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Early Development and Tissue Distribution of *Pseudoloma neurophilia* in the Zebrafish, *Danio rerio*

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Abstract

The early proliferative stages of the microsporidian parasite, *Pseudoloma neurophilia* were visualized in larval zebrafish, *Danio rerio*, using histological sections with a combination of an in situ hybridization probe specific to the *P. neurophilia* small-subunit ribosomal RNA gene, standard hematoxylin-eosin stain, and the Luna stain to visualize spores. Beginning at 5 days post fertilization, fish were exposed to *P. neurophilia* and examined at 12, 24, 36, 48, 72, 96, and 120 h post exposure (hpe). At 12 hpe, intact spores in the intestinal lumen and proliferative stages developing in the epithelial cells of the anterior intestine and the pharynx and within hepatocytes were observed. Proliferative stages were visualized in the pancreas and kidney at 36 – 48 hpe and in the spinal cord, eye, and skeletal muscle beginning at 72 hpe. The first spore stages of *P. neurophilia* were observed at 96 hpe in the pharyngeal epithelium, liver, spinal cord, and skeletal muscle. The parasite was only observed in the brain of larval fish at 120 hpe. The distribution of the early stages of *P. neurophilia* and the lack of mature spores until 96 hpe indicates that the parasite gains access to organs distant from the initial site of entry, likely by penetrating the intestinal wall with the polar tube.

Keywords

Microsporidia; in situ hybridization; infection; histology

THE microsporidium, *Pseudoloma neurophilia*, is an obligate intracellular parasite that infects the zebrafish, *Danio rerio*. The parasite generally results in a chronic infection of adult fish, with spore stages generally found in the anterior spinal cord and nerve root ganglia (Matthews et al. 2001; Kent and Bishop-Stewart 2003). Subclinical infections of zebrafish are problematic due to the potential for non-protocol induced variation when using infected fish in research (Kent et al. 2012). While much is known about the parasite distribution during later stages of infection, very little is known about the initial stages and,

more importantly, how the parasite is able to reach immune-privileged sites such as the spinal cord.

Cali et al. (2012) described the sequential development of *P. neurophilia* within zebrafish but there are still gaps in our understanding of the earliest stages of infection and how the parasite disseminates to extraintestinal tissues. As with most microsporidia, infection by *P. neurophilia* begins by the ingestion of the infectious spore stage. In the ultrastructural description of *P. neurophilia*, Cali et al. (2012) observed the parasite within skeletal muscle myocytes of larval fish at 108 h post exposure. Proliferation of the parasite occurs in direct contact with the host cytoplasm, beginning with several rounds of karyokinesis, resulting in the formation of a multinucleate plasmodial cell. This is followed by cytokinesis and the formation of uninucleate cells which eventually undergo sporogony, forming mature spores. This development is fairly rapid with the first mature spores observed at 8 d post infection in both spinal cord and skeletal muscle (Cali et al. 2012).

In subclinically infected adult fish, *P. neurophilia* is most commonly observed in immune-privileged sites such as the spinal cord, ventral nerve roots, and anterior brain (Matthews et al. 2001), however, free spores are also often seen in the kidneys and ovaries with the use of chitin-binding fluorescent stains such as Fungi-Fluor (Kent and Bishop-Stewart 2003). The use of special stains such as Fungi-Fluor and the Luna stain (Peterson et al. 2011) have also enabled the visualization of spores in other tissues, most notably the skeletal muscle of fish with clinical infections due to severe myositis (Kent and Bishop-Stewart 2003) and in the ovigerous stroma and within the developing ova of healthy-appearing females (Sanders et al. 2012).

While these special stains provide more sensitive detection of the spore stages of Microsporidia in tissues, the visualization of presporogonic stages of these parasites is much more difficult. In situ hybridization techniques have been used to detect presporogonic stages of microsporidian parasites in a few fish species such as *Glugea plecoglossi* in rainbow trout (Lee et al. 2004), an unknown species in amberjack (Miwa et al. 2011), and *Loma salmonae* in rainbow trout (Sanchez and Speare 2001). Sanchez and colleagues used this technique to track the initial stages of the parasite, finding proliferative stages of the parasite in the cells underlying the endocardium, which was present prior to the appearance of xenomas containing mature spores in the gills of infected fish (Sanchez and Speare 2001).

We infected newly hatched larval fish with *P. neurophilia* and tracked the infection at various time points post exposure. With the small size of the larvae, we were able to visualize all organs throughout the infection in whole-body coronal sections stained with either hematoxylin and eosin (HE), the Luna stain, or our in situ probe based on the small subunit rDNA gene of the parasite.

MATERIALS AND METHODS

Parasite exposure

Exposures were performed using AB line fish obtained from the *P. neurophilia* specific pathogen free (SPF) colony located in the Sinnhuber Aquatic Research Laboratory (SARL)

at Oregon State University (Kent et al. 2011). Embryos were held in sterile system water at 28° C and checked twice daily. At 5 days post-fertilization, fish were divided into two separate 250 ml glass beakers in 100 ml of sterile system water each and fed concentrated paramecia twice daily. Spores of *P. neurophilia* were collected from donor fish using the method previously described (Ramsay et al. 2009). Briefly, adult fish infected with *P. neurophilia* were killed by an overdose of tricaine methanesulfate (MS-222), their hindbrains and spinal cords were removed and placed in sterile water containing 100 units each of penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA), and then macerated by forcing the material through sequentially smaller gauge needles attached to a 5 ml syringe. Spores were then quantified using a hemocytometer and added to one beaker of larval fish at a concentration of 1.5×10^6 / 100 ml. Larvae in the remaining beaker were maintained as an unexposed control group.

In a study of the initial developmental stages of *Loma salmonae* in rainbow trout, Sanchez et al. (2001) first observed intracellular parasite DNA beginning at 12 h post exposure (hpe). A preliminary study in which we examined larval zebrafish at 1 and 6 hpe confirmed the presence of only extracellular spores in the gut lumen. Thus, exposed larval fish were collected at the following time points in hours post exposure: 0, 12, 24, 36, 48, 72, 96, and 120. Collected larval fish were euthanized by inducing instantaneous fatal hypothermia (ice bath immersion), immediately placed in Dietrich's fixative and fixed overnight at 4° C. After fixation, embryos were placed in 70% ethanol and embedded in 7 × 4 agarose arrays (Sabaliauskas et al. 2006). The arrays were processed for histology, paraffin-embedded, and 5 µm serial sections cut with alternating sections stained with HE or Luna, and unstained sections which were examined using in situ hybridization. The parasites observed were categorized as being either proliferative or spore stages based on morphology and staining characteristics (i.e., spore stages stain red with the Luna stain).

In situ hybridization

Two oligonucleotide probes previously developed for a real-time PCR based assay (Sanders and Kent 2011) and specific to the *Pseudoloma neurophilia* small subunit ribosomal RNA gene were used: P10F (5' - GTAATCGCGGGCTCACTAAG - 3') and P10R (5' - GCTCGCTCAGCCAAATAAAC - 3'). These oligonucleotides were labeled with digoxigenin (DIG) using the 3' DIG Oligonucleotide Tailing Kit (Roche Applied Science, Indianapolis, IN, USA) following the kit protocol. Tissue sections were deparaffinized by three 10 minute washes in xylene followed by a 3 minute wash in 100% ethanol and rehydration by sequential 3 min washes in progressively lower concentration ethanol solutions (95%, 80%, 70%, 50%) and then 3 min in deionized water. Tissue sections were then washed in Tris-CaCl₃ buffer for 3 min and permeabilized by incubating with proteinase K in Tris-CaCl₃ buffer (50 µg/ml) for 15 min at 37°C. After permeabilization, the sections were washed three times in phosphate buffered saline (PBS) for 10 min each.

Prehybridization was performed by incubating the tissues at 37 °C in hybridization solution (100 µl 20x saline-sodium citrate buffer, 10 µl salmon sperm, 5 mg dextran sulfate, 50x Denhardt's, 250 µl deionized formamide) without the addition of the digoxigenin-labeled probes for two hours. After two hours, the prehybridization solution was poured off and 60

µl of hybridization solution with 500 ng digoxigenin labeled probes was added to each tissue section. The slides were covered with Hybri-Slips (Sigma-Aldrich, St. Louis, MO, USA), denatured for 10 min at 95 °C, and then incubated overnight at 37 °C in a MicroProbe slide heater (Fisher Biotech, Fair Lawn, NJ, USA).

After incubation, stringency washes were performed using two 30 minute washes in 2x saline-sodium citrate (SSC, Sigma-Aldrich, St. Louis, MO, USA) buffer at 37 °C, three 10 minute washes in 1x SSC at 37 °C, and one 10 minute wash in 0.5x SSC at room temperature. Following the stringency washes, the tissue sections were washed in Wash Buffer (Roche Applied Science, Indianapolis, IN, USA) for 10 min at room temperature and then soaked in maleic acid Blocking Buffer (Roche Applied Science, Indianapolis, IN, USA) for one hour at room temperature. The sections were then incubated for two hours with anti-DIG antibody (Roche Applied Science, Indianapolis, IN, USA) diluted 1:1000 in Blocking Buffer at room temperature. The antibody solution was then poured off and the slides were washed twice for 15 min each in Wash Buffer on a shaker. They were then soaked in Detection Buffer (Roche Applied Science, Indianapolis, IN, USA) for 10 min after which they were drained and substrate, nitroblue tetrazolium NBT/BCIP Ready-to-Use Tablets (Roche Applied Science, Indianapolis, IN, USA) dissolved in Detection buffer was added for 1 – 2 hours. After examination that the blue reaction had occurred, the slides were washed twice in deionized water.

Tissue sections were counter-stained using Nuclear Fast Red (Vector Laboratories, Inc, Burlingame, CA, USA) for 5 min, followed by rinsing in deionized water and air drying. The tissue sections were then dehydrated by subsequent washing in 70% ethanol for 3 min, 95% ethanol for 3 min, and two changes of 100% ethanol for 3 min each. Finally, the tissue sections were soaked in two changes of xylene for 3 min each and coverslipped using Cytoseal XYL (Richard Allan Scientific, Kalamazoo, MI, USA) permanent mounting medium.

RESULTS

The chronological sequence of *Pseudoloma neurophilia* progressive infection in the larval zebrafish is presented in Table 1. Occasional intraluminal loose aggregates of individual mature intact spores were observed within the anterior intestine by Luna stain at 12 h post-exposure (hpe), likely reflecting ingestion by larval fish (Fig. 1A). The observation of mature spores within the intestinal lumen declined during the later time points and was no longer observed after 72 hpe. The initial observation of mature spores developing within host tissues was noted at 96 hpe. No parasites were observed in the unexposed negative control fish.

Intestine

Presporogonic proliferative stages of *P. neurophilia* were initially observed in the digestive tract at 12 hpe in both the pharyngeal (Fig. 1B) and intestinal epithelia (Fig. 1C, 1D) by in situ hybridization and Luna stain. Developing spores were localized within the apical cytoplasmic compartment of infected cells and tended to be found in the anterior segment of the intestine. Proliferative stages of the parasite continued to be observed in fish collected at

all later time points; however, mature spore stages were only observed in the pharyngeal epithelium at 96 hours post-exposure (hpe) and in the intestinal epithelium at 120 hpe.

Visceral organs and kidney

Within the liver (Fig. 2A, 2B), intrahepatocytic presporogonic developmental stages of *P. neurophilia* were initially observed at 12 hpe by in situ hybridization (Fig. 2C), followed by the appearance of mature spores at 96 hpe, which were detected by the Luna stain. Beginning at 36 hpe, similar presporogonic proliferative stages of *P. neurophilia* were observed associated with endothelial cells lining an intrapancreatic blood vessel (Fig. 2D) and intracellular mature spores were seen in pancreatic acinar cells of a larval fish at 120 hpe. Presporogonic developmental stages in the kidney were confined to occasional histiocytes within the renal interstitium (Fig. 2E) beginning at 48 hpe.

Muscle and neural

Presporogonic stages of *P. neurophilia* were first observed in the spinal cord, skeletal muscle, and eye at 72 hpe. In the spinal cord, small aggregates of presporogonic stages were distributed among ependymal cells forming the lining of the central canal as highlighted by in situ hybridization (Fig. 3A). Mature spores were observed in the spinal cord 96 hpe. Intrasarcolemmal dense aggregates of proliferative stages were observed within individual myofibers of skeletal muscle (Fig. 2, 3B), with mature spores first observed 96 hpe. Within the extraocular choroid rete, small aggregates of *P. neurophilia* presporogonic stages were observed in the non-vascular stroma immediately adjacent to the retinal pigmented epithelium (Fig. 3C–E) and within the retinal pigmented epithelium, extending into the photoreceptive layer, (Figs. 3F–H) by in situ hybridization and Luna stain. Mature spores were observed in these locations at 120 hpe. Brain neuropil contained both proliferative and mature spore stages (Fig. 3I, 3J), which were observed at 120 hpe only.

DISCUSSION

Determining the mechanisms of initiation of infection and the distribution of parasites within the host in the early stages of microsporidian infections is important to the understanding of systemic microsporidiosis. Using HE and Luna stains in combination with ISH enabled the observation of the earlier, presporogonic stages of *Pseudoloma neurophilia* and its distribution in tissues. Additionally, the use of larval zebrafish enabled us to examine several individual whole animals on a single slide. By performing serial sections, virtually all organs of each animal were examined. This method allows for the comprehensive analysis of the early development and tissue progression of *P. neurophilia* in the larval zebrafish, therefore expanding the understanding of initial infection and parasite distribution and development beyond our previous studies (Kent and Bishop-Stewart 2003; Cali et al. 2012; Sanders et al. 2012).

The following summarizes our understanding of the sequential development of *P. neurophilia*: Spores are ingested and germinate in the anterior intestine. By 12 hpe presporogonic proliferative stages are observed in the intestinal and pharyngeal epithelia, and liver. Beginning at 36 hpe, presporogonic proliferative stages are found in the pancreas,

and shortly thereafter in the kidney. At 72 hpe, presporogonic proliferative stages are first seen in the spinal cord, eye, and skeletal muscle. The first time developed spores are observed is at 96 hpe in the visceral organs, followed shortly thereafter in the CNS and the skeletal muscle.

It is well-recognized that Microsporidia initiate infection of host cells by extrusion of their polar tube and infection of the sporoplasm into host cells (Cali and Takvorian 1999). Following ingestion, spores adhere to gastrointestinal epithelia associated with sulfated glycans (Hayman et al. 2005). Spores may then extrude their polar tube and infect adjacent intestinal cells. Alternatively, spores may be phagocytosed by host cells in the gut, then extrude their polar tube and infect the same host cell (Couzinet et al. 2000). Polar tubes range in length from 50 to over 100 μm , and Cox et al. (1979) proposed a third mechanism; injection of the polar tube through the intestine to more distant tissues.

Pseudoloma neurophilia initially infects the host by ingestion of the infective spore stage, with spores being observed in the gut lumen of exposed larval fish at 3 hpe (Cali et al. 2012). Presporogonic and sporogonic stages can be observed in the skeletal muscle at 4.5 days post-exposure (Cali et al. 2012). That observation was confirmed by the current study in which the first stages observed in skeletal muscle were found at 72 hpe (3 dpe). In addition, we found numerous other tissues that were infected shortly after exposure, notably, the pancreas, liver, and kidney. Infections in these tissues and the intestinal epithelium appeared to occur simultaneously and the first mature spores were observed at 96 hpe, suggesting that autoinfection (i. e., newly developed spores infecting adjacent cells within the host) does not occur at this early stage of the infection. Hence, our study supports the mechanism proposed by Cox et al. (1979), piercing of the intestinal wall by the polar tube to infect distant tissues. The sites of initial parasite development that we observed are within the range of the polar tube, which is greater than 100 μm in length. This indicates that the spore germinates with the apical cap oriented facing the intestinal epithelium, firing the polar tube and acting as a syringe to penetrate the intestinal wall and infect distant tissues, such as the liver or pancreas, and injects the sporoplasm at these sites. Indeed, far more developing parasites were observed in the liver, kidney, and pancreas during early stages of infection, rather than within the intestinal tissues.

Hayman and colleagues (2005) showed that *Encephalitozoon intestinalis* spores bind to sulfated glycans on the surface of host cells and that this adherence was important to the infectivity of those spores. There is some evidence to support this, such as the specificity of germination triggers possessed by different species of the Microsporidia. The tissue tropism of a particular microsporidian species could be controlled by the environmental cue for germination (usually in the gastrointestinal tract), resulting in a spore firing only when this cue is sensed. The exact trigger for *P. neurophilia* is unknown and we have never observed firing of the polar tube except when spores are treated with a highly alkaline, chitin binding stain (Fungi-Fluor) and exposed to the UV light of a fluorescence microscope (Ferguson et al. 2007), a situation not encountered within live zebrafish tissues. The tight control of spore germination by a mechanism such as adhesion to host surface factors would prevent or limit unsuccessful infections by spores.

In an in vitro study of the early development of the microsporidium, *Anncalia algerae*, in rabbit kidney cells, Takvorian et al. (2005) did not observe mature spore formation in 48 hpe cells, but they did observe intracellular sporoplasms and early stages of the parasite in cell cultures incubated for up to 48 h, suggesting that these were new infections. They attributed these new infections observed several hours post inoculation to delayed spore germination and suggested that delayed spore activation was possibly an adaptation, allowing a population of parasites to infect various sites of the host (Takvorian et al. 2005). As this observation was made in cultured cells, this is likely true in their study. Although we observed presporogonic stages several days after the initial exposure, these were in tissues distant from the intestinal epithelium. There could be a number of mechanisms responsible for this observation, such as the transport of the parasite within a motile host cell (e.g., a macrophage), or the piercing of the intestinal epithelium by the polar tube of the parasite and the injection of the sporoplasm directly into the blood or the cytoplasm of the host cell in which the earliest stages of the parasite were observed.

As the name implies, *P. neurophilia*, is most often found in the neural tissue, mainly the ventral nerve root ganglia, metencephalon and myelencephalon (together comprising the hindbrain) of chronically infected zebrafish. Kent and Bishop-Stewart (2003) performed a histological survey of the tissue distribution of *P. neurophilia* in adult zebrafish and compared the distribution between subclinical and clinically infected fish. Using a chitin-specific fluorescent stain, Fungi-Fluor, they were able to increase the sensitivity of detection of the spore stage of the parasite in tissue sections over the use of the standard HE stain. Peterson and colleagues (Peterson et al. 2011), found that the use of the Luna stain similarly increased the sensitivity of the detection of spores in histological sections without the need for fluorescence microscopy.

Whereas the parasite is seen in the skeletal muscle in the early stages of infection, in chronic infections of ostensibly immunocompetent zebrafish hosts, *P. neurophilia* is generally isolated in immune-privileged sites such as the spinal cord, hindbrain, and developing ova (Matthews et al. 2001; Sanders et al. 2012). We observed *P. neurophilia* proliferative stages in the spinal cord and eye as early as 72 hpe and in the brain at 120 hpe. Therefore, a logical explanation for changes in parasite distribution over time is that the parasite initially infects, and even sporulates, in various organs throughout the fish in early infections. Then the parasite only persists in presumably immunologically privileged sites such as the CNS and ova, ostensibly due to effective host immune responses controlling the parasites in other tissues.

The observation of *P. neurophilia* developing in the choroid rete and pigmented retinal epithelia of the eyes of several zebrafish is a heretofore unreported site of infection for *P. neurophilia*. Other microsporidian species infecting humans have been documented to cause ocular infections (Friedberg and Ritterband 1999). In immunocompetent patients, these infections generally occur deep in the corneal stroma, occasionally associated with prior trauma, and are not associated with systemic microsporidiosis (Weber et al. 1994). In immunosuppressed patients, while infections are generally limited to the superficial epithelium of the cornea, they are often associated with systemic infection (Weber et al. 1994).

The exact mechanism of the movement of *P. neurophilia* within the body of the zebrafish after initiation is still not completely elucidated, as was the case with experimental infection studies with other systemic microsporidia (Cox et al. 1979). Sanchez et al. (2001) used in situ hybridization to describe the development of the microsporidium, *Loma salmonae* in the Atlantic salmon, *Salmo salar*, and found that shortly after infection, which begins in the intestinal epithelium, presporogonic proliferative stages can be seen within the intertrabecular spaces of the ventricular spongy myocardium of the heart and along the endocardial lining of the ventricular trabeculae at 2 dpe. Consistent with our findings, Sanchez et al. (2001) first observed proliferative stages of *Loma salmonae* in the intestinal epithelium at 12 hpe. Whereas we observed mature (Luna-positive) spores of *P. neurophilia* in various tissues as early as 96 hpe, the first spores of *L. salmonae* were observed at 4 wk post exposure and localized to the gills (Rodríguez-Tovar et al. 2003). The authors hypothesized that the parasite moved from the intestinal epithelium to the heart by infecting mobile leukocytes, such as monocytes. As the endocardial cells in the heart function as phagocytic cells (i.e., macrophages) in teleost fishes, it is possible that these cells are “grabbing” and sequestering the parasite as it enters systemic circulation. Both *Loma* and *Pseudoloma* have been observed within macrophages, adding support to this hypothesis. The use of real-time live imaging of an infection of a larval zebrafish by labeled *P. neurophilia* would likely enable us to definitively determine the mode of transport. Unfortunately, current lack of tools to produce transgenic microsporidia prevents this type of experiment.

In conclusion, we expanded our understanding of the early development, organ distribution, timing and location of sporulation of *P. neurophilia* in larval zebrafish. Most notably we observed first sporulation concurrently in the visceral organs and the CNS, whereas the latter has been previously considered the primary site of infection. Additionally, we have observed for the first time the parasite developing in the choroid rete and pigmented retinal epithelium of the eye. The retina is an extension of the central nervous system, thus is consistent with the neurotropism of this microsporidium. Both larval and post-larval fish are susceptible to natural transmission of the parasite (Kent and Bishop-Stewart 2003; Ferguson et al. 2007; Sanders et al. 2013), including maternal transmission to embryos and fry (Sanders et al. 2013). In chronic infections of older fish, *P. neurophilia* is most commonly found in immune-privileged sites such as the spinal cord, nerve root ganglia, hindbrain, and occasionally developing ova. The eye could be another target site for latent infection. A comparison of our findings in larval zebrafish to the early stages of progression and development of *P. neurophilia* in juvenile or adult fish is warranted.

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Figure 1.

Early stages of *Pseudoloma neurophilia* infection in the intestinal tissue of larval zebrafish at 12 – 72 h post-exposure. Bars = 10 μ m. **A.** Luna-stained section of a larval fish after 12 h post-exposure to *P. neurophilia*. Several individual mature intact spores (red) are visible in the lumen of the anterior intestine. **B.** Section of a larval fish after 36 h post-exposure to *P. neurophilia* stained using an in situ hybridization probe (ISH) specific to *P. neurophilia*. Presporogonic proliferative stages are visible (arrow) developing in the pharyngeal epithelium. **C.** ISH stained section of a larval fish after 48 h post-exposure. A single proliferative stage is visible (arrow) in the cytoplasm of an intestinal epithelial cell. **D.** Hematoxylin and eosin stained section of a larval zebrafish at 72 h post-exposure. Presporogonic proliferative stages in the cytoplasm of an intestinal epithelial cell (arrow).

Figure 2.

Early stages of *Pseudoloma neurophilia* infection in extraintestinal organs of larval zebrafish. Bars = 10 μ m. **A.** Hematoxylin and eosin stained section showing the liver of a larval zebrafish at 72 h post-exposure. Nucleated erythrocytes (e), hepatocyte nuclei (h) and a capillary (c) can be observed. **B.** High magnification of the boxed area in **A**. A cluster of presporogonic proliferative stages (arrow) can be seen developing in a hepatocyte. Note the proximity to a capillary (c). **C.** Section of a larval zebrafish at 72 h post-exposure, stained with an in situ hybridization (ISH) probe specific to *P. neurophilia*. Presporogonic proliferative stages (arrow) developing within a hepatocyte. **D.** ISH stained section of a larval zebrafish at 72 h post-exposure. Three presporogonic proliferative stages (arrows) can be seen associated with endothelial cells lining an intrapancreatic blood

vessel. **E.** ISH stained section of a larval zebrafish at 72 h post-exposure. A single presporogonic proliferative stage (arrow) can be seen developing within the cytoplasm of a kidney histiocyte.

Figure 3.

Early stages of *Pseudoloma neurophilia* in neural tissues of larval zebrafish. Bars = 10 μ m. **A.** Section of a larval zebrafish at 120 h post-exposure stained with an in situ hybridization (ISH) probe specific to *P. neurophilia*. Presporogonic proliferative stages (blue) and spores (arrow) developing among ependymal cells lining the central canal of the spinal cord. **B.** ISH stained section of a larval zebrafish at 120 h post-exposure. A dense aggregate of proliferative stages (blue) developing within an individual myofiber. **C–H.** Serial sections of an individual larval zebrafish with *P. neurophilia* infection of the retina. **C.** ISH stained section of a larval zebrafish at 72 h post-exposure. Proliferative stages and spores (arrow) within the extraocular choroid rete adjacent to the retinal pigmented epithelium. **D.** Adjacent section of the previous fish stained with the Luna stain. Red

staining mature spores (arrow) are more apparent within the extraocular choroid rete. **E.** Adjacent section of the previous fish stained with hematoxylin and eosin (HE). Mature spores (arrow) are faintly visible in the extraocular choroid rete. **F.** Adjacent section of the previous fish stained with ISH. Proliferative stages are visible (arrow) in the retinal pigmented epithelium extending into the photoreceptive layer. **G.** Adjacent section of the previous fish stained with the Luna stain. Red-staining mature spores (arrow) are visible in the retinal pigmented epithelium. **H.** Adjacent section of the previous fish stained with HE. No presporogonic nor spore stages are visible. **I.** ISH stained section of a larval zebrafish at 120 h post-exposure. A proliferative stage (arrow) developing within the brain neuropil. **J.** An HE stained section of a larval zebrafish 120 h post-exposure. A cluster of proliferative stages (arrow) within the brain neuropil.

Results of histological examination of larval zebrafish at various times post exposure to *Pseudoloma neurophilia*

Larval zebrafish were exposed to *P. neurophilia* spores and collected at 12–120 h post exposure. Numbers represent total individual fish in which the parasite was detected by either in situ hybridization, hematoxylin and eosin, or Luna stained histological sections. Spore stages of the parasite were determined based on the presence of Luna-positive (red) staining structures with morphology consistent with spores of *P. neurophilia*. HPE = hours post exposure, P = presporogonic proliferative stages, S = spore stages.

HPE	Total fish examined	Intestinal lumen		Intestinal epithelium		Pharynx		Liver		Pancreas		Kidney		Spinal Cord		Eye		Muscle		Brain	
		P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S
12	28	0	20	6	0	3	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
24	28	0	9	4	0	2	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
36	28	0	6	8	0	5	0	4	0	4	0	0	0	0	0	0	0	0	0	0	0
48	28	0	3	12	0	9	0	12	0	0	0	2	0	0	0	0	0	0	0	0	0
72	28	0	1	11	0	13	0	17	0	6	0	4	0	1	0	3	0	8	0	0	0
96	21	0	0	2	0	3	2	3	2	2	0	0	0	1	1	0	0	1	1	0	0
120	22	0	0	8	2	4	1	15	7	7	1	13	0	7	3	12	9	17	14	4	4