REVIEWS IN Aquaculture

Reviews in Aquaculture, 1–12 doi: 10.1111/raq.12545

Reviewing Pseudoloma neurophilia infections in the popular zebrafish model

Marta Caballero-Huertas[1], Manu Soto[2] and Laia Ribas[3]

1 Department of Environmental Sciences, Faculty of Sciences, Institute of Aquatic Ecology (IEA), University of Girona (UdG), Girona, Spain
2 CBET Research Group, Department of Zoology and Animal Cell Biology, Faculty of Science and Technology, Research Centre for Experimental Marine Biology and Biotechnology PiE-UPV/EHU, University of the Basque Country UPV/EHU, Leioa, Spain
3 Institut de Ciències del Mar, Spanish National Research Council (ICM-CSIC), Barcelona, Spain

Correspondence
Laia Ribas, Institut de Ciències del Mar, Spanish National Research Council (ICM-CSIC), Passeig Marítim de la Barceloneta, 37–49, 08003 Barcelona, Spain. Email: lribas@icm.csic.es

Declaration of interest
The authors declare no competing interests.
Received 9 September 2020; In Revised form 26 January 2021; accepted 26 January 2021.

Abstract

Zebrafish is a worldwide used animal model to better understand cultured fish species; thus, in the last two decades, zebrafish facilities have been created in many aquaculture research centres. However, and despite the attempts of rigorous aseptic conditions for animal husbandry in wet laboratory facilities, the presence of parasites cannot be underestimated. The microsporidium *Pseudoloma neurophilia*, which mostly affects the central nervous system, has been listed as the most prevalent parasite in reared zebrafish. The emergence of *P. neurophilia* is usually detected when the individual presents irreversible morphological and/or behavioural symptoms, and consequently, the spread of the disease is inevitable, indicating that the infection is expanded across rearing tanks. This review attempts, for the first time, to collect and discuss the current information on *P. neurophilia* life cycle, target tissues and symptomatology of the infection occurred in zebrafish. Due to its vertical transmission together with the increasing presence of zebrafish as a model in reproduction-related problems found in aquaculture, it is highlighted, among others, the sexual dimorphism of the pathogenic effects and the possible transgenerational implications of parasitic infections. Further, to date none effective treatments have been described, so here we provide in detail available diagnostic methods, discussing the importance of the early detection, giving safety-rearing recommendations to decrease the presence of *P. neurophilia*. Our intention is to minimize parasite outbreaks in zebrafish facilities, which, on the one hand, can compromise zebrafish welfare, and on the other hand, could interfere furtively in the resulting research previously designed for improving aquaculture production but also other biomedical-related research.

Key words: aquaculture, immune, infection, parasite, protocol, reproduction.

Introduction

During the last two decades, the use of model fish species in improving aquaculture research has been increased, and zebrafish (*Danio rerio* (Hamilton 1822)) is one of the fish model species with a higher expansion (Ribas & Piferrer 2014; Piferrer & Ribas 2020). Zebrafish has been traditionally used in many research fields, from development to toxicology, with biomedical purposes. This usefulness can be seen in the large number of available manuscripts related to zebrafish in the Web of Science Core Collection. Thus, the search for the topic "[research field] AND [zebrafish]" returns ~19,400 and ~3,300 documents for development and cancer, respectively. In aquaculture research, the increase in the number of publications using zebrafish has also been evidenced by the fact that by doing the same search, in six years it has passed from 204 manuscripts (Ribas & Piferrer 2014) up to 685. This is because zebrafish offers many advantages from biological and technical points of view, such as its short generation time in comparison with a long life cycle of some cultured fish species (e.g. European sea bass, *Dicentrarchus labrax* (Linnaeus 1758), which needs about 2-3 years for gonadal maturation (Blázquez *et al.* 1995)). Further, zebrafish is easy to maintain as freshwater animal, with small size, extrauterine fertilization and transparency that allows easy observation

during the embryonic development (Idilli *et al.* 2017; Santos *et al.* 2017). Further, zebrafish has many research resources available and it is well-suited to high-throughput applications (Miscevic *et al.* 2012). Thus, it has become an essential tool for developing cultured fish species and so, currently, many aquaculture institutions around the world host zebrafish facilities.

Pseudoloma neurophilia was firstly detected by De Kinkelin (1980) as a microsporidian parasite able to cause morphological abnormalities and mortality in zebrafish. Nevertheless, the microsporidium genus and species were not assigned until 2001 in zebrafish facilities at the Zebrafish International Resource Center (ZIRC), University of Oregon, Eugene (Oregon) (Matthews *et al.* 2001). About 40 years have passed since the first publication, but still few information regarding the infection has been reported. Consequently, there is a lack of standardized protocols to prevent infections. Only few screening protocols have been developed so far (e.g. those found in Kent *et al.* (2011), Spagnoli *et al.* (2015a) and Peneyra *et al.* (2018)). It can be outlined that infection outbreaks may depend on the zebrafish laboratory strain as differences in the occurrence of aggregates and parasite-associated lesions were observed. For example, myositis (e.g. inflammation in muscles) was not observed in the WIK strain when compared to AB and 5D strains infected by *P. neurophilia* (Sanders *et al.* 2020). Thus, rearing certain zebrafish strains in the fish facilities could explain the easiness of the parasitic dispersion (Whipps & Kent 2006).

The infection by *P. neurophilia* begins by the ingestion of surrounding infected tissues or spores present in the water, a typical infection route for microsporidia (Sanders *et al.* 2013; Sanders *et al.* 2014). Typically, this parasite affects the central nervous system (CNS) but it is also found in other organs and tissues, such as gonads or muscle. The parasitic transmission can be both horizontal and vertical (Sanders *et al.* 2013), the latter via female oocytes. Due to the importance of infection of this parasite in the reproductive tissues, here we further report its effects of infestation and alterations in the reproduction system. Further, we describe the applicability of current detection approaches (e.g. direct visualization, histology, *in situ* hybridization (ISH) or PCR), as well as the development of detection methods and the lack of effective treatments, highlighting the future of selective-breeding programmes based on transgenerational immune priming. Finally, recommendations are provided to abolish the infection of *P. neurophilia* parasite that could compromise final results in aquaculture research, but also, in other fields of investigation.

Characteristics of *Pseudoloma neurophilia*

Pseudoloma neurophilia is a spore-forming unicellular parasite of the large phylum Microsporidia. Phylogenomic reconstructions showed that Microsporidia has branched from the Rozellomycota, forming together either the most basal lineage of Fungi or its closer relative (Corsaro *et al.* 2016). Spores from microsporidia are commonly found in surface water and debris, and they are able to infect almost all invertebrates and vertebrates, including humans (Corsaro *et al.* 2016), as well as some protists (Han & Weiss 2017). Their life cycle consists of two general developmental stages: merogony and sporogony to later develop mature spores (Figure 1). In the case of *P. neurophilia*, meronts (early development proliferative stages (Cali *et al.* 2012)) multiply inside the infected host cell, eventually constituting sporonts (Sanders *et al.* 2012), which are developed within intracellular sporophorous vacuoles. Then, sporonts begin the metamorphosis into spores, resulting in aggregates of 8–16 spores per sporophorous vacuole (Cali *et al.* 2012). These spores will be released to the environment at some point, gaining access to cells of new hosts (adults and eggs) by penetrating their membranes with the use of a long coiled polar tube or by phagocytosis (Franzen 2004), what makes up a new spore-forming cycle (Figure 1). This is what could be considered horizontal transmission. However, some studies revealed the potential of *P. neurophilia* to be vertical transferred (Ramsay *et al.* 2009; Sanders *et al.* 2013) from the female to the offspring, having found spores within developing embryos (Sanders *et al.* 2013).

Microsporidia infections have been identified in cultured, marine and freshwater species of food commercial interest. Some examples of these microsporidia infections are found in Atlantic salmon (*Salmon salar* Linnaeus 1758) (Nylund *et al.* 2011; Matthews *et al.* 2013) and sea bream (*Sparus aurata* Linnaeus 1758) cultures (Picard-Sánchez *et al.* 2020). Indeed, the decline in fisheries production has also been attributed to microsporidiosis infections (Troemel 2011; Kent *et al.* 2014), as it was the case of *Pleistophora macrozoarcides* in 1946, responsible for the collapse of the North American ocean pout (*Zoarces americanus* (Bloch & Schneider 1801)) fishery, and the decline in the rainbow smelt (*Osmerus mordax* (Mitchill 1814)) fishery of New Hampshire caused by *Glugea hertwigi* (Franzen 2008).

Currently, in fish research facilities there are two main fish microsporidia infections, *Glugea anomala* of stickleback species (*Gasterosteus* spp.) and *P. neurophilia* of zebrafish (*Danio rerio* Hamilton 1822) (Kent & Fournie 2007; Monaghan *et al.* 2009). *Pseudoloma neurophilia* is the most common pathogen found in zebrafish in research facilities (Ferguson *et al.* 2007; Ramsay *et al.* 2009). Importantly, this parasite is mostly unique for zebrafish species. Nevertheless, under experimental conditions, *P. neurophilia* is also capable of infecting seven species of fish from five families by cohabitation with infected zebrafish: siamese fighting fish (*Betta splendens* Regan 1910), southern platyfish (*Xiphophorus maculatus* (Günther 1866)), giant danio

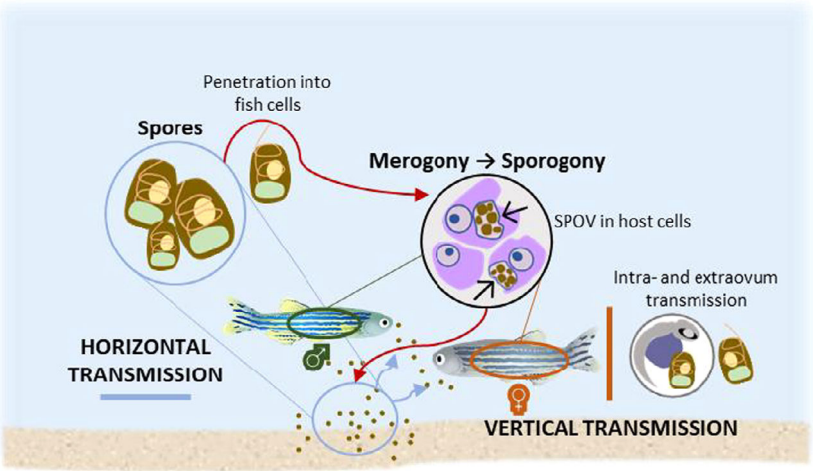

Figure 1 Types of routes of infection and life cycle of *Pseudoloma neurophilia* in zebrafish. Horizontal transmission via spores ingestion/penetration from debris and water, as well as vertical transmission are shown. Abbreviation: SPOV, sporophorous vacuoles.

(*Devario aequipinnatus* (McClelland 1839)), fathead minnow (*Pimephales promelas* (Rafinesque 1820)), medaka (*Oryzias latipes* (Temminck & Schlegel 1846)), goldfish (*Carassius auratus* (Linnaeus 1758)) and neon tetra (*Paracheirodon innesi* (Myers 1936)) (Sanders *et al.* 2016). Also naturally acquired *P. neurophilia* infections were recorded in a laboratory population of *P. promelas* (Sanders *et al.* 2016).

Diseases caused by *P. neurophilia* infection in zebrafish can have a wide spectrum of symptomatology, from benign effects to emaciation, skeletal deformities and even death (Peneyra *et al.* 2018; Sanders *et al.* 2020). Nevertheless, many infected fish are asymptomatic, in particular at early stages of infection, thus causing hidden infections that can generate outbreaks and cause serious sanitary problems in the fish facilities. Many aspects of *P. neurophilia* infections are still not well understood; thus, no effective treatment to abolish the infection has been developed yet. In fact, there are unsolved questions, such as the debate about whether *P. neurophilia* is a natural pathogen of zebrafish (Sanders *et al.* 2016).

Target organs for *Pseudoloma neurophilia* infestation

Pseudoloma neurophilia is an intracellular parasite commonly observed in the fish adult central nervous system (CNS). Although CNS is known as an immune-privileged site, this parasite has been localized at early stages of development in the spinal cord, ventral nerve roots and anterior brain, including the colonization of the choroid rete and pigmented retinal epithelium of the eye (Figure 2a) (Franzen 2004; Sanders *et al.* 2014). Infections of this microsporidium form clusters that are able to colonize different cell groups. It is documented that in adult zebrafish (Figure 2b), these parasite clusters are always observed within the axon rather than within neuron cell bodies, glial cells or capillary endothelial cells, being specifically located within the axonoplasm and producing gradual to abrupt swellings in the axon (Spagnoli *et al.* 2015b). Besides, cell shrinkage, pyknosis (i.e. irreversible condensation of chromatin which commonly occurs in both apoptotic and necrotic cell death), karyolysis (i.e. dissolution of a cell nucleus which usually occurs during necrosis processes) and cytoplasmic vacuolation, have been observed within either hindbrain or spinal cord grey matter (even when distant from the parasitic cluster) causing encephalitis or myelitis (Matthews *et al.* 2001). Despite the fact that intraneuronal clusters appear in axons, that in last term could disrupt neuron signalling (Sanders *et al.* 2020) or trigger neuronophagia (i.e. the invasion and destruction of neurons by phagocytic cells), neuron cell bodies were apparently free of spores (Spagnoli *et al.* 2015b). *Pseudoloma neurophilia* has no capacity for motility; thus, the uptake of material is by the axon terminal and the transport of it is by a centripetal or retrograde direction to the cell body (Lavail & Lavail 1972). This suggested a retrograde axonal transport along microtubules, what is also found in some viruses, toxins, organelles and proteins (Spagnoli *et al.* 2015b).

Apart from CNS, *P. neurophilia* is able to infect fish gonads and, consequently, transmitting the infection to the subsequent fish generation. Gonads are immune-privileged sites as they need to prevent from those immune responses

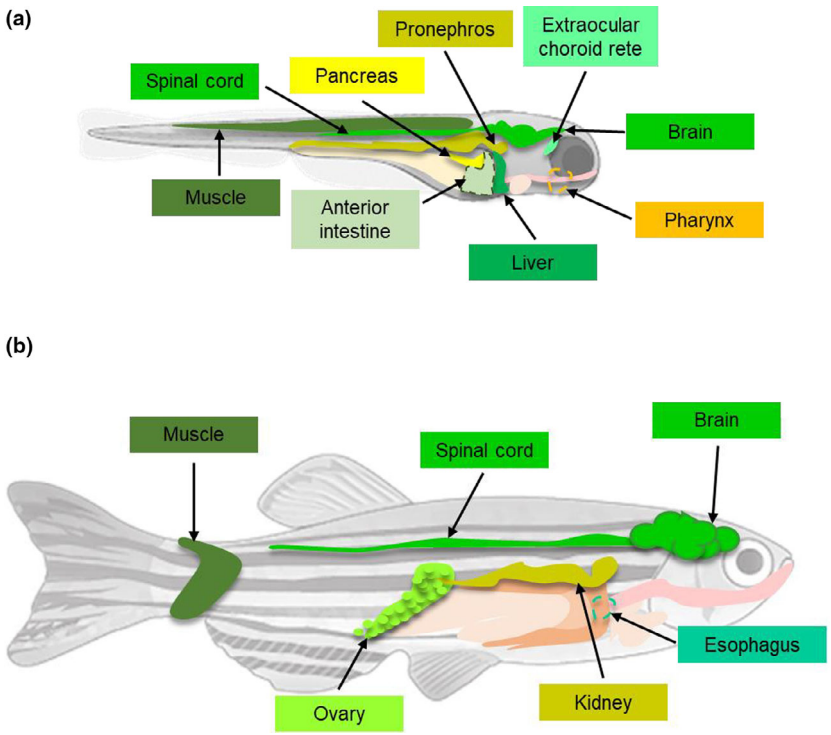

Figure 2 Schematic representation of zebrafish showing those target tissues and organs in which the parasite *Pseudoloma neurophilia* has been identified based on current publications. (a) Zebrafish larvae (infections detected from 12 to 120 hours post-fertilization, hpf). Infection documented in the represented tissues by Sanders *et al*. (2014). Larvae outline adapted from Lizzy Griffiths. (b) Adult zebrafish. Muscle: Matthews *et al*. (2001), Kent and Bishop-Stewart (2003), Murray *et al*. (2011); ovary: Kent and Bishop-Stewart (2003), Murray *et al*. (2011); kidney: Murray *et al*. (2011); Spinal cord: Matthews *et al*. (2001), Kent and Bishop-Stewart (2003), Whipps and Kent (2006), Murray *et al*. (2011), Spagnoli *et al*. (2015b); brain: Matthews *et al*. (2001), Kent and Bishop-Stewart (2003), Murray *et al*. (2011), Spagnoli *et al*. (2015b); and oesophagus: Murray *et al*. (2011).

against meiotic and haploid germ cells (Chaves-Pozo *et al*. 2010). Free spores were observed in ovarian tissue in fish (Figure 2b), generating ovarian granulomas (Sanders *et al*. 2014; Sanders *et al*. 2020). To date, contrary to what happens in ovary, no evidence of spores within the testicular parenchyma has been found (Murray *et al*. 2011; Peterson *et al*. 2011; Sanders & Kent 2011; Sanders *et al*. 2013; Kreul 2020). Murray *et al*. (2011) found positive results after PCR amplification related to its presence on testes and sperm, although it was attributed to a potential contamination of the samples from nearby tissues (Murray *et al*. 2011).

Further, in adults, spores have been observed in other organs such as the skeletal muscle, oesophagus and kidney (Figure 2b). In addition, spores are also observed in the pancreas, intestine, liver and pharynx of larvae (Figure 2a) (Matthews *et al*. 2001; Sanders *et al*. 2014). Kent *et al*. (1999) showed that in chinook salmon (*Oncorhynchus tshawytscha* (Walbaum 1792)) kidney stored *Loma salmonae* spores by entrapment of fixed macrophages after the end of the infection, providing immunologic stimuli to reinforce the resistance to new *L. salmonae* infections. In fact, head kidney is considered the principal immune organ in fish (Rauta *et al*. 2012) and it is developed from larval pronephros, where also *P. neurophilia* presence has been recorded (Figure 2a) (Sanders *et al*. 2014). Moreover, pronephros has been recently described as a location where lipopolysaccharide (LPS) is recruited after bath immune stimulation in zebrafish larvae (Moraleda-Prados *et al*., 2021). Therefore, future studies on both head kidney and pronephros roles in *P. neurophilia* infections are of interest to cope with the disease by developing immunity.

Alterations in the reproductive system

Reproduction system in zebrafish

Zebrafish is a gonochoristic teleost of undifferentiated type, meaning that the development of the ovaries or testes of all individuals occurs from immature ovarian tissue independently of the genetic constitution (Yamamoto 1969; Piferrer 2001). Zebrafish has polygenic sex-determining system in which environmental factors are able to drive the final sexual phenotype (Ospina-Álvarez & Piferrer 2008; Liew

et al. 2012). Pradhan and Olsson (2016) described that the first sign of the gonad differentiation process is found at 10 days post-fertilization (dpf), completing at 60–90 dpf (Santos *et al.* 2017). Between 14–16 dpf, juvenile zebrafish enter a stage in which oocytes are present in all individuals, but it is around 23 dpf when the generation of testicle is triggered, being apoptotic routes those responsible for the testicular development and, therefore, for the degeneration of the oocytes of approximately half of the individuals in the population (Pradhan & Olsson 2016).

During sex differentiation, sensitive windows to external environmental biotic and abiotic factors have been described in zebrafish such as high temperature (Ribas *et al.* 2017a), gamma rays (Kamstra *et al.* 2018), hypoxia (Shang *et al.* 2006) or high density (Ribas *et al.* 2017b) with a shift towards masculinization. Nevertheless, zebrafish populations treated with thermocycles (Villamizar *et al.* 2012) or chemical compounds, that is demethylation agent 5-aza-2′-deoxycytidine (5-aza-dC) (Ribas *et al.* 2017c), or with biotic factors such as *Escherichia coli* heat-killed bacteria (Pradhan *et al.* 2012) during sex differentiation showed sex ratio bias towards females. Recent findings have revealed that juvenile zebrafish treated along gonadal differentiation with immunostimulants (i.e. different LPS strains) feminized zebrafish populations in dose and LPS-strain-dependent manner (Moraleda-Prados *et al.* 2021). Therefore, and due to the plasticity of the sexual phenotype in early stages, it would not be surprising that the sex ratio of zebrafish populations was altered by the effects of pathogen colonization, including microsporidia.

In fish, there is a lack of data of the sexual phenotypic consequences of parasitic infections suffered along gonadal differentiation. It was described that in E. sea bass, infections with parasites showed ovo-testes and infertility (Sitjà-Bobadilla 2009), and in *Rutilus rutilus* (Linnaeus 1758), hermaphroditism was related to microsporidian infection in female ovaries (Wiklund *et al.* 1996). Nevertheless, few data explain the interactions between the reproduction and the immune system, and to our concern, inexistent data are present regarding whether parasitic infections are able to skew sex ratios in fish.

Sexual dimorphism in *Pseudoloma neurophilia* parasitic infections

Some studies reported a higher prevalence of infection (i.e. proportion of infected hosts out of the total number of individuals examined (Rózsa *et al.* 2000)) together with higher infection rates in male rather than in female individuals (Spagnoli *et al.* 2015a; Chow *et al.* 2016). The sexual dimorphic pattern of parasitic infection was similarly observed in *R. rutilus*, in which male gonads affected by microsporidian parasite *Pleistophora mirandellae* only contain small cysts while in females the microsporidian came to cause the absence or destruction of the whole ovary (Wiklund *et al.* 1996).

Sex bias in *P. neurophilia* infection has been related to pre-existing behavioural phenotypes, making male individuals more susceptible to infection due to their higher levels of cortisol under stressful conditions (Chow *et al.* 2016). These physiological responses could be due to the male aggressiveness and hierarchical behaviours found in the fish tanks (Spagnoli *et al.* 2015a). Moreover, sex-specific behavioural outputs after *P. neurophilia* infection were observed with a decrease in exploration in female zebrafish hosts compared with infected males (Midttun *et al.* 2020a). Further sex-specific effects in the organism involved a significant decrease in body condition in female fish when compared with males, as well as less gonadal area in female comparing to unexposed fish (Sanders *et al.* 2020).

Overall, sexual dimorphism needs to be taken into account when analyzing immune responses in fish, since reproductive and immune systems are interconnected playing sexual-dependent molecular functions. In fact, epigenetic sexual dimorphic differences were found in the DNA methylation levels of the promoters of two immune genes in which males showed a hypermethylation in the testes when compared to ovaries (Caballero-Huertas *et al.* 2020). Nevertheless, *P. neurophilia* reduced fecundity in a similar manner for male and female infected fish (Ramsay *et al.* 2009; Sanders *et al.* 2020), being vertical transmission one of the main concerns regarding the surviving progeny. Parasitic infections decrease hatching rate of the embryos, and it has been linked with the intensity of the infection and the stress response of infected offspring (Ramsay *et al.* 2009).

Possible transgenerational effects of parasitic infections

Transgenerational immune priming refers to the transfer of the parental immunological experience to its progeny, resulting in offspring protection from repeated encounters with pathogens that persist across generations (Tetreau *et al.* 2019) (or at least in F2 generation). This process has been mostly documented in insects, evidencing that epigenetic mechanisms operating before and after transcription contribute to the inherited transcriptional reprogramming of immune-related genes, generating the parasitic resistance evolution (Vilcinskas 2017; Villagra & Frías-Lasserre 2020). Effects of transgenerational immunity are seen on both innate and adaptive immune responses of teleost, since fish females deposit antibodies into eggs, with primed offspring having increased body weight, lysozyme activity, complement system efficiency and anti-protease activity (Roth *et al.* 2018). In pipefish (*Syngnathus typhle* Linnaeus 1758), immune priming was observed in the progeny after submitting parental and grandparental generations (both male

and female) to bacterial treatments (Menon & Kumar 2016; Beemelmanns & Roth 2017). In fact, in Atlantic salmon, embryos stimulated with LPS showed an alteration of the methylome and the transcriptome when adults, although the epigenetic inheritance was not specifically studied (Uren Webster *et al.* 2018). Since this transgenerational immune priming involves ecological and evolutionary consequences and can modify disease dynamics and epidemic spreading (Beemelmanns & Roth 2017), it is interesting to address whether exposure to *P. neurophilia* in past generations can give a clue on potential heritable trait in fish. However, there is a lack of data in fish species and most of the studies refer to transgenerational effects rather than transgenerational immune priming effects. Examples of this kind are done in zebrafish in which temperature altered epigenetic patterns in testis of unexposed F1 generation (Valdivieso *et al.* 2020). Due to the lack of data in this research field, we believed that a better comprehension of parasitic infections by *P. neurophilia* will help to develop screening methods for coping this parasitic infection that could be transferred in aquaculture production, incorporating, for example, innate disease resistance into selective-breeding programmes.

Detection methods

Since the detection and recognition of this parasite in 2001 in ZIRC (Oregon), diagnostic services were based on histopathology of the whole fish as primary method for disease identification (Chow *et al.* 2016). However, apart from techniques based on histology, other methods have been implemented to detect *P. neurophilia*. Here, we have analyzed the reliability and applicability of the different techniques available to date to detect *P. neurophilia* in fish facilities. With the aim to facilitate the revision of the status of the detection methods, published data are summarized in Table 1.

Direct visualization

As previously mentioned, *P. neurophilia* is mostly found in neural tissue of the posterior brain and spinal cord, and consequently, it may affect fish behaviour by reducing activity, alteration of swimming patterns and by showing high rates of freezing behaviour (i.e. stopping activity) (Sanders *et al.* 2012; Spagnoli *et al.* 2015b; Midttun *et al.* 2020a). Moreover, morphological alterations due to infections can compromise body weight and cause deformities in the spine, which can be easily visualized and measured as a diagnostic method (Spagnoli *et al.* 2015a). Thus, the identification of frequent behavioural patterns in infected individuals could be useful to identify its prevalence in the facilities. However, direct visualization methods are not fast in detecting damage, and their relevance in prognostic assays can be compromised, since when behaviour-related symptomatology appears, parasites are widespread throughout the fish facilities and health irreparable damages might arise. Furthermore, symptomatology (morphological and behavioural alterations) is not specific, which could lead to a false diagnosis. For instance, spinal curvature is the most common natural morphological deformity observed across many laboratory and aquaculture teleost species (Boswell & Ciruna 2017). Moreover, zebrafish frequently develop gross deformities of their vertebral column due to age-related degenerative changes (Hayes *et al.* 2013).

Behavioural alterations produced by *P. neurophilia* are the consequence when nervous system is infected. However, if the fish only houses the parasite in organs such as kidney or ovary, its presence could be expected to hardly behaviourally manifest. Thus, it is difficult to identify the infected fish as presporogonic and sporogonic *P. neurophilia* stages are difficult to be localized. Consequently, external manifestations could differ from behavioural ones. Hence, it can be outlined that histological and molecular diagnostic approaches should not be replaced by behavioural diagnosis because they are the main source of information regarding parasite prevalence, location and extension of the lesion. Even though both approaches exhibit some technical limitations, future approaches rely on the detection of the infection by direct visualization by early warning systems. These are tools based on computational methods that allow real-time monitoring by using behavioural/swimming patterns as biomarkers in zebrafish among other species (Bae & Park 2014), so they could be applied for *P. neurophilia* microsporidium diagnosis to some extent.

Histological analysis

Histochemical Luna staining was the histological stain method that obtained a better score into the reliability scale (Table 1) compared with Fungi-Fluor and haematoxylin and eosin stains (H&E). It was originally developed to detect cytoplasmic granules in eosinophils, Negri bodies, erythrocytes and phagocytes, and to highlight elastin in tissue sections, that is the *in situ* detection and assessment of the elemental composition of cells and tissues (Peterson *et al.* 2011). Among its tinctorial properties, the minimal background interference has to be outlined since allows a better visualization of spores. This issue is problematic in the case of H&E stain since spores can be obscured against a similar background (Peterson *et al.* 2011). Unlike Luna staining, H&E and Fungi-Fluor have been mostly applied in adult fish, although H&E can be used for the microsporidian detection in larvae. Besides neither of these two methods are reliable for testing the presence of presporogenic stages of *P. neurophilia*. In contrast, Luna

Table 1 Compilation of the detection methods of *Pseudoloma neurophilia* in zebrafish

Method	Stage	Tissues/Organs	Comments	Reliability	Reference
Direct visualization					
Neurobehavioural assay	Adult	Brain, spinal cord, muscle	It only detects late-stage neural and muscular microsporidiosis		Spagnoli *et al.* (2015a), Spagnoli *et al.* (2015b), Midttun *et al.* (2020a)
Histological analysis					
Haematoxylin and eosin (H&E) stain	Larva, adult	Central nervous system, nerves, muscle, kidney, oesophagus	Not easily applied to screening of individual eggs nor do they reliably detect presporogonic stages of the parasite		Matthews *et al.* (2001), Kent and Bishop-Stewart (2003), Murray *et al.* (2011), Spagnoli *et al.* (2015b)
Fungi-Fluor stain	Adult	Central nervous system, nerves, kidney, ovaries, somatic muscle	More sensitive than H&E histology for detecting the infection in tissue sections Not easily applied to screening of individual eggs or larval fish, nor do they reliably detect presporogonic stages of the parasite		Kent and Bishop-Stewart (2003)
Luna stain	Egg, larva, adult	Extraocular choroid rete, anterior intestine, visceral organs and kidney, muscle, ovaries and developing oocytes	It increases the sensitivity of the detection of spores in histological sections without needing fluorescence microscopy Combined with ISH allows the observation of presporogonic stages		Murray *et al.* (2011), Peterson *et al.* (2011), Sanders *et al.* (2013), Sanders *et al.* (2014), Spagnoli *et al.* (2015b)
Molecular analysis					
Polymerase chain reaction (PCR) (specific primers to small-subunit rDNA gene)	Egg, larva, adult	Whole individual	Useful to efficiently screen large numbers of fish. Require very little tissue, it can be adapted to screen water sources, and detect all life stages of the pathogen As assay is specific to only a single pathogen, it is used as a follow-up to histology		Whipps and Kent (2006), Murray *et al.* (2011), Spagnoli *et al.* (2015b), Miller *et al.* (2019)
In situ hybridization (ISH) probe (small-subunit rDNA gene)	Larva	Liver, anterior segment of the intestine, spinal cord, brain, extraocular choroid rete, muscle, pharynx, pancreas	To detect presporogonic stages of microsporidian parasites		Sanders *et al.* (2014)

Method, stage of the fish in which the parasite has been found, tissues/organs in which it was present, further comments, reliability scale and references are shown. Reliability scale based on the fulfilment of the following requirements by the method: (A) useful in all live stages of the fish; (B) reliability in detecting mature spores; (C) reliability in detecting presporogonic stages of the parasite; (D) none or small amount of tissue sample/environmental (i.e. tank debris, water) sample required; and (E) detection in tissues other than nervous. Dark green-completed circles correspond to the satisfied requirements. Half-dark green-completed circle indicates that the requirement is partially accomplished. Question mark (?) represents the potential of the method to accomplish the requirement but still not fully confirmed.

staining combined with molecular technique *in situ* hybridization (ISH) is useful to detect early presporogony (Sanders *et al.* 2014).

Molecular analysis

On the other hand, molecular PCR assays allow identifying parasitic infections in all stages of zebrafish (from embryos to adults), having a rapid and high detection capacity at all stages of the pathogen (Whipps & Kent 2006) from the entire fish individual body (Table 1). Moreover, it is very sensitive since it is based on the amplification of the parasite's DNA, and thus, very small amount of tissue sample is required (Murray *et al.* 2011).

Furthermore, a technique developed by mixing *in situ* hybridization (ISH) with adapted PCR analysis on tissue

sections is able to detect presporogenic stages of the microsporidian in various tissues of zebrafish larvae (Peterson *et al.* 2011). However, studies have been only performed at larval stage (Sanders *et al.* 2014), despite the fact that it may be applied to adult tissues too. The search for early detection methods lay on molecular techniques that detect the pathogen even prior the infection. Recently, and to complement PCR analysis of fish, Miller *et al.* (2019) tested the detection of *P. neurophilia* by using real-time PCR taking environmental samples such as water, sludge or detritus from the tanks. However, this approach was not sensitive enough for *P. neurophilia* detection, contrary to what happened with mycobacteria and *Myxidium streisingeri*, which were mainly identified in environmental samples (Miller *et al.* 2019).

Another potential molecular method for fast detection would be the identification of the host proteins involved in microsporidia infection through forward genetic screenings (i.e. identification of a gene(s) responsible for a particular phenotype of an organism) (Troemel 2011). This can be fully feasible in the case of a research model species as zebrafish in which all proteins are fully characterized. Further, transcriptomic approaches to better understand the immune responses will allow developing suitable diagnosis methods. Recently, Midttun *et al.* (2020b) described that in *P. neurophilia* infected zebrafish no differentially expressed genes or enriched pathways were specific for nervous system functions but classical upregulation of immune-related cascades such as pro-inflammatory Th1 response was observed.

Summarizing, there is no single approach offering irrefutable results for the early detection of the parasite in tissues from zebrafish or other fish species. From our perspective, the most accurate detection of the presence of *P. neurophilia* would be combining methodologies and approaches. Accordingly, Sanders *et al.* (2014) confirmed the efficacy of ISH probe specific to the small-subunit ribosomal RNA gene of the microsporidian, standard H&E stain, and Luna staining to detect and visualize presporogenic and mature stages of *P. neurophilia* in the same study. Nevertheless, when several methodologies are not technically possible to be performed, the preference would be adjusting to the better necessities of the research. For instance, rapid detection would involve PCR strategies in contrast to histology by Luna stain which in turn will give more accurate information on the localization and degree of damage, despite being more time-consuming. A feasible and simple set-up to detect the microsporidium in zebrafish facilities can be based on the continuous monitoring of healthy sentinel zebrafish maintained in tanks which receive the incoming water (prior filtration) before being pumped towards the facility (Kent *et al.* 2011; Kent *et al.* 2012). These fish can be routinely processed with the available detection methods in search of the presence of *P. neurophilia*.

Treatments against microsporidian infections in fish

Although to date no efficient treatment has been described to cope with *P. neurophilia* infection, there are some treatments in order to fight against microsporidiosis caused by different species of pathogens. Preliminary experiments showed that an elevated water temperature of 27–29°C was effective in controlling the microsporidian *Glugea plecoglossi* in ayu *Plecoglossus altivelis* (Takahashi & Ogawa 1997). Moreover, one treatment with dietary monensin (a polyether antibiotic) initiated at the time of exposure or one week before to microsporidian *Loma salmonae* showed a great reduction in xenoma production in rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) when compared with untreated fish (Becker *et al.* 2002). Conversely, it has been demonstrated the failure of using up to 200 mg/L iodine to control the microsporidian *Loma salmonae* (Shaw *et al.* 1999). Also, spores of *P. neurophilia* are highly resistant to common disinfectant reagent chlorine (Ferguson *et al.* 2007), and antimicrobial agent fumagillin, widely usually with good success as an oral treatment for cultured fish with microsporidiosis, does not seem to control or reduce *P. neurophilia* infections in zebrafish (ZIRC 2016), at least in preliminary studies.

Adaptive immunity plays an essential part for dealing with microsporidian infections in fish (Rodríguez-Tovar *et al.* 2011); thus, vaccine development is a promising method that could eradicate *P. neurophilia* infections. Unfortunately, and although microsporidian parasitic diseases are common in cultured fish species, vaccines against parasitic colonization by this taxon are still not commercially available. Important findings towards vaccine development are in progress with a spore-based vaccine applied to prevent microsporidial gill disease caused by *L. salmonae* in salmonids (Speare *et al.* 2007; Rodríguez-Tovar *et al.* 2011). Unfortunately, to date, no single therapeutic intervention (e.g. chemotherapeutics, vaccines) seem to be enough for limiting the infections caused by complex eukaryotic pathogens (e.g. Microsporidia) (Frenette *et al.* 2020).

Recommendations for zebrafish rearing conditions

Due to the important role that zebrafish plays in many research areas, including aquaculture, it is imperative that research facilities monitor their fish stocks to find the potential presence of *P. neurophilia* in order to avoid interference in the final research results. It is highly recommended to perform quarantine procedures and egg

disinfection before fish are transferred to the rearing water systems (Whipps & Kent 2006; Murray *et al.* 2011), and to have different independent fish chambers to allow moving fish in different quarantine procedures, minimizing the risk of pathogen introduction. As previously mentioned, the installation of a tank with sentinel fish is highly recommended to check routinely the state of the system as a whole. When there is a suspicious of infected fish (from the sentinels or from the experimental units), for example by changes in behaviour or spine deformation, it is required to separate this individual from the rest of the fish, not only to avoid the dissemination of the diseases, but also for animal welfare standard procedures. Following, the identified fish needs to be euthanized and the presence or absence of the *P. neurophilia* parasite needs to be analyzed.

As transmission mostly occurs through the consumption of environmentally resistant spores either free in the water or embedded in fish carcasses (Figure 1), filtration is an essential component when avoiding microsporidia spreading. Moreover, taking into account that *P. neurophilia* spores can survive bleaching at the standard concentration (25–50 ppm) used in most facilities for embryo sterilization (Ferguson *et al.* 2007), filtering seems to be the most effective way to combat the colonization of the facilities by this organism. A developed microsporidia-free facility disposes of well water, charcoal filtered, particulate filtered and UV treated at target 30,000 $\mu Ws\ cm^{-2}$, which incoming water is filtered by reverse osmosis, and discharged to the sump of the recirculating system (Kent *et al.* 2011). Wastewater is filtered by a particulate bead filter, a fluidized biofilter and a 10-μm bag filter, and then, water is passed through biological UV treatment (100,000 $\mu Ws\ cm^{-2}$) before being returned to aquaria (Kent *et al.* 2011). Post-filtration UV sterilizers have been also described as useful against other species of taxonomic division microsporidian (Peneyra *et al.* 2018).

Crowding stress and associated high cortisol were related with more severe *P. neurophilia* infections (Chow *et al.* 2016) and an increase in mortality in zebrafish (Ramsay *et al.* 2009). Thus, the number of individuals in the tanks needs to be revised. In order to avoid masculinization due to a stress response, the initial number of larvae along gonadal differentiation (10–45 dpf) in the 2.8-L tanks should not be higher than 13–20 fish (Ribas *et al.* 2017b). As described above, sometimes zebrafish males display aggressive behaviour towards other males, experiencing greater levels of cortisol when exposed to stressors in comparison to females (Chow *et al.* 2016). Therefore, a balanced number of males and females in each tank, or even a higher rate of females, could lead to lower levels of stress in the tank compared to one with a high number of males.

Mating is another critical point that should be taken into consideration. Peneyra *et al.* (2018) confirmed that disinfection of mating tanks before spawning did not decrease *P. neurophilia* transmission when infected fish remained present in the breeding population so breeding individuals should be free of infections and thus diagnosis methods should be performed periodically in broodstock populations.

Finally, it needs to be said that some private companies offer diagnosis services (e.g. histopathological and PCR analysis) for the detection of traditional zebrafish pathogens, including *P. neurophilia*. Therefore, it is highly recommended to routinely perform a pathogenic screening to avoid serious problems in zebrafish research facilities.

Final conclusions

Pseudoloma neurophilia is a common parasite in zebrafish, the most important fish model for aquaculture research. Most of the time, it cannot be easily identified, thus being compromised the resulting research but also fish welfare. Sexual dimorphism of zebrafish should be taken into account on the management of this microsporidium. As discussed, there are differences in *P. neurophilia* infection prevalence and infection rates (Spagnoli *et al.* 2015a; Chow *et al.* 2016), higher in males, but significantly decreasing female body condition (Sanders *et al.* 2020), more invasive in female gonad, and differentially affecting behaviour among sexes (Midttun *et al.* 2020a). With this in mind, we suggest a low number of males in the tanks to decrease the parasite load per tank.

Despite the fact that behavioural and morphological alterations are usually the most evident factors of parasitism due to this microsporidium (Spagnoli *et al.* 2015a; Spagnoli *et al.* 2015b; Midttun *et al.* 2020a), other more reliable diagnostic methods should be used regularly. *Pseudoloma neurophilia* can be located in other tissues different from nervous ones (e.g. ovary and kidney), without external evidences reflected in fish morphology, biometry or behaviour. Consequently, appropriate histological staining, ISH approaches and PCR technology, alone or desirably in combination, should be performed as routinely controls in fish and mainly in sentinel fish placed in tanks inside the installation. It has to be noted that early and rapid detection methods based on PCR analyses of parasitic DNA in environmental samples (debris and water) (Murray *et al.* 2011), identification of parasite proteins (Troemel 2011) and differential gene expression (Midttun *et al.* 2020b), stand as promising methods, although they deserve further research efforts prior routinely applied. Other strategies like better understanding the molecular bases of the infection or studying the transgenerational immune priming involved in parasitic resistances will help to decipher underling mechanisms to fight against *P. neurophilia*.

Acknowledgements

Authors' research funding is supported by the Spanish Ministry of Science and Innovation grants AGL2015-73864-JIN 'Ambisex' and RYC2018-024017-I and by Spanish National Research Council (CSIC) grant 202030E004 'Interomics' to LR. We would like to thank Dr. Francesc Padrós from Universitat Autònoma de Barcelona (UAB) for his comments on *P. neurophilia* infections in zebrafish.

Data Availability Statement

Data openly available in a public repository that issues datasets with DOIs.

References

Bae MJ, Park YS (2014) Biological early warning system based on the responses of aquatic organisms to disturbances: a review. *Science of the Total Environment* **466**: 635–649.

Becker JA, Speare DJ, Daley J, Dick P (2002) Effects of monensin dose and treatment time on xenoma reduction in microsporidial gill disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **25**: 673–680.

Beemelmanns A, Roth O (2017) Grandparental immune priming in the pipefish *Syngnathus typhle*. *BMC Evolutionary Biology* **17**: 44.

Blázquez M, Piferrer F, Zanuy S, Carrillo M, Donaldson EM (1995) Development of sex control techniques for European sea bass (*Dicentrarchus labrax* L.) aquaculture: effects of dietary 17 α-methyltestosterone prior to sex differentiation. *Aquaculture* **135**: 329–342.

Boswell CW, Ciruna B (2017) Understanding idiopathic scoliosis: a new zebrafish school of thought. *Trends in Genetics* **33**: 183–196.

Caballero-Huertas M, Moraleda-Prados J, Joly S, Ribas L (2020) Immune genes, *IL1β* and *Casp9*, show sexual dimorphic methylation patterns in zebrafish gonads. *Fish & Shellfish Immunology* **97**: 648–655.

Cali A, Kent M, Sanders J, Pau C, Takvorian PM (2012) Development, ultrastructural pathology, and taxonomic revision of the microsporidial genus, *Pseudoloma* and its type species *Pseudoloma neurophilia*, in skeletal muscle and nervous tissue of experimentally infected zebrafish *Danio rerio*. *Journal of Eukaryotic Microbiology* **59**: 40–48.

Chaves-Pozo E, Liarte S, García-Ayala A (2010) Immune and reproductive interaction: an essential clue for understanding gonad functions in gilthead seabream. In: García-Ayala A, Meseguer-Peñalver J, Chaves-Pozo E (eds) *Recent Advances in Fish Reproductive Biology*, pp. 127–139. Research Signpost, Kerala, India.

Chow FW, Xue L, Kent ML (2016) Retrospective study of the prevalence of *Pseudoloma neurophilia* shows male sex bias in zebrafish *Danio rerio* (Hamilton-Buchanan). *Journal of fish diseases* **39**: 367.

Corsaro D, Michel R, Walochnik J, Venditti D, Müller KD, Hauröder B *et al.* (2016) Molecular identification of *Nucleophaga terricolae* sp. nov (*Rozellomycota*), and new insights on the origin of the Microsporidia. *Parasitology Research* **115**: 3003–3011.

De Kinkelin P (1980) Occurrence of a microsporidian infection in zebra danio *Brachydanio rerio* (Hamilton-Buchanan). *Journal of Fish Diseases* **3**: 71–73.

Ferguson JA, Watral V, Schwindt AR, Kent ML (2007) Spores of two fish microsporidia (*Pseudoloma neurophilia* and *Glugea anomala*) are highly resistant to chlorine. *Diseases of Aquatic Organisms* **76**: 205–214.

Franzen C (2004) Microsporidia: how can they invade other cells? *Trends in Parasitology* **20**: 275–279.

Franzen C (2008) Microsporidia: a review of 150 years of research. *The Open Parasitology Journal* **2**: 1–34.

Frenette AP, Harrold T, Bentzen P, Paterson IG, Malenfant RM, Nardi G *et al.* (2020) *Loma morhua* infections in Atlantic cod (*Gadus morhua*) reveal relative parasite resistance and differential effects on host growth among family lines. *Aquaculture* **522**: 735111.

Han B, Weiss LM (2017) Microsporidia: obligate intracellular pathogens within the fungal kingdom. In: Heitman J, Howlett BJ, Crous PW, Stukenbrock EH, James TY, Gow NAR (eds) *The Fungal Kingdom*, pp. 97–113. American Society for Microbiology, Washington, DC.

Hayes AJ, Reynolds S, Nowell MA, Meakin LB, Habicher J, Ledin J *et al.* (2013) Spinal deformity in aged zebrafish is accompanied by degenerative changes to their vertebrae that resemble osteoarthritis. *PLoS One* **8**: e75787.

Idilli AI, Precazzini F, Mione MC, Anelli V (2017) Zebrafish in translational cancer research: insight into leukemia, melanoma, glioma and endocrine tumor biology. *Genes* **8**: 236.

Kamstra JH, Hurem S, Martin LM, Lindeman LC, Legler J, Oughton D *et al.* (2018) Ionizing radiation induces transgenerational effects of DNA methylation in zebrafish. *Scientific Reports* **8**: 1–13.

Kent ML, Bishop-Stewart JK (2003) Transmission and tissue distribution of *Pseudoloma neurophilia* (Microsporidia) of zebrafish, *Danio rerio* (Hamilton). *Journal of fish diseases* **26**: 423–426.

Kent ML, Buchner C, Watral VG, Sanders JL, LaDu J, Peterson TS *et al.* (2011) Development and maintenance of a specific pathogen-free (SPF) zebrafish research facility for *Pseudoloma neurophilia*. *Diseases of Aquatic Organisms* **95**: 73–79.

Kent ML, Dawe SC, Speare DJ (1999) Resistance to reinfection in chinook salmon *Oncorhynchus tshawytscha* to *Loma salmonae* (Microsporidia). *Diseases of aquatic organisms* **37**: 205–208.

Kent ML, Fournie JW (2007) Parasites of fishes. In: Baker DG (ed) *Flynn's Parasites of Laboratory Animals*, pp. 69–117. Blackwell Scientific Publications, Oxford, UK.

Kent ML, Harper C, Wolf JC (2012) Documented and potential research impacts of subclinical diseases in zebrafish. *ILAR Journal* **53**: 126–134.

Kent ML, Shaw RW, Sanders JL (2014) Microsporidia in fish. In: Weiss LM, Becnel JJ (eds) *Microsporidia: Pathogens of Opportunity*, pp. 493–520. Willey Blackwell, Oxford, UK.

Kreul TG (2020) *Comparison of three diagnostic assays in the detection of Pseudoloma neurophilia in zebrafish*. Oregon State University, Corvallis, OR. [Cited 5 Sep 2020.] Available from URL: https://ir.library.oregonstate.edu/concern/honors_college_theses/n009w818g

Lavail JH, Lavail MM (1972) Retrograde axonal transport in the central nervous system. *Science* **176**: 1416–1417.

Liew WC, Bartfai R, Lim Z, Sreenivasan R, Siegfried KR, Orbán L (2012) Polygenic sex determination system in zebrafish. *PLoS One* **7**: e34397.

Matthews CGG, Richards RH, Shinn AP, Cox DI (2013) Gill pathology in Scottish farmed Atlantic salmon, *Salmo salar* L., associated with the microsporidian *Desmozoon lepeophtherii* Freeman et Sommerville, 2009. *Journal of Fish Diseases* **36**: 861–869.

Matthews JL, Brown AM, Larison K, Bishop-Stewart JK, Rogers P, Kent ML (2001) *Pseudoloma neurophilia* ng, n. sp., a new microsporidium from the central nervous system of the zebrafish (*Danio rerio*). *Journal of Eukaryotic Microbiology* **48**: 227–233.

Menon P, Kumar MS (2016) Trans-generational immune priming in aquaculture-disease combating potential. *International Journal of Fisheries and Aquatic Studies* **4**: 126–130.

Midttun HL, Vindas MA, Nadler LE, Øverli Ø, Johansen IB (2020a) Behavioural effects of the common brain-infecting parasite *Pseudoloma neurophilia* in laboratory zebrafish (*Danio rerio*). *Scientific Reports* **10**: 1–9.

Midttun HL, Vindas MA, Whatmore PJ, Øverli Ø, Johansen IB (2020b) Effects of *Pseudoloma neurophilia* infection on the brain transcriptome in zebrafish (*Danio rerio*). *Journal of Fish Diseases* **43**: 863–875.

Miller M, Sabrautzki S, Beyerlein A, Brielmeier M (2019) Combining fish and environmental PCR for diagnostics of diseased laboratory zebrafish in recirculating systems. *PLoS One* **14**: e0222360.

Miscevic F, Rotstein O, Wen XY (2012) Advances in zebrafish high content and high throughput technologies. *Combinatorial Chemistry & High Throughput Screening* **15**: 515–521.

Monaghan SR, Kent ML, Watral VG, Kaufman RJ, Lee LE, Bols NC (2009) Animal cell cultures in microsporidial research: their general roles and their specific use for fish microsporidia. *Vitro Cellular & Developmental Biology-Animal* **45**: 135.

Moraleda-Prados J, Caballero-Huertas M, Valdivieso A, Joly S, Ji J, Roher N *et al.* (2021) Epigenetic differences in the innate response after immune stimulation during zebrafish sex differentiation. *Developmental and Comparative Immunology* **114**: 103848.

Murray KN, Dreska M, Nasiadka A, Rinne M, Matthews JL, Carmichael C *et al.* (2011) Transmission, diagnosis, and recommendations for control of *Pseudoloma neurophilia* infections in laboratory zebrafish (*Danio rerio*) facilities. *Comparative Medicine* **61**: 322–329.

Nylund S, Andersen L, Sævareid I, Plarre H, Watanabe K, Arnesen CE *et al.* (2011) Diseases of farmed Atlantic salmon *Salmo salar* associated with infections by the microsporidian *Paranucleospora theridion*. *Diseases of Aquatic Organisms* **94**: 41–57.

Ospina-Álvarez N, Piferrer F (2008) Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. *PLoS One* **3**: e2837.

Peneyra SM, Cardona-Costa J, White J, Whipps CM, Riedel ER, Lipman NS *et al.* (2018) Transmission of *Pseudoloma neurophilia* in laboratory zebrafish (*Danio rerio*) when using mass spawning chambers and recommendations for chamber disinfection. *Zebrafish* **15**: 63–72.

Peterson TS, Spitsbergen JM, Feist SW, Kent ML (2011) Luna stain, an improved selective stain for detection of microsporidian spores in histologic sections. *Diseases of Aquatic Organisms* **95**: 175–180.

Picard-Sánchez A, Piazzon MC, Ahmed NH, Del Pozo R, Sitjà-Bobadilla A, Palenzuela O (2020) *Enterospora nucleophila* (Microsporidia) in Gilthead Sea Bream (*Sparus aurata*): pathological effects and cellular immune response in natural infections. *Veterinary Pathology* **57**: 565–576.

Piferrer F (2001) Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture* **197**: 229–281.

Piferrer F, Ribas L (2020) Chapter 10. Zebrafish as a research tool for improving finfish aquaculture. In: Benfey TJ, Farrell AP, Brauner CJ (eds) *Aquaculture. Fish Physiology*, Vol. **38**, pp. 273–313. Academic Press, Cambridge, MA. https://doi.org/10.1016/bs.fp.2020.10.003

Pradhan A, Khalaf H, Ochsner SA, Sreenivasan R, Koskinen J, Karlsson M *et al.* (2012) Activation of NF-κB protein prevents the transition from juvenile ovary to testis and promotes ovarian development in zebrafish. *Journal of Biological Chemistry* **287**: 37926–37938.

Pradhan A, Olsson PE (2016) Regulation of zebrafish gonadal sex differentiation. *AIMS Molecular Science* **3**: 567–584.

Ramsay JM, Watral V, Schreck CB, Kent ML (2009) *Pseudoloma neurophilia* infections in zebrafish *Danio rerio*: effects of stress on survival, growth, and reproduction. *Diseases of Aquatic Organisms* **88**: 69–84.

Rauta PR, Nayak B, Das S (2012) Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. *Immunology Letters* **148**: 23–33.

Ribas L, Liew WC, Díaz N, Sreenivasan R, Orbán L, Piferrer F (2017a) Heat-induced masculinization in domesticated zebrafish is family-specific and yields a set of different gonadal transcriptomes. *Proceedings of the National Academy of Sciences of the United States of America* **114**: E941–E950.

Ribas L, Piferrer F (2014) The zebrafish (*Danio rerio*) as a model organism, with emphasis on applications for finfish aquaculture research. *Reviews in Aquaculture* **6**: 209–240.

Ribas L, Valdivieso A, Díaz N, Piferrer F (2017b) Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response. *Journal of Experimental Biology* **220**: 1056–1064.

Ribas L, Vanezis K, Imués MA, Piferrer F (2017c) Treatment with a DNA methyltransferase inhibitor feminizes zebrafish and induces long-term expression changes in the gonads. *Epigenetics & Chromatin* **10**: 59.

Rodríguez-Tovar LE, Speare DJ, Markham RF (2011) Fish microsporidia: immune response, immunomodulation and vaccination. *Fish & Shellfish Immunology* **30**: 999–1006.

Roth O, Beemelmanns A, Barribeau SM, Sadd BM (2018) Recent advances in vertebrate and invertebrate transgenerational immunity in the light of ecology and evolution. *Heredity* **121**: 225–238.

Rózsa L, Reiczigel J, Majoros G (2000) Quantifying parasites in samples of hosts. *Journal of Parasitology* **86**: 228–232.

Sanders JL, Kent ML (2011) Development of a sensitive assay for the detection of Pseudoloma neurophilia in laboratory populations of the zebrafish *Danio rerio*. *Diseases of Aquatic Organisms* **96**: 145–156.

Sanders JL, Monteiro JF, Martins S, Certal AC, Kent ML (2020) The Impact of *Pseudoloma neurophilia* Infection on Body Condition of Zebrafish. *Zebrafish* **17**: 139–146.

Sanders JL, Peterson TS, Kent ML (2014) Early development and tissue distribution of *Pseudoloma neurophilia* in the zebrafish, *Danio rerio*. *Journal of Eukaryotic Microbiology* **61**: 238–246.

Sanders JL, Watral V, Kent ML (2012) Microsporidiosis in zebrafish research facilities. *ILAR Journal* **53**: 106–113.

Sanders JL, Watral V, Clarkson K, Kent ML (2013) Verification of intraovum transmission of a microsporidium of vertebrates: *Pseudoloma neurophilia* infecting the zebrafish, *Danio rerio*. *PLoS One* **8**: e76064.

Sanders JL, Watral V, Stidworthy MF, Kent ML (2016) Expansion of the known host range of the microsporidium, *Pseudoloma neurophilia*. *Zebrafish* **13**: S-102.

Santos D, Luzio A, Coimbra AM (2017) Zebrafish sex differentiation and gonad development: a review on the impact of environmental factors. *Aquatic Toxicology* **191**: 141–163.

Shang EH, Yu RM, Wu RS (2006) Hypoxia affects sex differentiation and development, leading to a male-dominated population in zebrafish (*Danio rerio*). *Environmental Science & Technology* **40**: 3118–3122.

Shaw R, Kent ML, Adamson ML (1999) Iodophor treatment is not completely efficacious in preventing *Loma salmonae* (Microsporidia) transmission in experimentally challenged chinook salmon, *Oncorhynchus tshawytscha* (Walbaum). *Journal of Fish Diseases* **22**: 311–313.

Sitjà-Bobadilla A (2009) Can myxosporean parasites compromise fish and amphibian reproduction? *Proceedings of the Royal Society B: Biological Sciences* **276**: 2861–2870.

Spagnoli S, Xue L, Kent ML (2015a) The common neural parasite *Pseudoloma neurophilia* is associated with altered startle response habituation in adult zebrafish (*Danio rerio*): implications for the zebrafish as a model organism. *Behavioural Brain Research* **291**: 351–360.

Spagnoli ST, Xue L, Murray KN, Chow F, Kent ML (2015b) *Pseudoloma neurophilia*: a retrospective and descriptive study of nervous system and muscle infections, with new implications for pathogenesis and behavioral phenotypes. *Zebrafish* **12**: 189–201.

Speare DJ, Markham RJF, Guselle NJ (2007) Development of an effective whole-spore vaccine to protect against microsporidial gill disease in rainbow trout (*Oncorhynchus mykiss*) by using a low-virulence strain of *Loma salmonae*. *Clinical and Vaccine Immunology* **14**: 1652–1654.

Takahashi S, Ogawa K (1997) Efficacy of elevated water temperature treatment of ayu infected with the microsporidian *Glugea plecoglossi*. *Fish Pathology* **32**: 193–198.

Tetreau GH, Dhinaut J, Gourbal B, Moret Y (2019) Trans-generational immune priming in invertebrates: current knowledge and future prospects. *Frontiers in Immunology* **10**: 1938.

Troemel ER (2011) New Models of Microsporidiosis: Infections in Zebrafish, *C. elegans*, and Honey Bee. *PLoS Path* **7**(2): e1001243.

Uren Webster TM, Rodríguez-Barreto D, Martin SA, Van Oosterhout C, Orozco-terWengel P, Cable J *et al.* (2018) Contrasting effects of acute and chronic stress on the transcriptome, epigenome, and immune response of Atlantic salmon. *Epigenetics* **13**: 1191–1207.

Valdivieso A, Ribas L, Monleón-Getino A, Orbán L, Piferrer F (2020) Exposure of zebrafish to elevated temperature induces sex ratio shifts and alterations in the testicular epigenome of unexposed offspring. *Environmental Research* **186**: 109601.

Vilcinskas A (2017) The impact of parasites on host insect epigenetics. *Advances in Insect Physiology* **53**: 145–165.

Villagra C, Frías-Lasserre D (2020) Epigenetic Molecular Mechanisms in Insects. *Neotropical Entomology* **49**(5): 615–642. https://doi.org/10.1007/s13744-020-00777-8

Villamizar N, Ribas L, Piferrer F, Vera LM, Sánchez-Vázquez FJ (2012) Impact of daily thermocycles on hatching rhythms, larval performance and sex differentiation of zebrafish. *PLoS One* **7**: e52153.

Whipps CM, Kent ML (2006) Polymerase chain reaction detection of *Pseudoloma neurophilia*, a common microsporidian of zebrafish (*Danio rerio*) reared in research laboratories. *Journal of the American Association for Laboratory Animal Science* **45**: 36–39.

Wiklund T, Lounasheimo L, Lom J, Bylund G (1996) Gonadal impairment in roach *Rutilus rutilus* from Finnish coastal areas of the northern Baltic Sea. *Diseases of Aquatic Organisms* **26**: 163–171.

Yamamoto TO (1969) 3 Sex Differentiation. *Fish Physiology* **3**: 117–175.

ZIRC (2016) Protozoan diseases. *Diseases of Zebrafish in Research Facilities*. [Viewed 15 July 2020] Available from URL: health/disease_manual/protozoan_diseases.txt.