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## Review of *Pseudoloma neurophilia* (Microsporidia); a common neural parasite of laboratory zebrafish (*Danio rerio*)

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### Abstract

Zebrafish (*Danio rerio*) is now the second most commonly used animal model in biomedical research. As with other vertebrate models, underlying diseases and infections often impact research. Beyond mortality and morbidity, these conditions can compromise research endpoints by producing non-protocol induced variation within experiments. *Pseudoloma neurophilia*, a microsporidium that targets the central nervous system, is the most frequently diagnosed pathogen in zebrafish facilities. The parasite undergoes direct, horizontal transmission within populations, and is also maternally transmitted with spores in ovarian fluid and occasionally within eggs. This transmission explains the wide distribution amongst research laboratories as new lines are generally introduced as embryos. The infection is chronic, and fish apparently never recover following the initial infection. However, most fish do not exhibit outward clinical signs. Histologically, the parasite occurs as aggregates of spores throughout the midbrain and spinal cord and extends to nerve roots. It often elicits meninxitis, myositis, and myodegeneration when it infects the muscle. There are currently no described therapies for the parasite, thus the infection is best avoided by screening with PCR-based tests and removal of infected fish from a facility. Examples of research impacts include reduced fecundity, behavioral changes, transcriptome alterations, and autofluorescent lesions.

### Keywords

Fish; infection; parasite; transmission; diagnostics; pathogenesis

## 1. INTRODUCTION

Here we review various aspects of the biology, pathogenesis, diagnostics, control, and impacts on research caused by *Pseudoloma neurophilia* (Microsporidia), the most frequently diagnosed pathogen in zebrafish research facilities. The benefit of aquatic models, notably zebrafish (*Danio rerio*), as part of an integrative approach to improving human health is being recognized by the scientific community (Phillips and Westerfield 2014). Zebrafish are now the second most commonly used animal model in biomedical research (Kinter et al., 2021). The NIH sponsored Zebrafish International Resource Center (ZIRC) listed 1,880 academic and 39 commercial users of zebrafish between 2016 and 2024 (Zoltan Varga, Director of ZIRC, pers. comm.). The use of zebrafish as a model for biomedical research began with investigations in developmental genetics, in which experimental endpoints involved primarily embryos or larval fish (Eisen 2020; Phillips and Westerfield 2020), and this line of research continues to advance with the application of CRISPR technologies (Hans et al., 2021). Adult zebrafish are now also used extensively as models throughout the biomedical research arena, for applications as varied as infectious disease susceptibility and immune system function (Torraca, and Mostowy 2018), toxicology (Tanguay 2018), behavior (Gerlai 2019), and oncology (Völkel et al., 2018; Yan et al., 2019). As with rodent models, underlying, often subclinical infections in zebrafish are a concern due to their potential and documented impacts on research. These underlying pathogens and diseases can cause significant non-protocol induced variation, yielding physiological impacts that confound study endpoints (Kent et al., 2012).

The typical zebrafish vivarium is comprised of multiple small aquaria on a recirculating system from commercial “racks” with about 30–50 tanks to large systems supporting hundreds of tanks. Regardless of size, the general design is recirculating effluent water through mechanical and biological filtration, followed by ultraviolet sterilization. The water source is usually provided by reverse osmosis in which salts are added to achieve a conductivity of 250 to 1,700 microsiemens (Hammer 2020). In their natural habitats in India, zebrafish generally thrive in much softer water (Lee et al. 2022), while the flow-through vivarium at Oregon State University holds them at less than 150 microsiemens. The initial supply water in the vivarium is presumed to be free of important zebrafish pathogens and fish are generally introduced to research vivaria in the form of chlorine or iodine disinfected embryos; however, infectious diseases are surprisingly common in research zebrafish (Kent et al., 2020a). Maternal transmission, including intraovum, transmission, is one way that pathogens circumvent biosecurity barriers, and pathogens may also be introduced when “eggs only” policies are ignored in research facilities. Moreover, pet stores also represent a potential source for the introduction of pathogens in research colonies because as some researchers introduce zebrafish from these stores directly into research facilities. Pet store zebrafish are exposed to a myriad of fish pathogens, including those from other fish species, with no pathogen screening.

*P. neurophilia* is readily transmitted vertically (Sanders et al., 2013), which likely explains why this microsporidium is the most prevalent pathogen diagnosed in zebrafish facilities (Kent et al., 2020a). We have reported two other microsporidia in zebrafish facilities, *Pleistophora hyphessobricolis* (Sanders et al., 2010) and a putative *Nucleospora* sp. (Kent

et al., 2021), but these infections are rare. We have been investigating *P. neurophilia* in zebrafish since our first description (Matthews et al., 2001), and here we review studies on the parasite over the last two decades. This review covers primary papers, as well as reviews by our group (Kent et al., 2020c; Schuster et al., 2022c; Sanders et al., 2012; 2014) and others (Caballero-Huertes et al., 2021). We provide updated data on the prevalence and distribution of the parasite in zebrafish facilities, review of the pathogenesis and impacts on research, diagnostic methods, our recent findings on the phylogeny of *P. neurophilia* and recently discovered relatives, control and treatment, and potential uses of the parasite as a model in biomedical research.

## 2. DISTRIBUTION IN RESEARCH FACILITIES

*Pseudoloma neurophilia* and *Mycobacterium* spp. are the most frequently diagnosed pathogens in fish submitted to the Zebrafish International Resource Center (ZIRC) diagnostic pathology service (<https://zebrafish.org/wiki/health/submission/report>). Our report on occurrence of *P. neurophilia* infections was based on ZIRC data obtained between 2006–2023 (Figure 1). Submissions to the diagnostic service ranged from 20–68 facilities and 277–1,441 fish/year, with a total of 12,927 zebrafish examined. Over this time period, the parasite was detected in 19–74% of facilities, and 3–30% of the total fish submitted.

## 3. TRANSMISSION AND DEVELOPMENT

The parasite is transmitted both horizontally (fish to fish) and vertically (mother to progeny) (Sanders et al., 2013) and as with all microsporidia, *P. neurophilia* replicates intracellularly within the zebrafish host. The sequential development of *P. neurophilia* within larval zebrafish was described in Cali et al. (2012) and Sanders et al. (2014), where it was shown that infection initially begins with the ingestion of infectious spores in the environment.

With horizontal transmission, spores are ingested and then make their way through the intestine and into the coelomic organs and muscle, where they continue to undergo karyokinesis. In this case, this is the proliferative stage of the life cycle (Cali et al., 2012), which is in direct contact with the cytoplasm of host cells. In larval studies, proliferative stages were sequentially visualized in the pancreas and kidney, spinal cord, eye, and skeletal muscle (Sanders et al., 2014). The first spore stages of *P. neurophilia* were observed sequentially in the pharyngeal epithelium, liver, spinal cord, and skeletal muscle (Sanders et al., 2014). The parasite was visualized in the central nervous system (CNS) after many other organ systems were infiltrated. The distribution of the early stages of *P. neurophilia* and the lack of mature spores early in infection, indicates that the parasite gains access to organs distant from the initial site of entry. In adult zebrafish, the primary mode of transmission occurs horizontally, where zebrafish contract the infection by ingestion of spores shed from live fish in excretory material, spawning, and by ingestion of infected dead tissue or eggs.

Of note, the parasite is also commonly found in the ovaries, but has not been found in the testes. Vertical transmission can occur both inside the developing embryo (intraovum) and outside the egg in ovarian fluids (extraovum) further contributing the periodic release of

resistant, infectious spores into the environment. Thus, spawning events can also represent a significant contributor to transmission (Sanders et al., 2013; Schuster et al., 2022a).

### Environmental Transmission Dynamics.

Table 1 reviews transmission results from various experiments and observations. Often wild-type zebrafish populations at the ZIRC exhibit no clinical symptoms of infection, nor evidence of infection by histology early in the infection (e.g., 4 mo), but the parasite is then found in the same populations when fish are about 8 months old. Laboratory transmission experiments accelerate the process (Table 1). In this case, the lack of histological evidence of infection could be a result of the failure to sample infected fish because of the low prevalence of infection in a given sampling unit. Alternatively, histological changes indicative of infection may not develop until more mature stages of infection.

In a longitudinal exposure experiment, a population of fish were infected with a low concentration of spores (8,000 spores/fish), which was more reflective of natural infections than previous transmission studies, and it was shown that prevalence in the sample population was minimal until about 3 mo post exposure (Schuster et al., 2022b). In this experiment, a subset of individual fish from a population were analyzed by PCR and histology, while also accompanied by analysis of tank water with an eDNA assay (Schuster et al., 2022b; 2023). While the prevalence of infection in the population remained minimal for the first 3 mo, inflammation (myositis) was observed at 5 wks after exposure, while at this same time the parasite was not detected in the tank water. This further supports the hypothesis that the parasite gains access to areas of the body distant from the initial site of entry, as seen in larval fish (Sanders et al., 2014) but is not being constantly shed into the environment. Following the establishment of active infection around 3 mos, spore detection in the water was sporadic, likely corresponding to periodic shedding of mature spores related to spawning events as the parasite proliferated in the population. Moreover, there is a positive correlation with the abundance of spores in the water and the prevalence in fish populations held in the same tank (Schuster et al., 2022a; 2023).

## 4. PHYLOGENY AND TAXONOMY

*Pseudoloma neurophilia* was first reported in zebrafish *Danio rerio* collected from a pet store over 40 years ago (de Kinkelin 1980), and two decades later it was assigned to a new genus and species (Matthews et al., 2001). At that time, phylogenetic analysis based on SSU rDNA placed it closest to *Ichthyosporidium*. Cali et al. (2012) redescribed *P. neurophilia* and assigned it to the family Ichthyosporidae. They conducted more detailed electron microscopy and showed that its early development occurs in direct contact with the host cell. They described a character that is unique to the genus; a surface layer reminiscent of the glycocalyx of gut epithelial cells, excluding the direct contact of host organelles with the parasite plasmalemma, sporogonic development proceeds in intracellular sporophorous vesicles (SPOVs), which are the outermost structures interfacing the host cytoplasm. Xenoma formation was absent.

SSU rDNA sequence has recently become available from three other microsporidia that appear to be related to *P. neurophilia* based on phylogenetic analysis. Brocklebank et al.

(1995) described *Microsporidium cerebralis* from the CNS of Atlantic salmon (*Salmo salar*), and then Jones et al. (2017) provided sequence on the salmon parasite and *Microsporidium lucoppercae* from pike-perch (*Sander lucioperca*). We recently identified a genetically related parasite – a microsporidium infecting the brain of the swamp guppy (*Micropoecilia picta*) from Grenada (Schuster et al., 2024). These fish microsporidia form a clade of related species based on SSU rDNA (Fig. 2). We did not assign these all to the genus *Pseudoloma* as details of ultrastructure are lacking for some of these microsporidia, and only *P. neurophilia* has matching signature sequences, which are regions of the SSU gene that are genetically distinct from other Microsporidia, for the genus established by Cali et al. (2012). The observation of a microsporidium similar to *P. neurophilia* in a field survey suggests that there are many more species related to *P. neurophilia*. Whereas the swamp guppy microsporidium is clearly a distinct species from *P. neurophilia*, the former was detected by a conventional PCR test designed for *P. neurophilia* (Murray et al., 2016), but was not detected by a commonly used qPCR test targeting a 4 times smaller amplicon (Sanders et al., 2011). Larger amplicon size has been reported to decrease amplification efficiency, increasing the likelihood of non-specific amplification, as there may be more template available for off-target amplification (Dieffenbach et al. 1993, Suzuki & Giovannoni 1996). *P. neurophilia* infects a wide variety of fishes in different families, several of which are used as laboratory fish (Sanders et al., 2016). It is conceivable that there are other related microsporidia from other tropical freshwater fishes that would be capable of infecting zebrafish and other laboratory fishes and may cross react with diagnostic tests presently used for *P. neurophilia* in zebrafish facilities.

## 5. CLINICAL SIGNS AND PATHOBIOLOGY

Based on various laboratory transmission studies (Table1), fish show histologically visible infections about 2–4 mo post-exposure, with increasing prevalence per sampling unit well over 50% as the disease progresses over many months to a year. Observations from natural exposures performed at the ZIRC show detection later (Murray et al., 2011; 2016), but the time of initial exposures in this case is unknown. Regardless, most infected fish show no clinical signs until much later in the course of the infection. As the infection progresses, fish within the population increasingly appear emaciated, and less frequently they may exhibit spinal deformations such as lordosis, kyphosis, and scoliosis. At the ZIRC, Matthews et al. (2001) noted the infection was more prevalent in fish with these clinical signs. Spagnoli et al. (2015) reviewed histologic slides of 550 infected fish submitted to the ZIRC diagnostic service from 86 laboratories between the years 2000 and 2013. Both apparently normal, sentinel fish and fish with clinical signs were submitted. For approximately 70% of these infected fish, no clinical signs were noted by the submitter, indicating that most infected fish do not show outward signs of disease. There was, however, no difference between clinical and normal fish in terms of severity of infection in the CNS, but the myositis associated with infection was linked to clinical disease. In another study, mortality was greatly increased in infected fish that were immunosuppressed by gamma irradiation (Spagnoli et al., 2016), while the groups of fish with the parasite or gamma irradiation exposure alone showed minimal mortality. Experimentally infected female fish, but not males, had reduced body condition scores due to ovarian mass being reduced by about one third (Sanders et al., 2019).

## Microscopy.

The primary site of *P. neurophilia* infection is the CNS. In wet mounts of the neural tissue, the spores show the typical microsporidian morphology; they are about 5 µm in length and have a prominent posterior vacuole (Figure 3). They generally occur in clusters, comprised of multiple groups of 8–16 spores, and they do not form xenomas. Spagnoli et al. (2015) retrospective review of zebrafish diagnostic cases included review of tissue distribution of *P. neurophilia* based on histology (Figure 4). Parasite clusters (PCs), which appear somewhat like xenomas, generally occur in distinct axonal swellings, frequently with no associated inflammation. Inflammation may be observed in viable cell bodies distant from PCs. Multiple PCs occasionally occur within a single axon, suggesting axonal transport. PCs occur most frequently in the spinal cord ventral white matter and the spinal nerve roots, as well as the vagus nerve. In the rhombencephalon, PCs are common in the primary descending white matter tracts. Within the gray matter of the rhombencephalon, PCs occurred most frequently in the reticular formation and the griseum centrale. The potential impacts of high numbers of PCs within brain and spinal cord structures that are associated with the infection as discussed below.

Inflammation associated with *P. neurophilia* infection is common, and infections in the CNS are associated with encephalitis/myelitis when present in the neuropil of the brain or spinal cord, or meninxitis (corresponding to meningitis in mammals) when it is associated with the membranes surrounding the central nervous system (Fig. 4C). Muscle infections occur in about 10–15% of the diagnostic cases and show a similar pattern with experimental infections (Ramsay et al., 20008; Sanders et al., 2020). Lesions present as a distinctive pattern characterized by multifocal to coalescing areas of granulocytic to granulomatous inflammation with concurrent myodegeneration (Fig. 4D). Only a few free spores or PCs are usually detected within the usually extensive muscle lesions (Sanders et al., 2012). These muscle lesions are more common in zebrafish that have been immune compromised by either crowding stress (Ramsay et al., 2009) or gamma irradiation (Spagnoli et al., 2016b). Some muscle infections are more granulomatous and were first noted as a confounding variable as autofluorescing nodules in a line of zebrafish with the lck:EGFP transgene conferring T cell-specific GFP expression (West et al., 2014). Spores were visualized within the granulomas with special stains and *P. neurophilia* infection was confirmed with PCR. The ovary is another common site of infection, with spores occurring in the stroma and occasionally within developing oocytes (Fig. 4G) (Kent and Bishop 2003; Sanders et al., 2012). Disseminated infections can occur in other organs (Kent and Bishop-Stewart 2003), which is particularly evident in fish that are immune compromised by crowding (Ramsay et al., 2009) or immunosuppressed with gamma irradiation (Spagnoli et al., 2016b).

## Impacts beyond morbidity.

Zebrafish are increasingly used in experiments measuring behavioral endpoints (Gerlai 2019). Hence, *P. neurophilia* is a potential confounding factor with these endpoints, which is of particular concern given that the infection is so common and infected fish often do not exhibit obvious clinical changes. Chronic neural infections by *P. neurophilia* have been demonstrated to result in significant behavioral changes attributed to a hyper-vigilant phenotype, leading to behavior associated with higher anxiety such as the formation of

tighter shoals and a lack of habituation to startling stimuli (Spagnoli et al., 2015b). Consistent with this change, in a study by Spagnoli et al. (2016a) infected fish formed tighter shoals and were less likely to be caught by netting compared to uninfected fish, of which the latter should be considered when sampling populations for the presence of the infection. Midttun et al. (2020b) found that although sociability and aggression remained unaltered, infected fish exhibited reduced activity, elevated rates of freezing behavior, and sex-specific effects on exploration. Overall, results generally support consistent changes in behavior, resulting in a stress and anxiety prone phenotype. The same laboratory (Midttun et al. 2020a) demonstrated that *P. neurophila* infections cause alterations in the brain transcriptome profiles, specifically downregulating key host immune genes, such as MHC II, which is critical for adaptive immunity. This suggests a novel method for immune evasion that could explain why aggregates of spores in the neural tissues often do not elicit an inflammatory response. Moreover, 14 gene ontology terms were enriched by the parasite in infected fish, eight of which were associated with immune response and circadian rhythms. Other potential non-protocol induced variations caused by the parasite in research zebrafish should be considered. For example, in addition to behavior, the CNS has fundamental control of heart rate, and damage has been connected with heart arrhythmias and other electrical problems (Tahsili-Fahadan and Geocadin 2017). The Cardiac Autoregulation and Arrhythmias Laboratory (Dalhousie University, Canada) employs the zebrafish to study the electrical function of the heart (Stoyek et al., 2018) and they observed potential links of *P. neurophila* infections to bradycardia and some profound arrhythmias. Hence, moving forward this laboratory made efforts to avoid the infection in their experimental animals.

Finally, *P. neurophila* infections may hamper zebrafish breeding efforts. A constant and reliable source of embryos produced by females within a laboratory is important for most zebrafish research programs. Thus, another important potential impact of subclinical *P. neurophila* is that infection may decrease fecundity. Infected females have fewer developed eggs in the ovary (Sanders et al., 2020), and Ramsay et al. (2009) showed that severity of infection in both males and females is negatively correlated with egg production at spawning.

## 6. DIAGNOSTICS

### Microscopy.

Microsporidian spores may be observed and identified using a wet mount identification technique by placing fresh neural tissue on a microscope slide. However, this method is often only informative for late-stage or heavier infections, where the parasite has formed aggregates in the neural tissues. Histopathology is one of the most informative diagnostic tools available for zebrafish as it allows for diagnosis of multiple pathologies from a single fish, and this approach is efficient because sagittal sections from an entire adult fish can be processed onto one slide (Kent et al., 2020b; Murray et al., 2011). Thus, the ZIRC diagnostic service is largely based on histopathology of whole fish as the predominant diagnostic tool to detect the *P. neurophila* in fish tissues. While PCR may allow for earlier detection of the parasite (Schuster et al., 2022a, b), histology allows evaluation of the degree of infection, as well as the potential detection of other pathogens. The stains available for detection by

histology include hematoxylin and eosin (H&E), Luna, Gram, acid fast, and Fungi-Fluor stains. H&E allows for detection of the parasite in both larval and adult fish, and careful examination may reveal presporogonic stages. The special stains (e.g., the Fungi-Fluor and Luna) highlight only spores, and thus these are used mostly for adult fish. The Fungi-Fluor stain can be applied to fresh tissues or paraffin sections and produces a fluorescence in the presence of the microsporidian chitinous spore wall (Kent and Bishop-Stewart 2003). The Luna stain increases sensitivity of the detection of spores as they stain red on a blue background of other tissues (Peterson et al., 2011). Furthermore, the Luna stain has been shown to be particularly useful as a follow-up to H&E staining as it can detect single or rare spores in non-neural tissues and in areas of inflammation where microsporidiosis is suspected (Murray et al., 2011). In-situ hybridization (ISH) allows for the detection of the presporogonic stages of the parasite in histological sections that would otherwise be missed (Sanders et al., 2014).

### Molecular.

Several molecular assays have been developed for the detection of *P. neurophila* in fish including those based on conventional PCR (Whipps & Kent 2006), quantitative PCR (qPCR) (Sanders et al., 2013; Crim et al., 2017), digital PCR (dPCR) (Schuster et al., 2022a; 2023), and in situ hybridization (ISH) using formalin-fixed, paraffin-embedded tissue sections (Sanders et al., 2014). The latter allows for detection and differentiation of all stages of the parasite in adult and larval zebrafish. However, this method is low-throughput relative to PCR-based methods and, as a result, has not been implemented in routine diagnostics as far as the authors are aware.

Initially, PCR tests were performed on spinal and brain tissue dissected from adult fish, or pools of larval fish. Indeed, the systematic screening of larval fish and neural tissue from broodstock using a PCR-based assay was instrumental in the development of the first *P. neurophila*-free zebrafish facility (Kent et al., 2011). One limitation of testing specific tissues is the large amount of time and expertise required to necropsy and adequately sample neural tissue from adult zebrafish. As a result, several laboratories have moved to the homogenization and testing of whole adult fish, both individually and in pools, for various pathogens, including *P. neurophila* (Crim et al., 2017, Norris et al., 2020). In a study performed by Schuster et al. (2022b), the authors compared the detection probability of CNS versus whole body homogenates of adult fish for *P. neurophila* using a qPCR-based detection system and found the two sample types to be comparable.

Estimating prevalence of *P. neurophila* in populations by post-mortem testing of individuals and tissues requires euthanasia of a significant subset of the population. The increased interest in health screening of research animals, along with the need to reduce the numbers of animals used in research has driven a great deal of interest in the use of environmental samples (e.g., tank water) to detect zebrafish pathogens. The purification and detection of DNA from environmental samples, referred to as environmental DNA (eDNA), has been widely employed in aquatic systems, both natural and artificial. A qPCR-based method was developed and demonstrated to detect *P. neurophila* in both spiked samples and water from a group spawn of infected fish (Sanders and Kent 2011). This technique was later used to

detect *P. neurophilia* in water samples collected from fish spawned in pairs to elucidate the vertical transmission of the parasite (Sanders et al., 2013).

The ability to detect *P. neurophilia* in water can be inconsistent (Crim et al., 2017; Miller et al., 2019), and it was suggested that this may be because spores sink in the water column. However, what appears most probable for this inconsistency is related to the structure of the microsporidian spore itself. The spores contain a thick endospore wall composed of chitin, and isolation and purification of DNA from spores in the environment often requires aggressive disruption techniques. For example, Phelps and Goodwin (2007) observed 500 times increase in sensitivity by briefly sonicating *Ovipleistophora ovariae*, compared to simple protease K digestion. There is no defined standard for disruption of spores in environmental samples for *P. neurophilia*. Whereas Crim et al. (2017) used stainless steel beads for mechanical disruption of spores, Sanders and Kent (2011) performed a comparison of various mechanical disruption techniques, showing sonication resulted in a near 3-log increase in *P. neurophilia* DNA. Moreover, Peneyra et al. (2018) also utilized the methods outlined in Sanders and Kent (2011), showing that there were no inconsistencies in detection of the parasite in environmental samples obtained in that particular study. It would be expected that *P. neurophilia* present in the environment would be in the form of spores, and thus any environmental sampling techniques must incorporate sonication or a comparable disruption method in order to obtain the sensitivity necessary to be diagnostically relevant.

Advancement in PCR technology has improved nonlethal eDNA assays for aquatic pathogen detection and quantification. Schuster et al. (2022a) developed an eDNA assay that enabled the detection of the parasite in tank water during rare events (low concentrations) and was used to track the progression of infection over a 10-month period (Schuster et al. 2022b). The hallmark of the dPCR assay is that it allows zebrafish facilities to detect the parasite early in infection without the need to euthanize a significant subset of valuable fish lines. The main challenge with environmental detection overall is the requirement for a framework to interpret the diagnostic data in the context of different environments. For example, it was shown that leaving tanks static for 8 h significantly improved the probability of detection in zebrafish aquaria. Thus, understanding facility specific dynamics as they relate to flow, temperature, conductivity, etc., is important for integrating the eDNA assay into zebrafish facility monitoring (Schuster et al., 2022a, b; 2023).

While the advancements in the detection of *P. neurophilia* since it was first recognized in zebrafish populations in 2001 are noteworthy, there is no single approach recommended for early detection of the parasite. Populations at the ZIRC often are not seen to be positive by histology until 4 months post exposure, whereas with eDNA assays, detection can occur shortly after initial exposure (Schuster et al., 2022b). The optimized eDNA protocol for detection of *P. neurophilia* in tank water complements histopathology-based diagnostic methods, moreover, integrating these approaches not only identify infections earlier, but also provides valuable information on the pathological changes associated with the progression of disease. As eDNA tests are further developed and validated, we envision that in the future that they will be universally used for both *P. neurophilia* and other zebrafish pathogens. Whereas there is an effort to publish assays that are readily available to the zebrafish community, there are also commercial diagnostic

services for the detection of the parasite offered by a variety of commercial laboratories – e.g., IDEXX (<https://www.idexxbioanalytics.com/zebrafish-health-monitoring>), Charles River (<https://www.criver.com/products-services/research-models-services/animal-health-surveillance/animal-health-monitoring/zebrafish-health-monitoring?region=3601>), VRL Animal Health Diagnostics (<https://vrlsat.com/animal-health-monitoring/aquatics/>), and a collaboration between the Oregon Veterinary Diagnostic Laboratory (OVDL) and ZIRC (<https://zebrafish.org/health/>).

## 7. TREATMENT, DISINFECTION AND CONTROL

Various therapeutic drugs, particularly oral fumagillin, have been effectively used to treat several microsporidian infections in fishes (Kent et al., 2014). However, these treatments have not proved effective with *P. neurophilia*. For example, Lavin et al. (2023) evaluated oral treatments with fumagillin and albendazole for *P. neurophilia* infection, and neither were efficacious. Therefore, at this time, the infection is best controlled by avoidance.

Disinfection to prevent transmission of *P. neurophilia* is also challenging. Spores survive the typical concentrations of chlorine used for surface disinfection of embryos, i.e., 25–50 ppm unbuffered sodium hypochlorite (bleach) for 10 min (Ferguson et al., 2007). Higher doses are more effective, with about 80% of spores neutralized by 100 ppm unbuffered bleach. Complete neutralization is achieved by 200 ppm bleach and 100 ppm buffered to pH 7. However, these doses are also toxic to embryos and larvae (Kent et al., 2014). Crim et al. (2017) presented preliminary evidence that simply rinsing embryos with disinfected (UV-irradiated) fish water may reduce the likelihood of pathogen contamination of progeny. However, no amount of rinsing or surface disinfection will eliminate spores transmitted vertically within the egg (Sanders et al., 2013). High doses of bleach, i.e., >100 ppm, with sufficient rinsing or neutralization with sodium thiosulfate can be used for equipment disinfection. Peneyra et al (2018) tested different approaches with spawning chambers to reduce transmission using the Sanders and Kent (2011) PCR with sonication as a detection method. They found that 100 ppm bleach soak (pH 7.0), and a soak or spray with 75 ppm Wescodyne (an iodine product) (Steris Corp, Mentor, OH) was 100% effective at eliminating detection of the parasite. Disinfection of spawning chambers before spawning did not decrease *P. neurophilia* transmission when infected fish remained present in the breeding population, and hence they recommend not using infected brood fish to avoid infection. Mendoza et al. (2022) reviewed effects of various combinations of iodine or sodium hypochlorite on zebrafish egg and embryo viability, providing a useful guideline when developing disinfectants for killing *P. neurophilia* spores associated with eggs.

The spores are also resistant to freezing with cryoprotectant, a strategy commonly used to store zebrafish sperm (Norris et al., 2018). *P. neurophilia* spores have been detected by PCR in dissected testes and stripped sperm (Murray et al., 2011). What remains to be quantified is the risk of recovering infected progeny from in vitro fertilization using thawed sperm that was naturally contaminated with *P. neurophilia* prior to cryopreservation.

Ultraviolet sterilizers are essential for controlling the spread of infectious pathogens on recirculating aquaculture systems. Specific studies of the dose of UV irradiation required

to neutralize *P. neurophilia* are not published. However, for several years ZIRC observed the pathogen in pre-filtration sentinel fish but not in sentinels receiving post-filtration water treated with 45,000  $\mu\text{Wsec}/\text{cm}^2$  UV irradiance, evidence that the dose effectively prevented recirculation of infectious spores (Murray et al., 2011).

As the *P. neurophilia* life cycle and modes of transmission are well understood and sensitive diagnostic tests for the pathogen are available for in-house application and from diagnostic laboratories, *P. neurophilia* meets the criteria for exclusion Specific Pathogen Free (SPF) facilities (Murray et al., 2022). Indeed, the Sinnhuber Aquatic Research Laboratory (SARL) has successfully maintained a zebrafish colony that is SPF for *P. neurophilia* for over 10 years (Kent et al., 2011; Barton et al., 2016). As the parasite is maternally transmitted, the SARL strategy involves screening broodstock and progeny by PCR and a three-step quarantine to ensure that only *Pseudoloma*-free stocks are propagated. In addition, thorough testing of sentinel, moribund, and aged fish by histopathology and PCR is evidence that the colony remains SPF.

To avoid the potential negative impacts of *P. neurophilia* on research outcomes, from our conversations with lab animal veterinarians and facility managers it is apparent to us (K.M.) that several new zebrafish facilities have an intent to exclude the parasite. To achieve this objective, facilities may decline importation of fish from facilities with *P. neurophilia* or import lines into quarantine where progeny and broodstock are screened for the parasite before the line is established in the main facility. This strategy, while proven successful at SARL, requires time and resources that may be limiting factors when investigators need fish for grant-funded research.

*Pseudoloma neurophilia* has been found in 30% or more of zebrafish facilities submitting samples to the ZIRC diagnostic service (Figure 1). The persistence of the parasite likely reflects the difficulty of eliminating it from facilities once infections are established. While exclusion protocols are available, how to minimize and eliminate established endemic infections is a different and more complicated problem. One approach is to rederive individual contaminated fish lines by in vitro fertilization (IVF) using sperm from contaminated males and eggs from *Pseudoloma*-free females SPF AB wild-type fish are available for purchase from SARL (Ventura-Fernandes et al., 2021). Progeny must then lethally be tested by PCR for the infection, and the procedure may need to be repeated several times to obtain sufficient fish numbers to ensure statistical confidence that there is not occurrence in the rederived progeny, while ensuring that there is still a sufficient number of fish within the population that are not subjected to this lethal test screening. Additionally, sufficient numbers are needed to replace progeny that are infected from contaminated sperm samples. Progeny resulting from this cross will be heterozygous and must be backcrossed to establish a homozygous population. Furthermore, this strategy of relying on sperm to maintain a germline cannot be used for wild-type lines, where both males and females are required to preserve the line-specific genome, as opposed to preserving a genetic feature of a mutant or transgenic line.

The proportion of facilities with infected fish that have submitted samples to ZIRC appears to have decreased since a peak in 2010 (Figure 1), but conclusions as to the apparent cause

should be accepted with caution as the submitters (facilities) are not selected at random from the overall research community. Nevertheless, it is possible that more researchers now appreciate the potential negative effects of the parasite beyond morbidity, and hence are taking measures to avoid the infection. These efforts include obtaining new fish from sources documented to be free of the infection and carrying out more rigorous quarantine procedures for these imports beyond simply introducing new stocks directly into the main facilities as surface disinfected eggs. In addition, more institutions are requiring their facilities to have health reports on zebrafish imports (Mocho et al., 2022).

The ZIRC's strategy for minimizing *P. neurophilia* infections has evolved in tandem with our understanding of the parasite. To maintain genetic diversity, every third generation of mutant and transgenic lines is outcrossed to wild-type AB fish. Since 2006, the ZIRC outcrossing protocol has specified that AB fish cannot be used to outcross more than one stock (a specific generation of a genetic line), nor can they be returned to the original AB population after being used for outcross. This has prevented contaminated AB fish from spreading the parasite to other fish lines in the facility. In addition to robust biosecurity measures to prevent parasite spread between tanks, ZIRC has been identifying infected fish stocks by in-house PCR (Murray et al., 2011) and histopathology on moribund and randomly sampled colony fish since 2008. Infected stocks are euthanized, when possible, i.e., when a younger generation is sufficiently close to maturity, and genetically related stocks are monitored for the parasite. For a large facility, this is a slow process, but the ZIRC was able to decrease the parasite to below detectable levels by PCR and histopathology on moribund, pre-filtration sentinels, and randomly sampled colony fish from October 2010 to February 2013 ([https://zebrafish.org/wiki/health/health\\_reports/start](https://zebrafish.org/wiki/health/health_reports/start)).

Prior to the establishment of new biosecurity measures, ZIRC imported new fish lines into quarantine and their surface-sanitized embryos were moved into the main fish colony. In addition, in 2011 ZIRC began importation of thousands of fish lines as cryopreserved sperm generated from large mutagenesis projects in the research community. These lines were regenerated at ZIRC by IVF to amplify sperm samples when they became depleted by customer orders. The combination of untested new fish lines in the facility and a 10-hour failure of UV sterilizers in September of 2013 had the unfortunate result of spreading *P. neurophilia* throughout the facility via untreated water in the recirculating system. In response, ZIRC intensified in-house testing and modified the importation strategy to include diagnostic testing and resulted in fewer new lines being moved into the main fish colony. As of 2016, all mutant and transgenic lines are imported to ZIRC via male fish. Sperm is collected in quarantine, cryopreserved, and stored in designated quarantine freezers. The males are then euthanized, fixed, and processed into paraffin blocks. Customer orders for these lines are filled by IVF using eggs from ZIRC AB females and shipped as surface-sanitized embryos. Before the line is regenerated and reared at ZIRC to reamplify sperm samples, histological sections are prepared from paraffin blocks of males that contributed the original sperm samples. Results of histological analysis for pathogens determines whether the line will be reared in quarantine or the main fish facility. By directing all imported lines through cryopreserved sperm, ZIRC avoids the risk of vertical and early horizontal transmission of *P. neurophilia* to progeny via infected females. The risk of contaminated sperm is mitigated by histological evaluation of males.

In 2018 ZIRC initiated a labor-intensive project to eliminate *P. neurophilia* from wild-type lines in the facility. For each line, at least 50 individual pair crosses were spawned. Clutches of 50 or embryos were surface-sanitized and held in isolation pending in-house PCR results for *P. neurophilia* on CNS dissected from both parents. Only embryos from *Pseudoloma*-free adults were pooled and reared to propagate the line. At least 25 clean clutches were required to maintain genetic diversity of each line. ZIRC continues to test pre- and post-filtration sentinel and moribund fish by histopathology. Every month, water from all 20-gallon tanks of 8-month fish is tested by dPCR for eDNA of *P. neurophilia* (Schuster et al., 2023). As of June 2022, *P. neurophilia* decreased below detection level. However, reintroduction of the parasite is a constant risk given the high rate of importation and IVF necessary to carry out the ZIRC mission as a stock center.

## 8. POTENTIAL MODELS

Sanders and Kent (2104) outlined potential uses of the *Pseudoloma*-infected zebrafish as a biomedical model for microsporidian infections. With the scalability (large sample sizes for low cost and space), optical clarity of the embryos, availability of SPF lines, and amenability for gene expression studies, both larvae and adult zebrafish provide excellent opportunities to study infectious diseases in this host. Other naturally occurring pathogens of zebrafish, such as *Mycobacterium marinum* (Myllymaki et al. 2016) and *Pseudocapillaria tomentosa* (Gaulke et al. 2109; Sharpton et al. 2020) have been leveraged into useful infectious disease models in zebrafish. Likewise, with its ease of transmission and readability of infection in all life stages, *P. neurophilia* has the potential to elucidate details of early development of microsporidia (Sanders et al. 2013) as well as facilitate investigations of downstream effects of specific pathogen-associated changes in the CNS. Moreover, with the publication of the genome of *P. neurophilia* (Ndikumana et al. 2016), there is potential to use the model to understand the evolution of the genome as it relates to the spliceosome, as it was shown that with only 14 predicted proteins, *P. neurophilia* may be the smallest spliceosome currently known (Whelan & Fast 2023).

## 9. CONCLUSION

*P. neurophilia* continues to be widespread in zebrafish vivaria, and it is clear now that this chronic neural infection of the CNS has many potential and demonstrated impacts on infected research animals. With recognition of the problem, and more recent implementation of control measures since a peak in detection in 2010 from facilities that report to ZIRC, the percentage of facilities where the parasite was detected has fallen below 50%. Sensitive and specific PCR-based tests are now available for screening, and we see even more utility of these tests as they transition into highly efficient eDNA tests that coupled with current surveillance efforts have the potential to help move facilities towards SPF status.

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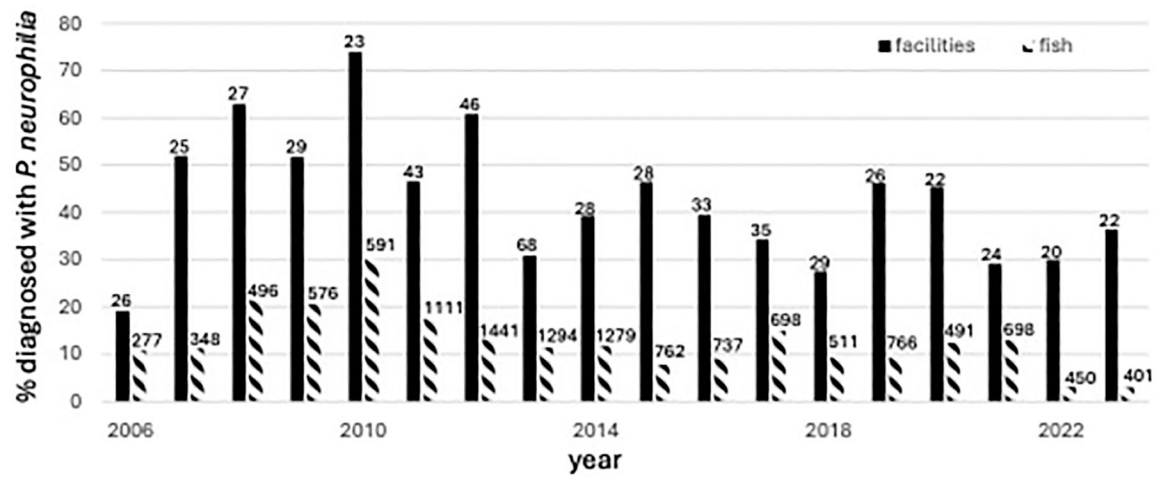


**Figure 1.**

Percentage of facilities and fish diagnosed with *P. neurophilia* by histopathology at the ZIRC diagnostic pathology service from 2006–2023. The number of submitting facilities or total number of fish is listed above each bar.

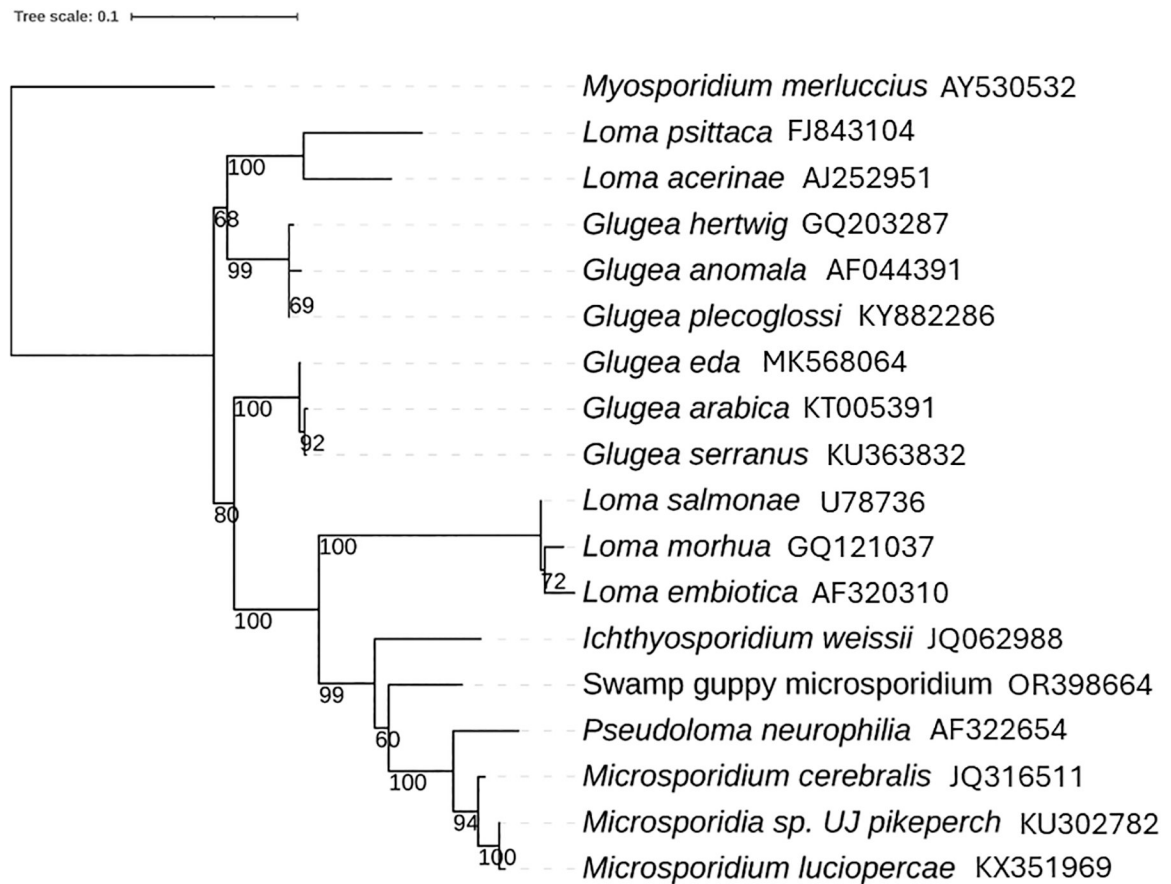


**Figure 2.**

Phylogenetic relationships among close relatives of *P. neurophilia*. The microsporidium clusters with other neurotropic microsporidia that do not form xenomas (swamp guppy microsporidium and *M. cerebralis*). Sequences were aligned using MAFFT v.7.520 (L-INS-i option) (Kato et al. 2019) and trimmed using trimAl v1.4rev22 (Capella-Gutierrez et al. 2009) to remove all columns with gaps in more than 20% of the sequences. The maximum-likelihood tree was generated using the TIM3+F+I+G4 substitution model determined to be the best-fit using ModelFinder (Kalyaanamoorthy et al. 2017) in IQ-Tree v1.6.11 (Nguyen et al. 2015), and bootstrap values were generated using UFBoot with 1,000 replicates (Hoang et al. 2018). The tree was visualized in iTol (Letunic and Bork 2021).

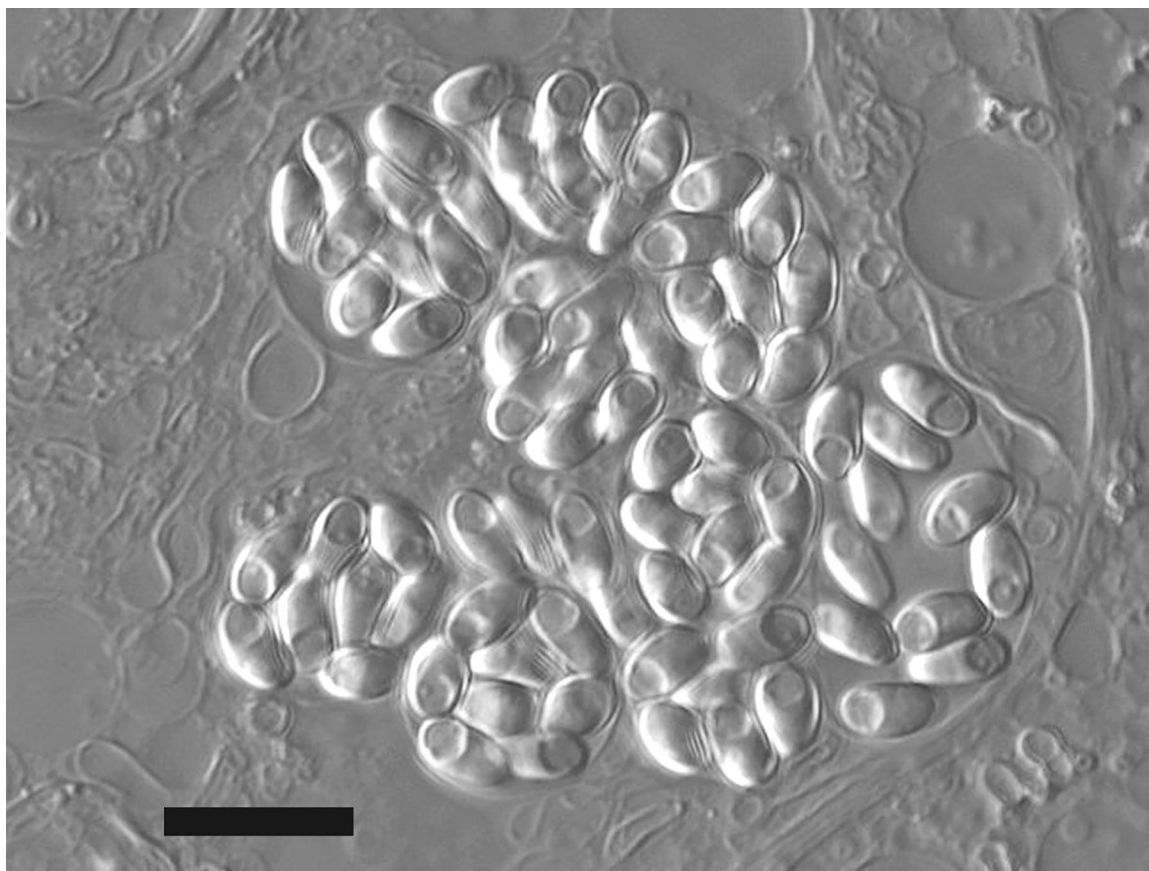


**Figure 3.**  
*P. neurophilia* spores in wet mount preparation of zebrafish (*Danio rerio*) spinal cord.  
Normarski phase contrast. Bar = 10  $\mu$ m.

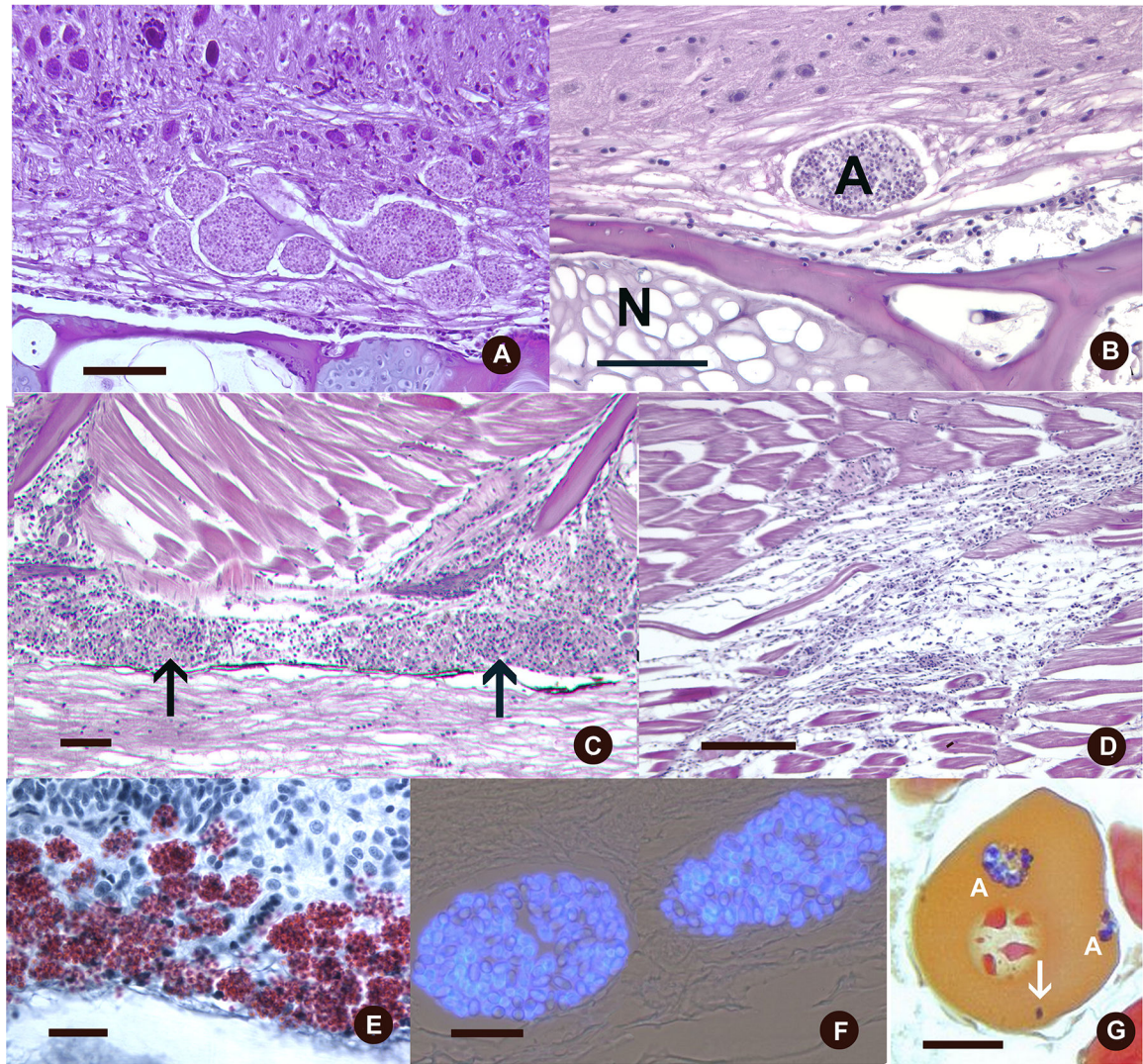


**Figure 4.**

*P. neurophilia* in histologic sections from zebrafish (*Danio rerio*). A, B, Several aggregates of spores (A) in ventral spinal cord. N = notochord. H&E. Bars = A, 200  $\mu$ m, B = 20  $\mu$ m. C. Chronic meninxitis (arrow) along dorsal aspect of spinal cord. H&E. Bar = 200  $\mu$ m. D. Focal myositis and degeneration. H&E. Bar = 200  $\mu$ m. E. Numerous spore aggregates, stained red with Luna stain, in spinal cord. Bar = 20  $\mu$ m. F. Two fluorescing aggregates of spores using DAPI filter and Fungi-Fluor stain. Bar = 20  $\mu$ m. G. Developing egg with spores. A = aggregates, Arrow = single spore. Gram stain. Bar = 25  $\mu$ m.

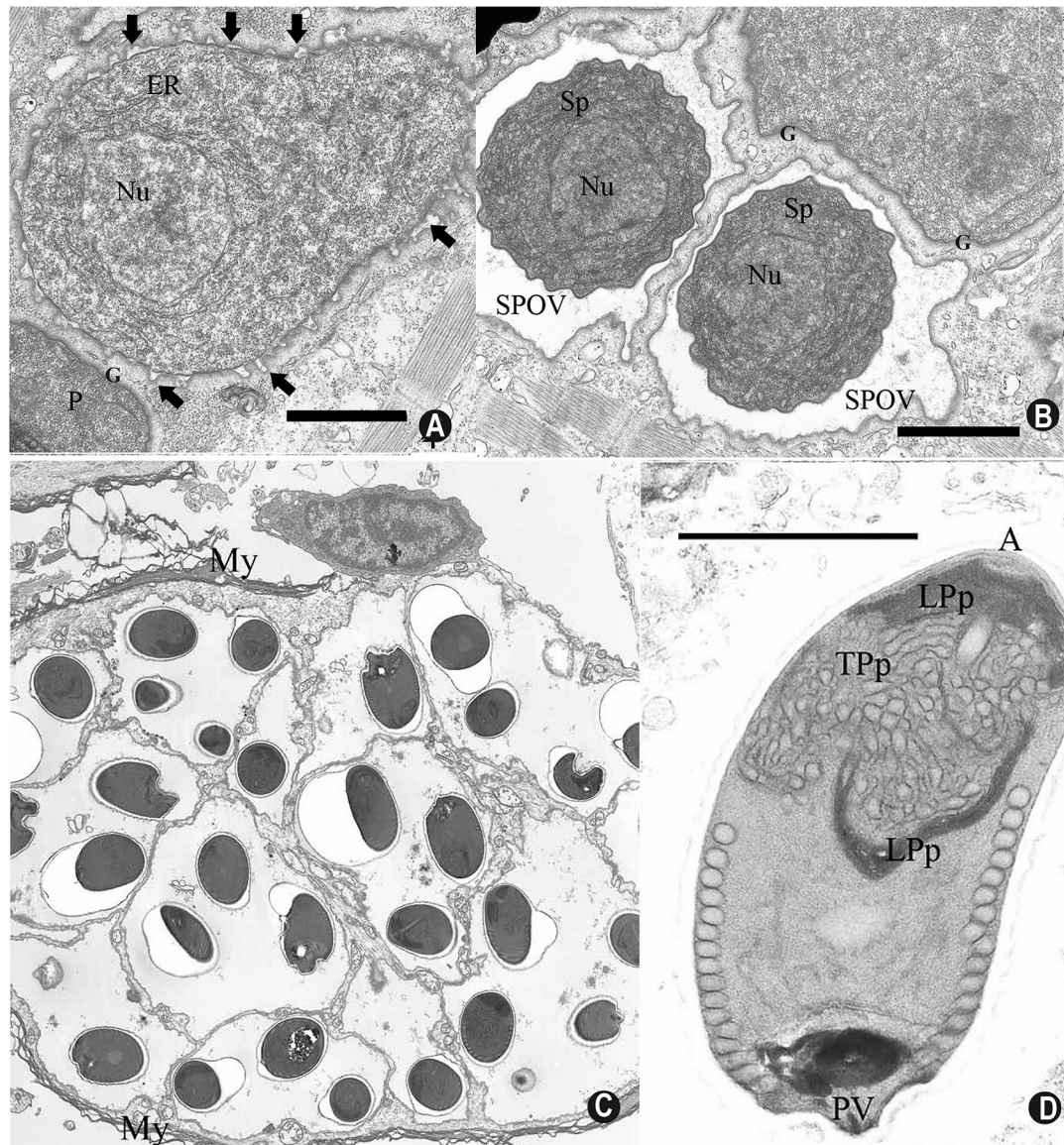


**Figure 5.**

Ultrastructural morphology and development of *P. neurophilia*. Images adapted from Cali et al. (2012). A, B) Parasite cell starting the transition from proliferative to sporogonic development. Note the difference between it and the abutting proliferative cell (P) membrane which is still covered with its glycocalyx-like surface coat (G). The plasmalemma has numerous “blisters” starting to emerge (bold arrows) from it and the new plasmalemma is now “thickened” in the areas under the blisters. The blistering continues until it forms an isolation chamber, a sporophorous vesicle (SPOV). Nu = nucleus, Sp = sporont. ER = endoplasmic reticulum. Scale bar = 1.7  $\mu$ m. C. Low-power image of a myelinated nerve with a long-term infection. The axoplasm filled with SPOVs containing spores; note the presence of a scant amount of axoplasm just inside the myelin (My) layers. Scale bar = 5  $\mu$ m. D. A spore in the SPOV. The anterior structure of the spore (A) is well-illustrated. The lamellar polaroplast (LPp) surrounds the tubular portion (TPp) and clearly extends approximately

half the length of the spore to the nucleus. The polar filament coil cross-sections are visible as 16 sections in a single row. PV = polar vacuole. Bar = 1  $\mu\text{m}$ .

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Table 1.

Review of transmission of *Pseudoloma neurophilia* in zebrafish in reports from research facilities and experimental studies. H = histology or wet mounts, PCR = polymerase chain reaction, Environmental DNA = eDNA, wpe = weeks post exposure, A = adult, L = larvae, NA = not available. Table expanded from Schuster et al. (2022b).

| Exposure                                            | Spores/mL | Spores/Fish | Diagnostic Test | Prevalence (%) | wpe       | Exposure A vs L | Notes                                                  | Reference                  |
|-----------------------------------------------------|-----------|-------------|-----------------|----------------|-----------|-----------------|--------------------------------------------------------|----------------------------|
| Natural Exposures                                   |           |             |                 |                |           |                 |                                                        |                            |
| Natural                                             | NA        | NA          | H               | NA             | 3 mo      | NA              | pet store fish, 15% morbidity                          | de Kinkelin 1980           |
| Natural                                             | N/A       | N/A         | H               | 10–30          | NA        | N/A             | Apparently healthy                                     | Matthews et al. 2001       |
| Natural                                             | N/A       | N/A         | H               | 90             | NA        | N/A             | Emaciated                                              | Matthews et al. 2001       |
| Natural                                             | NA        | NA          | H               | 90             | 74        | NA              | TU Line                                                | Murray et al. 2011         |
| Natural                                             | NA        | NA          | H               | 77             | 53        | NA              | TU Line                                                | Murray et al. 2011         |
| Natural                                             | NA        | NA          | H               | 2–10           | Up to 138 | NA              | Increasing over time,                                  | Borges et al. 2016         |
| Natural                                             | NA        | NA          | H               | 59             | 33        | NA              | AB line, based on 50 of 110 positive tanks. 2018–2020. | Murray pers comm. 2021     |
| Experimental Exposures                              |           |             |                 |                |           |                 |                                                        |                            |
| Screened neural tissues                             | NA        | NA          | H               | 30             | 8         | A               |                                                        | Kent & Bishop-Stewart 2003 |
| Screened neural tissues                             | NA        | NA          | H               | 100            | 20        | A               |                                                        | Kent & Bishop-Stewart 2003 |
| Tank detritus                                       | NA        | NA          | H               | 15             | 7         | A               | TU line                                                | Murray et al. 2011         |
| Parasite-laden water w/ continued effluent exposure | N/A       | N/A         | H               | 56             | 14        | A               | 5D                                                     | Spagnoli et al. 2015b      |
| Carcasses                                           | NA        | NA          | H               | 45             | 8         | A               | AB, 5D and WIK lines                                   | Sanders et al. 2020        |
| Neural tissue mixed with infected fish              | NA        | NA          | PCR             | 100            | 16        | A               |                                                        | Midtun et al. 2020         |
| Neural tissue, mixed with brine shrimp              | NA        | NA          | H               | 84             | 15        | A               |                                                        | Peneyra et al. 2018        |
| Spores                                              | 750       | 10,000      | H               | 55             | 8         | A               | Crowding                                               | Ramsay et al. 2009         |
| Spores                                              | 750       | 10,000      | H               | 16             | 8         | A               | Normal                                                 | Ramsay et al. 2009         |
| Spores                                              | 40        | 10,000      | H               | N/A            | N/A       | L               | Spore survival of chlorine treatment                   | Ferguson et al. 2007       |

| Exposure | Spores/mL | Spores/Fish | Diagnostic Test         | Prevalence (%)    | wpe                | Exposure A vs L | Notes                       | Reference                                   |
|----------|-----------|-------------|-------------------------|-------------------|--------------------|-----------------|-----------------------------|---------------------------------------------|
| Spores   | 100       | 10,000      | H & electron microscopy | NA                | 3h, 4.5 dpe, 8 dpe | L               | Larval exposure study       | Cali et al. 2012                            |
| Spores   | 300       | NA          | H                       | 6                 | 1                  | L               | Larval exposure study       | Sanders et al. 2013                         |
| Spores   | 500       | NA          | H                       | 32                | 1                  | L               | Larval exposure study       | Sanders et al. 2013                         |
| Spores   | 60        | 8,000       | H, PCR, eDNA            | H: >45% PCR; >90% | 13                 | A               | Longitudinal exposure study | Schuster et al. 2022b; Schuster et al. 2023 |