant *Bonamia* microcells in every tissue, with very high numbers observed both intracellularly (up to 32 *Bonamia* microcells per infected haemocyte) and extracellularly (up to 572 microcells per field of view; Fig. 9). Note that two individuals from cohort 1 did not have a *Bonamia* infection intensity observed for all tissues and thus a 'whole-animal grade' could not be calculated, however these two individuals had tissue grades of low or absent for all tissues that were observed.

All of the 8 infected (PCR positive) oysters in cohort 2 had very low

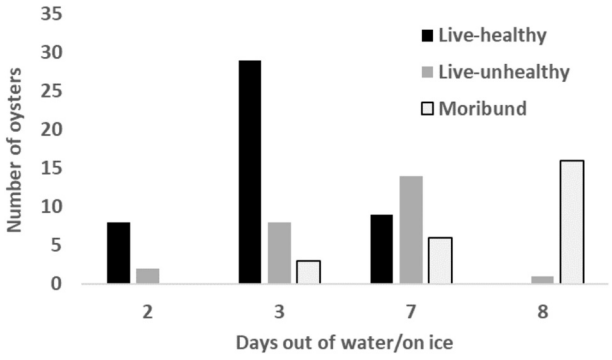


Fig. 7. Histological assessment of the apparent health status of oysters of all cohorts (either alive and healthy, alive and unhealthy, or moribund), relative to the number of days they were held out of water and on ice (days since collection) prior to dissection. Oysters that were dissected following 8 days of storage on ice were too moribund to be used for accurate assessment of *Bonamia* intensity.

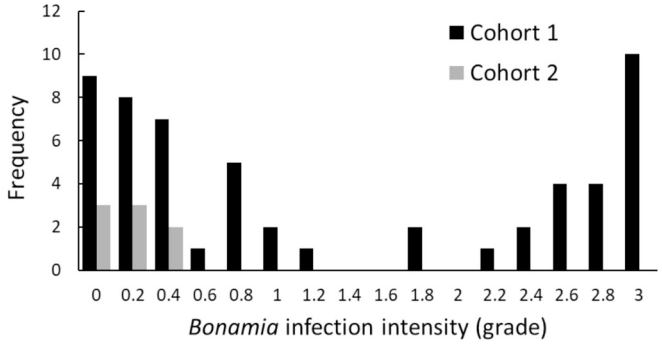


Fig. 8. Frequency and distribution of the histologically observed whole-animal grade describing *Bonamia ostreae* infection intensity (derived from the mean of the 5 tissue grades) in the 65 individual animals found to be *B. ostreae* PCR positive, from two cohorts of *Ostrea chilensis* obtained from an infected farm in Port Underwood, Marlborough Sounds, NZ in November 2017. Cohort 1 (black) early grow-out at higher stocking density ($n = 57$) and Cohort 2 (grey) later grow-out at lower stocking density ($n = 8$).

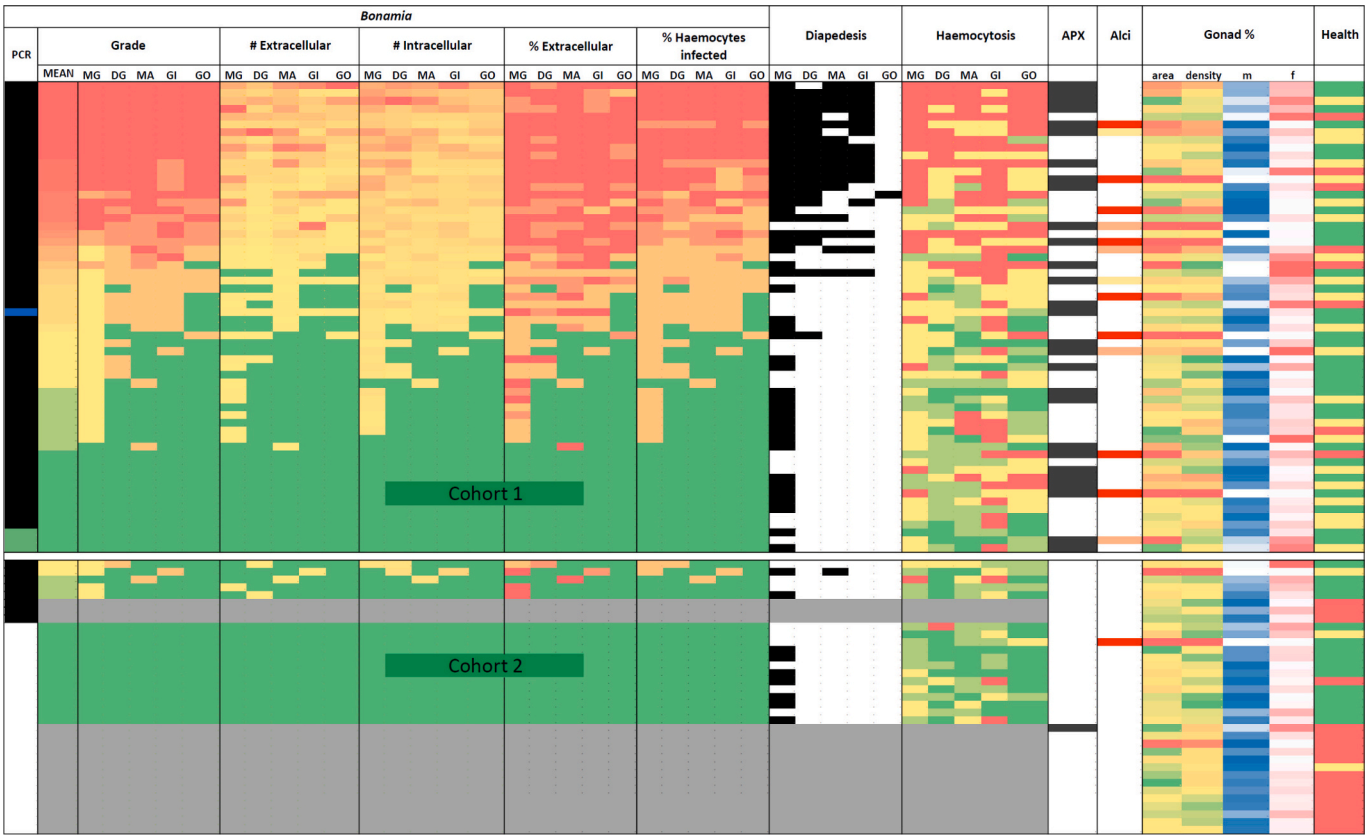


Fig. 9. Heatmap of all samples from the two different flat oyster cohorts (Cohort 1, $n = 60$ and cohort 2, $n = 35$) in which all tissues were present for analysis (total $n = 95$), ordered within cohort by whole-animal *Bonamia* score (MEAN) from highest to lowest. Black and white tiles represent samples that were positive and negative respectively for the presence of *Bonamia ostreae* (PCR), diapedesis via histology (Diapedesis) and Apicomplexan X via PCR (APX). The blue tile in the PCR column represents the single sample that was positive for both *B. ostreae* and *B. exitiosa.* Bucephalid parasite *Alcicornis longicornutus* infection intensity (Alci) is indicated on a scale of red to white, where red is heavy, orange is moderate, pink is light, and white is absent. Samples are ordered within cohort by the mean *Bonamia* infection intensity grade in the whole animal (MEAN) from red to green where red = highest mean *Bonamia* infection intensity (grade 3; *Bonamia* abundant in all tissues) to green = lowest (grade 0; *Bonamia* not observed in any tissue). Subsequent columns show infection intensity as the *Bonamia* grade per tissue type (GRADE) on the same red to green colour scale where red = 3 and green = 0 in midgut (MG), digestive gland (DG), mantle (MA), gill (GI) and gonad (GO); the maximum observed count of extracellular microcells in each of the tissue types (# extracellular) where red = 572 and green = 0; the maximum observed count of microcells per infected haemocyte in each of the tissue types (# Intracellular), where red = 32 and green = 0; the proportion of *Bonamia* microcells occurring extracellularly (% Extracellular) from red to green, where red is >50 % extracellular and green is no extracellular *Bonamia*; the percent of haemocytes infected (% Haemocytes infected) from red to green, where red is >50 % of haemocytes infected and green is no infection. Haemocytosis is presented on an intensity scale of 0 to 3 (green to red) where red indicates intense haemocytosis present (grade 3) and green indicates no haemocytosis (grade 0) in each tissue. Gonad scores (Gonad %) are percentage gonad area (area) and relative density (density) on a scale of 0–100 % where red is 0 % and green is 100 %. Proportion of male (m) and female (f) gametes are shown on a scale of white to blue or red, respectively, where 100 % male is blue and 100 % female is red, and 0 % of either is white. "Health" score (at time of dissection) is shown on a scale of green = alive and apparently healthy, yellow = alive and unhealthy, and red = moribund. Finally, greyed out sections indicate samples where tissues were so degraded (due to the animals being moribund) that it was not possible to accurately describe the *Bonamia* infection intensity. Those samples (n = 3 *Bonamia* PCR positive and $n = 14$ *Bonamia* PCR negative) were not included in statistical analyses of *Bonamia* infection intensity and its interactions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

infection intensities. Where these infections were observable via histology (n = 5) the *Bonamia* microcells only appeared in one or two tissue types in each individual, however the infections in these 5 samples included observations of *Bonamia* in all tissues except the gonad (Fig. 9).

Overall, when observations from both cohorts were combined, *B. ostreae* infections occurred most commonly in the midgut, followed by the digestive gland, gill, and mantle, and less frequently in the gonad. However, as mentioned for the animals in cohort 2, the infections in each individual did not always follow this pattern (Fig. 9). The infections were most often observed as *Bonamia* microcells within infected haemocytes, and extracellular *Bonamia* microcells occurred at a higher number and frequency in oysters with a higher infection intensity in general. However, in most instances, if *Bonamia* microcells were observed in haemocytes, they also occurred extracellularly in the same tissue. In contrast, in a few instances of very light infections, *Bonamia* microcells were only observed extracellularly in the midgut. In heavy infections (overall *Bonamia* infection intensity grade 2–3), more than half of all haemocytes were infected, and *Bonamia* microcells were predominantly extracellular (Fig. 9).

3.4. Detailed histological observations of *B. ostreae* infection in *O. chilensis*

Microcells of *B. ostreae* were observed within the haemocytes, as well as freely in the connective tissue, in and around the mid-gut, digestive tubules, mantle, gills and gonad of *O. chilensis* (Fig. 10 A-I). Intra- and extracellular microcells were also recorded in the heart of a heavily infected oyster, although this was only in one individual in which the heart tissue was present for observation.

In the mid-gut and digestive gland, at lower infection intensities, there were focal and multifocal areas of haemocytosis surrounding the stomach, intestine and digestive tubules, in which *Bonamia* cells were

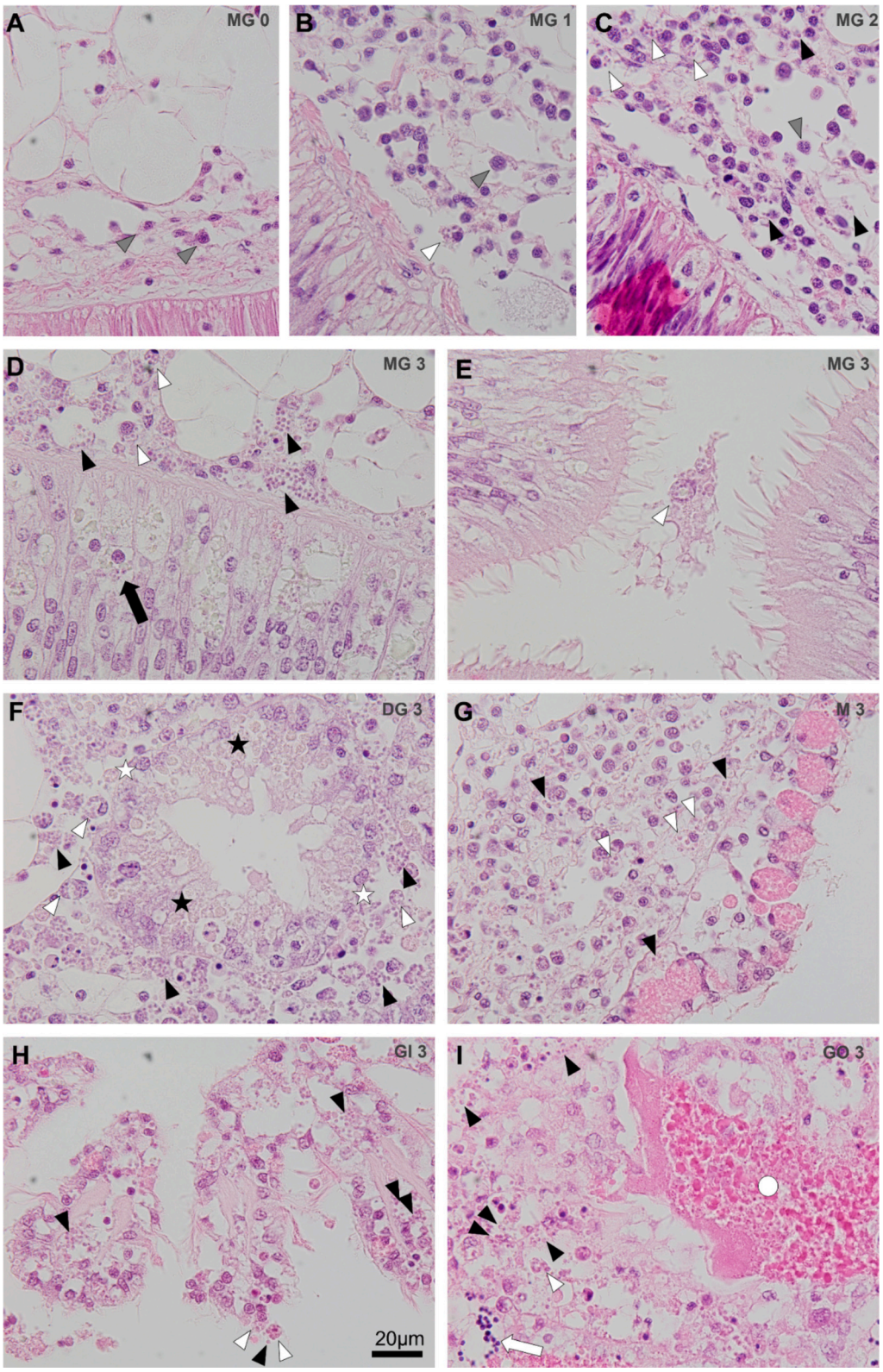


Fig. 10. Examples of *Bonamia* spp. infection intensities in five different tissues: midgut (mostly sub epithelial connective tissue), MG; digestive gland, DG; mantle, M; Gill, GI; and Gonad, GO. Images A-E show grade 0–3 infections in the MG and images F—I show grade 3 infections in the DG, M, GI and GO respectively. Grey triangles show examples of healthy, uninfected hemocytes while the white triangles show *Bonamia* spp. microcells inside hemocytes (intracellular) and black triangles show *Bonamia* spp. microcells loose in the connective tissue (extracellular). The black arrow (D) shows diapedesis of microcells into the lumen of the intestine, where there are other intracellular and extracellular microcells (E). In the DG (F), black stars in the tubule epithelium show intense vacuolization of digestive cells which are filled with grey, amorphous material and white stars show 'wearing down' of the basement membrane (from externally). In the GO (I), the white arrow shows spermatozoa and white circle, an atresic oocyte, surrounded by intracellular and extracellular microcells (examples shown by white and black triangles). All images at identical scale, bar = 20 µm.

often found. More often, microcells were found inside hemocytes but, at increasing infection intensities, more extracellular microcells were observed (Fig. 9). Diffuse, severe haemocytosis was observed at higher infection intensities, with rupturing of hemocytes releasing microcells (Fig. 9). At the highest infection intensity (grade 3), *Bonamia* microcells were predominantly extracellular and generally found in abundance outside the basement membrane of the stomach, intestine and digestive tubules (Fig. 10 D and F). At the same grade 3 infection intensity in the midgut, there was diapedesis of hemocytes, free, extracellular microcells (Fig. 10 D) and hemocytes containing microcells in the lumen of the stomach and intestine (Fig. 10 E). Meanwhile, in the digestive gland at grade 3 infection intensity, there was disintegration of the structure of the connective tissue and digestive tubules, with the basement membrane sometimes appearing broken from the outside of the tubules (Fig. 10F). There was also intense vacuolisation of digestive cells which were filled with grey, amorphous material (Fig. 10F) which was independent of the health status assigned during the health assessment (section 2.3.1).

In the mantle, in lower intensity infections, there was focal and multifocal haemocytosis and these areas often contained inter and extracellular *Bonamia* microcells. As the infection intensity increased, there was increasingly severe, diffuse haemocytosis and microcells of *B. ostreae* were detected within hemocytes in the blood spaces. At grade 3 infection intensity, the connective tissue structure became significantly disrupted, and both extra- and intracellular microcells were observed in abundance at the mantle edge and outside the animal (Fig. 10 G).

Lower intensity *Bonamia* infections in the gill were relatively difficult to detect given the number of hemocytes normally observed in this tissue (Fig. 9), however, predominantly intracellular microcells could be detected in areas of haemocytosis. At higher infection intensities, the gill structure became disrupted and microcells of *B. ostreae* were detected within haemocytes in the blood spaces. At grade 3 infection intensities, predominantly extracellular microcells were found towards the tips of the gill where there was rupture of microcells to the outside of the animal.

In the gonad, at lower infection intensities, intracellular and extracellular *Bonamia* microcells were observed in the connective tissue between developing gonadal follicles. As the infection intensity increased there was localised disruption of the connective tissue. At the highest infection intensity (grade 3), there was widespread degradation of the connective tissue surrounding the gonadal follicles (Fig. 10 I) and the membrane surrounding the gonadal follicles was sometimes broken, resulting in an influx of *Bonamia* and debris into the follicle. This stage was associated with an abundance of atresic oocytes and intra- and extracellular microcells were observed next to atresic oocytes (Fig. 10. I). Hemocytes and microcells were also observed gathered near the gonoducts.

Although high intensity infections were associated with intense inflammatory reactions in most of the tissues studied (i.e. haemocytosis, Fig. 9), at lower infection intensities, this was not the case. Diapedesis (the passage of haemocytes through the epithelium) in the digestive gland, mantle and gill were associated with high intensity *Bonamia* infections but not in the gonad- where diapedesis was only observed once. Moreover, in the mid-gut, diapedesis was observed frequently, regardless of *Bonamia* infection intensity (Fig. 9).

3.5. Other parasite prevalence and infection intensity, and interactions with B. ostreae

Aside from *Bonamia* spp., three other parasites were observed and/or detected using a combination of histological observation and PCR. The apicomplexan parasite Apicomplexan X (APX) was detected by PCR in 30 % of samples overall, and the bucephalid trematode *Alcicornis longicornutus* was observed via histology in 15 % of samples (Fig. 9). Finally, presumptive cysts of *Microsporidium rapu* (Jones 1981) were observed in the connective tissue near the gut of a single individual.

The prevalence of *B. ostreae*, APX and *A. longicornutus* differed significantly between cohorts, with the prevalence of all 3 parasite species significantly higher in cohort 1 compared with cohort 2 (Fig. 11; all $p < 0.001$). Similarly, in cohort 1, 45 % of oysters were PCR positive for APX, with just 3 % positive in cohort 2. For *A. longicornutus* the infection prevalence was 23 % and 3 % for cohort 1 and 2, respectively (Fig. 11). The odds ratios indicated that oysters were 47 times more likely to be infected with *B. ostreae,* 26 times more likely to be infected with APX and 21 times more likely to be infected with *A. longicornutus* if they were in cohort 1.

The correlation analysis indicated that the presence of APX correlated with many indicators of tissue level *Bonamia* and other histological measures including midgut and digestive gland *Bonamia,* proportion of extracellular *Bonamia* in the digestive gland and gills, maximum observed *Bonamia* microcells per haemocyte in all tissue types, proportion of intracellular *Bonamia* in the digestive gland, total count of *Bonamia* per field of view in the digestive gland, and midgut, digestive gland and gonad haemocytosis (Fig. 6; Supp. File 2). However, all of these correlations, although significant ($p = 0.001$–0.049) had relatively low r values (0.22–0.37) and APX was not significantly correlated with the overall whole-animal *Bonamia* score which represented the overall infection intensity in the individual (Fig. 6; Supp. File 2).

The linear model subsequently used to test the effect of cohort, presence of APX infection, presence of *Alcicornis* infection, % gonadal area and % gonad density on *Bonamia* infection intensity, showed that there was a significant effect of cohort on *Bonamia* infection intensity ($p < 0.001$), but neither APX, *Alcicornis* or percentage gonadal area or density had a significant effect ($p > 0.75$). The final model selected using a stepwise procedure based on the Akaike information criterion (AIC) included only cohort as a significant predictor of infection intensity and predicted a significant increase in infection intensity for oysters in cohort 1 (earlier grow-out at high density; $p < 0.001$).

3.6. Relationship between Bonamia prevalence and infection intensity, reproductive parameters and other parasites

Both cohorts were observed to be reproductive. The overall gonad condition of the oysters covered a very large range with the percent gonadal area ranging from 0 to 78 %, gamete density from 0 to 96 %, and

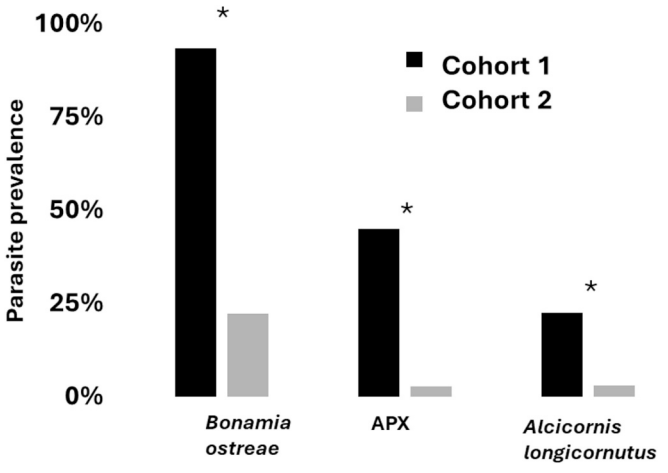


Fig. 11. Prevalence of the parasites *Bonamia ostreae* (via PCR), Apicomplexan X (APX; via PCR), and *Alcicornis longicornutus* (via histology), in two cohorts of *Ostrea chilensis* obtained from an infected farm in Port Underwood, Marlborough Sounds, NZ in November 2017. Oysters from cohort 1 were grown at higher stocking densities (up to 200 individuals per linear m) from earlier in the summer (December) and those from cohort 2 were grown at lower stocking densities (50–80 individuals per linear m) from later in the summer (February). Asterisks indicate a significant difference between pairs at $p < 0.001$.

both percent of male and female gametes ranging from 0 to 100 %. These ranges were almost identical in the two different cohorts, with the only differences being that cohort 2 (the slightly younger oysters) had a slightly lower maximum % gonad area of 68 % (vs 78 % for cohort 1), and a maximum % female gametes of 96 % (vs 100 % for cohort 1). All except one of the animals that had 0 % gonad area had a moderate to high intensity infection with *Alcicornus longicornutus* which is a trematode that infects the gonads and at high intensity renders the animal sterile. Two individuals, both from cohort 1 were observed to be brooding mid-stage 'grey-sic' larvae. Both brooding oysters were infected with *B. ostreae*; one had no histologically detectable infection and the other had a very high intensity infection (overall score 2.8/3).

Logistic regressions indicated that APX and *A. longicornutus* infections but not *B. ostreae* infections were associated with a smaller gonadal area ($p < 0.001$) and *Alcicornis* infections were also significantly associated with lower gamete density ($p < 0.01$).

4. Discussion

Two year old *Ostrea chilensis* who had been transferred to their grow-out site later in the NZ summer (Feb) and at reduced stocking density (including lower density per linear meter on each dropper, and increased spacing between droppers) showed significantly lower infection intensities of *B. ostreae* and significantly lower infection prevalence of all parasites compared to those moved to grow-out earlier in the summer (Dec) at higher stocking densities on the same farm. Although other factors that were not measured in this study, such as the localised inoculum pressure during the two-month period when only the first cohort were in the grow-out area of the farm, or possibly subtle differences in water flow in different areas of the farm, could have also contributed to the observed differences, and the final prevalence and intensity of infection in the two cohorts may be due to a combination of all of those factors, these data nonetheless indicate that adapted farming practices may indeed contribute to reducing parasite transmission within farms, which may lead to lower parasite burden and lower mortality.

Histopathological and molecular assessments of oyster tissues also provided novel insights into the progression of *B. ostreae* infection in *O. chilensis* in NZ, with low-level infections observed most commonly in the midgut, implying they occur there first, before next most commonly being observed in the tissues surrounding the digestive gland, then in the mantle and gills and finally in the gonad, suggesting this order as the progression of infection.

Possibly the most surprising finding of this study however, was that despite the parents of the two cohorts examined in this study having experienced very high mortalities due to *B. ostreae* in late 2014 to early 2015 (estimated to be over 95 %) and the cohort examined in this study that was grown at high density from 2015 to 2017 also experiencing approx. 95 % mortality prior to sampling, around *half* of the surviving oysters from this cohort (all of which were found interspersed among hundreds to thousands of dead oysters) were relatively healthy, and around one quarter of the total had no histologically observable *Bonamia* infection. Moreover, 5 % of the survivors of cohort 1 (3/62 individuals) were PCR negative. Unless the ~95 % mortality of this cohort were due to some other unidentified unexplained factor and not *B. ostreae,* it is impossible that these oysters were not exposed to *Bonamia* during their two-year grow-out phase, as a single dying infected oyster can release up to many hundreds of millions of *Bonamia* microcells into the surrounding water column (Lallias et al., 2008). Thus, the absence of infection in these survivors strongly indicates that this farmed population of oysters had individuals that exhibited true resistance to, as well as tolerance of *B. ostreae* infection.

4.1. Progression of B. ostreae infection

Although it has long been known that *O. chilensis* were susceptible to the exotic parasite *B. ostreae* outside of NZ (Bucke and Hepper, 1987; Morgan, 2012; Walne, 1963, 1974), it was not until *B. ostreae* was detected in NZ in 2015, that *O. chilensis* in their native environment were also shown to be extremely susceptible to the parasite (Lane, 2018; Lane et al., 2016). The detailed progression of *B. ostreae* infection however, remained largely unknown, particularly with respect to light or low-intensity infections. In the current study, we have inferred detailed insight into disease progression from the wide range of *Bonamia* infection intensities recorded in this one-off sample of the population. In light infections, *Bonamia* microcells were observed in the mid-gut (stomach and intestine). As infections became heavier, they were also observed in the digestive gland (digestive tubules and surrounding connective tissue), then mantle and gills, and were only consistently observed in the gonad in heavy infections. Consistent with the present study, Lane (2018), also observed *Bonamia* spp. microcells in the haemocytes surrounding the gut and digestive gland of lightly infected individuals, and least often in the connective tissues of the gonad. Conversely, in their study, microcells were also detected in the gills and mantle of *lightly* infected oysters, but differences may exist in the definition of a 'light' infection between these two studies. Studying the congeneric *B. exitiosa* infection in *O. chilensis*, Hine (1991a, 1991b) observed similarly to this current study, that light infections were most often observed around the gut, and only in other tissues at higher infection intensities.

Although the overall health status of flat oysters at the time of dissection ('healthy', 'unhealthy' or 'moribund') was not correlated with overall *Bonamia* or other parasite infection prevalence or intensity there was observed to be a weak but statistically significant correlation between poorer health status and both gill haemocytosis and the relative proportion of extracellular *B. ostreae* microcells in the gills. We thus hypothesise that it may be that once the infection becomes systemic and present extracellularly in the gills (but prior to infiltrating the gonads) that the host's health begins to be significantly negatively affected.

Early studies of *B. ostreae* infections in the closely related European flat oyster, *O. edulis,* showed the parasite induces high gill pathology (e.g. Balouet et al., 1983; Grizel et al., 1988; Bucke and Hepper, 1987). In the present study, *Bonamia* microcells did induce gill pathology but were only observed in the gills at higher infection intensities, at which point the parasite had become systemic and appeared in almost all other tissues simultaneously. As observed and described by Lane (2018, 2022) more recent studies on both *B. ostreae* and *B. exitiosa* in multiple hosts suggest that such differences are more likely to be due to seasonality, progression of disease and differences in host biology and susceptibility rather than inherent differences between the parasites themselves. The proportion of infected hemocytes increased as infection intensity increased, as did the proportion of extracellular *Bonamia* microcells, which is in accordance with previous studies of *B. exitiosa* progression in *O. chilensis* by Dinamani et al. (1987) Hine (1991a, 1991b) and Diggles et al. (2003) and also those of *B. ostreae* progression in *O. edulis* (Bucke, 1988) and *O. chilensis* (Lane, 2018).

The presence of *Bonamia* microcells in the gonad of highly infected individuals in this study is unsurprising given the observed infection cycles of both *B. ostreae* in the northern hemisphere and *B. exitiosa* in NZ. In addition, general health status was significantly correlated with the relative proportion of female:male gametes, where poorer health was associated with a higher proportion of female gametes. Although poorer general health could reflect the high energetic cost of gamete provisioning and gametogenesis in a species that broods just once annually, *Bonamia* microcells are also believed to use spent gonad material as an important energy source for their proliferation, and thus parasite proliferation and highest prevalence and intensity of infection are thought to generally occur in autumn or winter following the peak spawning period of the host when oysters are reabsorbing gametes (Engelsma et al., 2010; Hine, 1991a, 1991b; Jeffs and Hickman, 2000; Laing et al., 2014; Mérou et al., 2023). The prevalence and timing of spawning in *O. chilensis* in NZ is known to be very asynchronous within a population, and vary latitudinally (Brown et al., 2010; Jeffs, 1998; Jeffs et al.,

1997a; Jeffs et al., 1997b). The most northern populations may have some proportion of the population brooding year-round, whereas populations in cooler latitudes (including the Marlborough Sounds where the animals were sampled for the current study) have a marked peak spawning in spring and summer. The results of this current study, where animals were collected in November (the beginning of Austral spring), in what is the beginning of the known peak spawning period at this latitude (Brown et al., 2010) and when *B. exitiosa* infection intensity is generally predicted to be relatively low, suggests several potential hypotheses. Some animals may have already had very high infection intensities during winter but had not yet died from those infections. The subset of the population showing high infection intensities in November (spring) may have been early-season spawners, and were possibly already suffering from the predicted rapid proliferation of *B. ostreae* infection during this part of the reproductive cycle. Engelsma et al. (2010) have observed a peak in *B. ostreae* prevalence to occur in spring in European oysters (*O. edulis*). Or, since the two cohorts examined in the present study experienced very different levels of mortality, and *B. ostreae* prevalence and intensity, despite being obtained from the same broodstock, and being cultured in the same location at the same time, apart from the first 2 months of life for cohort 1, it could be that that the detailed epidemiology and impact of *B. ostreae* on each population may be quite distinct due to the interplay between timing and magnitude of exposure to infective doses, host age, population density and susceptibility as well as latitudinal variations in host biology.

Most mortality attributed to the putatively endemic *B. exitiosa* in these farmed stocks was previously observed in 3 yr old oysters during the autumn months after their spawning peak (A. Elliot and S. Webb unpublished data). In this study, the observation of presumably *Bonamia*-induced mortality already at age 2 yrs., suggests that *B. ostreae* may be more pathogenic in this host species than *B. exitiosa*, which is similar to observations in Europe where the arrival of *B. ostreae* was associated with high levels of mortality in populations of *O. edulis*, but the subsequent detection/arrival of *B. exitiosa* was not (Abollo et al., 2008; Longshaw et al., 2013; Ramilo et al., 2014). Likewise, Lallias et al. (2008) have demonstrated that when challenged through co-habitation experiments, even one year old oysters are susceptible to mortality from *B. ostreae*. However, it should be noted, that the initial intensity of an epizootic occurring in a naïve population, where many oysters are dying simultaneously and thus releasing many millions of *Bonamia* particles in the same area at more or less the same time, may cause an intense epizootic and mass mortality, but as observed in Europe after the arrival of *B. ostreae*, and indeed following the major epizootics in the Foveaux Strait of NZ with *B. exitiosa* in the 1980s, this pattern may not continue once the host-pathogen dynamic becomes more stable and/or the population density is reduced.

4.2. Changes in Bonamia spp. prevalence over time

The year 2015 is believed to have been early on in the incursion of *B. ostreae* to NZ, because archival material sampled from the infected farms in the Marlborough Sounds prior to 2015 did not detect *B. ostreae*, but did successfully detect *B. exitiosa* (Lane and Jones, 2020). We are thus able to compare the findings of the current study of the farm in 2017 to a previous study of the same farm in 2015 at the beginning of the epizootic (Lane and Jones, 2020; Lane et al., 2016), and this comparison suggests that between 2015 and 2017 after the arrival and ensuing epizootic of *B. ostreae*, there was a marked reduction in prevalence of the endemic *B. exitiosa* and of co-infections of *B. exitiosa* and *B. ostreae*. In oysters sampled from this farm in 2015, 50 % of *Bonamia* spp. infections were co-infections of *B. ostreae* and *B. exitiosa* and a further 42 % of individuals were infected with *B. exitiosa* alone. Just 8 % of individuals were infected with *B. ostreae* alone (Lane, 2018). At the same time on a nearby farm, the prevalences were different, with just 5 % of animals being infected by *B. exitiosa* alone, and 50 % with *B. ostreae* alone, but still 45 % of animals demonstrated a co-infection of both *Bonamia* species. Although *Bonamia* spp. infections may be patchy in both space and time in an individual and a population, the finding of the current study that in the same farm 2 years later, *B. exitiosa* was not observed as a unique infection among 98 sampled oysters, and was observed only once as a co-infection, suggests that *B. ostreae* may have essentially 'outcompeted' it's congener in this population. This further supports the hypothesis that *B. ostreae* is more pathogenic than *B. exitiosa*, at least in naïve populations of *O. chilensis*, as has also been speculated due to the general absence of mass mortality associated with the detection of *B. exitiosa* in *O. edulis* in Europe (e.g. Abollo et al., 2008; Longshaw et al., 2013; Ramilo et al., 2014).

4.3. Emerging resistance in this population?

Results of the current study indicate that this farmed population of *O. chilensis* in NZ had individuals that may have been resistant or resilient, and/or tolerant to *B. ostreae* infection. Holbrook et al. (2021) discussed the definitions of resilience, tolerance, and resistance of individuals and populations in the context of European flat oysters *O. edulis* infected with *B. ostreae*. They defined *resistance* as a state where the parasite is not able to infect the host, or not able to proliferate within the host. *Resilience* is where the host can neutralise the virulence of the pathogen or recover from infection, and *tolerance* is where the host is still able to survive and reproduce despite the parasite burden (Holbrook et al., 2021). In this study, cohort 1 (early grow-out at high density) had suffered extremely high mortality (~ 95 %) presumably from *B. ostreae*-induced bonamiosis. Yet, around a quarter of the survivors (approx. 1 % of the total population) although they were PCR positive for *B. ostreae*, had no visible infection detectable via histology, and were completely healthy, thus indicating potential tolerance or resilience. Furthermore, the existence of a small number of PCR negative individuals among the rare survivors of this cohort (approx. 0.25 % of the original population), in a population in which mass-mortality had occurred, and where the individuals sampled were surrounded at very high densities in extremely close proximity (Fig. 3 a and b) to thousands of dead conspecifics, strongly indicates that some true resistance was present in a small number of these survivors. Since an infected adult oyster when it dies can release many hundreds of millions of parasites into the surrounding water (Lallias et al., 2008) it is therefore virtually impossible to imagine how one of these individuals could have not been exposed to what should have been an infective dose of *B. ostreae*.

The cohorts sampled in the current study were bred on-farm from parents that included some survivors of the *B. ostreae*-induced mass mortalities of 2014–2015. We hypothesise that it is highly likely that genetic variation in resilience, tolerance, or both, to *B. ostreae*-induced bonamiosis exists within NZ *O. chilensis* populations, as has been demonstrated in European flat oysters (Launey et al., 2001). Although the levels of genetic and/or phenotypic variation in wild populations of *O. chilensis* may be expected to be quite low due to their very low fecundity (~ 50,000 eggs per female in a single brood per year), and lack of a significant planktonic phase, the farmed populations sampled in the current study may be expected to have had higher variation because farmers had intentionally brought stocks from different regions of NZ (including Banks Peninsula, Foveaux Strait, Tasman Bay and Cloudy Bay; Fig. 1) to use as broodstock to begin their farming operations in the Marlborough Sounds. Different populations of *O. chilensis* are known to be very genetically distinct, and the population from Foveaux Strait represents the most ancestral and genetically diverse. Thus, assuming the translocated stocks had inter-bred, the farmed populations may have had a greater genetic and/or phenotypic variation than the local wild populations.

4.4. Interactions with apicomplexan X and Alcicornis longicornutus

A limitation to the current study was that the infection intensity of the intra-haemocytic apicomplexan X parasite (APX) was not quantified,

so we could not directly address the hypothesis of Hine (2002) that higher infection intensities of APX predispose *O. chilensis* to *Bonamia* [*exitiosa*] infection. Furthermore, new primers were developed for the detection of APX via PCR in the current study to provide correctly sized amplicons from FFPE preserved samples. Whilst the minimisation of the chances of PCR primers annealing to non-target sequences were identified theoretically by alignment of the 18S rRNA sequences of APX (GenBank acc. no.: **KX774501**, **KX774502**); *Ostrea chilensis* (**KX977494**); *Crassostrea gigas* (**AB064942**); *B. ostreae* (**AF262995**) and *B. exitiosa* (**JF495410**) following Suong et al. (2018), a practical investigation to confirm the new primers were not annealing to non-target sequences, and not producing false negatives was outside of the scope of this study, and results were only confirmed by using positive controls.

These caveats notwithstanding, in this study, *B. ostreae* infection intensity did not differ between APX infected and uninfected animals ($p > 0.75$) and *B. ostreae* prevalence was not significantly associated with APX prevalence. Thus, we conclude that APX infection per se did not predispose these animals to *B. ostreae* infection or vice versa. However, the results did indicate that individuals with APX had smaller proportional gonad area than those without APX infection, as was observed by Hine, 2002. This suggests that the presence of APX may indeed reduce the energy available to invest in reproduction, in agreement with the results of Martin-Gomez et al. (2012) who demonstrated that the host energy reserves appear to be mobilised towards immune response, as they observed an upregulation of genes involved in metabolism such as immunoresponsive gene 1 when infected with the parasite (Martin-Gomez et al., 2012). Similarly, *A. longicornutus* infections were also associated with a significantly smaller gonadal area ($p < 0.001$) and lower gamete density ($p < 0.01$; Fig. 9). This makes complete sense as *A. longicornutus* is observed to infect the gonads, and very heavy infections do not just reduce gamete output, but actually render the oyster completely sterile, as was observed in some individuals.

4.5. Oyster stocking density

Flat oysters spawned and moved to grow-out later in the summer and at reduced densities had a significantly lower prevalence of all parasites, as well as lower intensity of *B. ostreae* infections compared with those who had been spawned and moved to grow-out earlier in the summer at a higher (normal pre-2015) density. Longline culture (as was used in the current study) and reduced stocking densities have long been among the measures advised in Europe for reducing the effects of Bonamiosis when growing flat oysters in *Bonamia*-infected areas (Culloty et al., 2004; Georgiades, 2015; Grizel et al., 1988; Hugh-Jones, 1994). Longline culture is thought to reduce inter-individual transmission rates because *Bonamia* parasites are negatively buoyant and will fall out of the water column since they are adapted to infecting a benthic host (Mialhe et al., 1988).

The relatively low overall prevalence of APX in this study (25 % prevalence) compared to the almost ubiquitous presence of APX in dredged benthic wild flat oysters from Foveaux Strait (where sometimes up to 100 % of individuals are infected with APX; Fidler unpublished data; Webb pers. comm. Hine, 2002; Suong et al., 2018) is also of note. This may also be the result of longline culture reducing the transmission rate of APX among individuals, compared to benthic farming or wild beds, just as it appears to do with *Bonamia* (Georgiades, 2015).

There are clearly other factors that differed between these cohorts such as their age, size and timing of deployment to the final grow-out area, as well as unknown factors such as potential differences in inoculum pressure during the first 2 months of life when only cohort 1 was at the grow-out site, or potential differences in inoculum pressure due to subtle differences in water velocities between sites on the farm. Engelsma et al. (2010) demonstrated that in European *O. edulis*, the impact of Bonamiosis was higher in bigger oysters, however this was in a population where the weight range varied 3-fold, compared to just 10 % difference in size in the current study. But Cáceres-Martínez et al. (1995) and Culloty and Mulcahy (1996) have also observed larger or older European oysters to be more susceptible to Bonamiosis. Lallias et al. (2008) in contrast found no significant effect of size on differences in mortality of *O. edulis* in lab co-habitation challenges. We argue that these other potential factors are unlikely to be strong enough to have caused the vastly different parasite prevalences and intensities that were observed in these 2 yr old cohorts on this farm. The current study, whilst not designed or carried out in a manner able to decisively state causation, suggest there may indeed be a significant advantage of making these husbandry adaptations, with potential to vastly reduce the impact from many different species of parasites.

4.6. The future of B. ostreae research and O. chilensis culture in NZ

Using modified farming practices, the farming of *O. chilensis* in *Bonamia*-infected areas in NZ may still be feasible without increasing the prevalence of the parasites in the local environment. The use of disease-free seed stock combined with appropriate disease surveillance and husbandry (i.e. density reduction, increased line-spacing, year-class separation, and harvesting oysters at an appropriate size prior to them experiencing potentially high levels of mortality) warrant further rigorous investigation as to the effects on parasite prevalence, infection intensity and subsequent mortality.

The current results also suggest that selective breeding for *B. ostreae* tolerance or resistance, as has been carried out successfully in Europe (Baud et al., 1997; Culloty et al., 2001, 2004; Lynch et al., 2014; Martin et al., 1993; Naciri-Graven et al., 1998), may offer additional benefits, and that variation for tolerance and/or resistance is likely present in the NZ populations of *O. chilensis*, as has been observed in *O. edulis* (Culloty et al., 2004; Elston et al., 1987; Hervio et al., 1995).

Future studies comparing the mechanisms of infection and host immune defence in this relatively new host-pathogen system would help to inform targeted selection for resistance, resilience, and/or tolerance, although it is likely that these would be highly polygenic traits (Pardo et al., 2016; Pardo et al., 2013). One mechanism observed to be at play in the resilience of *O. edulis* to infection by *B. ostreae* is that individuals more resilient to infection or more tolerant (displaying delayed mortality from bonamiosis) had different total and differential haemocyte counts (Cochennec et al., 1995; Naciri-Graven et al., 1998), and reduced phagocytic activity (Morga unpublished data cited in Engelsma et al., 2014) and thus, microcells were less likely to be internalised into haemocytes (Morga et al., 2012). This may be advantageous in defending against *B. ostreae* infection, but may increase susceptibility to other pathogens, and thus great care should be taken to examine holistically the response of oysters, considering multiple host-pathogen-environment interactions.

The results from the present study are based on just two contrasting cohort samples, taken at a single timepoint on a single farm. Nevertheless, they suggest there are interventions that may have the potential to greatly reduce the impact of *B. ostreae* in NZ. The characterisation of *O. chilensis* haemocytes (Rolton et al., 2020) along with the recent construction of a fully annotated reference genome and transcriptome for *O. chilensis* (Rodriguez Piccoli, 2024) will facilitate more detailed future research into the mechanisms of pathology and resilience, tolerance and resistance in this host-pathogen system, as well as the potential for intervention via selective breeding.

Changes in farming practices, in combination with the production of disease-free seed stocks and potential selective breeding, may enable the successful re-establishment of this important aquaculture industry, with potential positives for the long-term protection of fisheries and biodiversity in NZ.

CRediT authorship contribution statement

Zoë Hilton: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation,

Funding acquisition, Formal analysis, Data curation, Conceptualization. **Anne Rolton:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Farhana Muznebin:** Writing – original draft, Investigation. **Stephen C. Webb:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Andrew Fidler:** Writing – review & editing, Writing – original draft, Investigation. **Andrew Elliot:** Writing – review & editing, Resources. **Javier Atalah:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation. **Andrea C. Alfaro:** Writing – review & editing, Supervision, Funding acquisition. **Kate S. Hutson:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to acknowledge the assistance of doctoral candidates Suong Thao Nguyen and Thao Van Nguyen for their assistance with oyster sampling, and Dr. Henry Lane and all the staff of the Ministry for Primary Industries' Animal Health Facility at Wallaceville, NZ for their assistance with sampling of the oysters. In addition, we would like to thank the MPI staff and contractors involved in the *Bonamia ostreae* response and staff of Kono Ltd. who helped with at sea collection of the oysters from the farm. We sincerely thank Dr. Jessica Ericson for assistance with the statistical analyses, and P. M. Hine for his advice with histological interpretations, as well as two anonymous reviewers whose comments greatly improved the manuscript. This work was funded by the New Zealand Ministry of Business, Innovation and Employment, under the Programmes: Aquaculture Health to Maximise Productivity and Security (CAWX1707) and the Shellfish Aquaculture Research Platform (CAWX1801).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742683.

Data availability

Data associated with this manuscript will be available in Zenodo.

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