1 Microsporidiosis in zebrafish research facilities

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3 Justin L. Sanders*, Virginia Watral, Michael L. Kent

4 *Justin.Sanders@oregonstate.edu

5 Justin L. Sanders, B.S., Department of Microbiology, Oregon State University, Corvallis, OR

6 Virginia Watral, B.S., Faculty Research Assistant, Kent Laboratory, Department of

7 Microbiology, Oregon State University, Corvallis, OR

8 Michael L. Kent, PhD, Professor, Department of Microbiology, Biomedical Sciences, Oregon

9 State University, Corvallis, OR

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11 ## Abstract

12 *Pseudoloma neurophilia* (Microsporidia) is the most common pathogen detected in zebrafish
13 (*Danio rerio*) from research facilities. The parasite infects the central nervous system and
14 muscle, and may be associated with emaciation and skeletal deformities. However, many fish
15 exhibit subclinical infections. Another microsporidium, *Pleistophora hyphessobryconis,* has
16 recently been detected in a few zebrafish facilities. Here we review the methods for diagnosis
17 and detection, modes of transmission, and approaches used to control microsporidia in zebrafish,
18 focusing on *P. neurophilia*. The parasite can be readily transmitted by feeding spores or infected
19 tissues, and we showed that cohabitation with infected fish is also an effective means of
20 transmission. Spores are released from live fish at various points, including the urine, feces, and
21 sex products during spawning. Indeed, *P. neurophilia* infects both the eggs and ovarian tissues,
22 where we found concentrations ranging from (12,000 – 88,000 spores/ovary). Hence, various
23 lines of evidence support the conclusion that maternal transmission is a route of infection: spores
24 are numerous in ovaries and developing follicles in infected females, spores are present in
25 spawned eggs and water from spawning tanks based on PCR tests, and larvae are very
26 susceptible to the infection. Furthermore, egg surface disinfectants presently used in zebrafish
27 laboratories are ineffective against microsporidian spores. At this time, the most effective
28 method for prevention of these parasites is avoidance.

29 Key Words: *Danio rerio*, Microsporidia, *Pseudoloma neurophilia*, *Pleistophora hyphessobryconis*,
30 zebrafish

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Introduction

The dramatic increase in the use of zebrafish (*Danio rerio*) in biomedical research has led to a corresponding increased interest in the diseases affecting this important biological model. Many of the laboratory animal health and pathogen control principles developed for mice and rats are applicable to aquatic laboratory animals such as the zebrafish, however, there are special considerations in working with aquatic animals. Kent et al. (2009) provided a general review of the control of diseases in fish research colonies. The present review focuses specifically on the transmission and control of microsporidia in zebrafish facilities. We emphasize particularly *Pseudoloma neurophilia* as this microsporidium is very common in these fish (Murray 2011), and provide a discussion on *Pleistophora hyphessobryconis*, which was recently detected in a few facilities (Sanders et al. 2010).

Microsporidia

Microsporidia are obligate intracellular eukaryotic parasites with species infecting virtually all animal phyla. They have a relatively simple life cycle, consisting of two general developmental stages; mergony and sporogony. Meronts multiply inside the infected host cell, eventually forming sporonts and then spores, which are ultimately released from the host and transmit the infection. The infectious spore stage has a thick, chitinous endospore, making it extremely resistant to environmental stress and lysis, allowing the organism to maintain viability for extended periods in the aquatic environment (Shaw et al. 2000). Additionally, microsporidia are generally resistant to many standard forms of surface decontamination used for fish eggs such as chlorine and iodophores, complicating the control of these pathogens.

Microsporidia are common pathogens of numerous aquatic organisms including

55 crustaceans, amphipods, and members from some 18 genera of these parasites have been
56 described in fishes (Lom 2002; Lom and Nilsen 2003). The impacts of microsporidian infections
57 on fish populations in the wild, aquaculture, and laboratory have been documented in numerous
58 cases (reviewed in Shaw and Kent 1999). These often focus on the more acute effects of
59 microsporidian disease such as mortality, however, most microsporidian species infecting
60 aquatic animals result in chronic diseases with minimal associated host mortality (Murray et al
61 2011).

62 *Pseudoloma neurophilia*

63 *Pseudoloma neurophilia* was first reported by de Kinkelin (1980) in fish purchased from a pet
64 store for use in toxicological studies. The parasite was further described and assigned to a new
65 genus, *Pseudoloma neurophilia,* by Matthews et al (2001). *Pseudoloma neurophilia* is the most
66 commonly observed microsporidian parasite of zebrafish. For example, the infection was
67 detected in greater in 74% of the facilities examined through the Zebrafish International
68 Resource Center (ZIRC) diagnostic service in 2010 (Murray et al. 2011). It generally causes
69 chronic infections in zebrafish with clinical signs ranging from emaciation and obvious spinal
70 deformities (lordosis, scoliosis) to subclinical infections exhibiting no outward signs of disease
71 (Matthews et al. 2001). As with other animals used in research, experiments utilizing zebrafish
72 with these infections may be subject to non-experimental variation, potentially confounding
73 results as has been described in laboratory colonies of rabbits and mice infected with the
74 microsporidian parasite, *Encephalitozoon cuniculi* (Baker 2003). Furthermore, infected fish
75 without overt clinical disease have been shown to have reduced fecundity and size (Ramsay et al.
76 2009).

Pleistophora hyphessobryconis

The muscle-infecting microsporidium, *Pleistophora hyphessobryconis*, has also been observed and described in laboratory populations of zebrafish (Sanders et al. 2010). Commonly known as "neon tetra disease" for its type host, the neon tetra, *Paracheirodon innesi*, this parasite is very common in the aquarium trade, often resulting in considerable mortality. This microsporidium has been described in a broad range of fish hosts, and has been reported from many species of aquarium fishes in several families, including *Danio rerio* and *D. nigrofasciatus* (Steffens 1962). Similar to *Pseudoloma neurophilia*, *P. hyphessobryconis* can also be harbored by otherwise healthy appearing fish, which may show clinical signs of the infection or mortality after experiencing experimental or incidental immunosuppression (Sanders et al. 2010). The presence of *P. hyphessobryconis* infections in laboratory zebrafish colonies highlights the importance of obtaining fish used in research from reputable sources and also illustrates the potential for introduction of otherwise novel microsporidia with a broad host range to new hosts.

Current Methods of Detection

External Indicators of Infection

External indications of zebrafish infected by *P. neurophilia* include reduced growth, emaciation, spinal deformation (e.g. lordosis, scoliosis), or low-level mortalities with no grossly-visible lesions. Typically, indicators of infection and mortality become apparent only after a stress event (Ramsay et al. 2009), such as crowding or shipping. These general clinical presentations are not pathognomonic for *P. neurophilia*, making external examination of fish alone of little use in the diagnosis of this infection.

The skeletal muscle infecting microsporidium, *Pleistophora hyphessobryconis*, can also be harbored by otherwise healthy appearing fish. Similar to fish infected with *P. neurophilia*, immunosuppression by various means can result in acute infection, with affected fish displaying large, depigmented regions localized around the dorsal fin. Fish presenting severe signs of *P. hyphessobryconis* eventually die from the infection.

Microscopy

Microsporidian spores can often be seen in wet mount preparations from infected tissues. They are discernable by their generally refractile appearance and characteristic posterior vacuole. In suspected cases of infection by *P. neurophilia,* posterior brain and spinal cord tissue can be examined by wet mount for the presence of spores which are about 3 by 5 µm in size and pyriform in shape (Figure 1a). Wet mount preparations of tissue from opaque lesions present in the skeletal muscle can be examined for the presence of *P. hyphessobryconis* spores, which are 4 by 6-7 µm in size, also pyriform in shape and possess a very prominent posterior vacuole (Figure 1b).

In general, microsporidian spores can be readily detected in standard hematoxylin and eosin (H&E) stained tissue sections when they occur in aggregates. However, in light infections, when only single spores are, present within areas of inflammation, detection by H&E is difficult. Microsporidian spores appear Gram positive in Gram stains (Figures 2a, 2b, 2c, 2g) and are generally acid fast in various acid-fast staining methods (Figure 2e). The acid fast character of the spores can be variable depending upon the amount of decolorization. In cases where microsporidian infection is suspected, special stains such as the Luna stain or periodic acid Schiff (PAS) can greatly increase the visibility of spores allowing greater sensitivity of detection by histology (Peterson et al. 2011). Chitin specific fluorescent stains such as Fungi-Fluor

121 (Polysciences, Warrington, PA) also increase the sensitivity of spore detection by histology but
122 require the use of a fluorescence microscope (Kent and Bishop-Stewart 2003).

123 With *Pseudoloma neurophilia*, large aggregates of spores are primarily found in the
124 neural tissue of the posterior brain and spinal cord. Smaller groups or individual spores can also
125 be seen in the kidney, skeletal muscle, gut epithelium, and ovary (Kent and Bishop-Stewart
126 2003), or within developing follicles (Figure 2). Spores of *P. neurophilia* released from
127 aggregates within myocytes or peripheral nerves in the somatic muscle typically elicit a severe
128 inflammatory reaction (Ramsay et al. 2009).

129 In contrast, the muscle is the primary site of infection for *Pleistophora*
130 *hyphessobryconis*. Massive infection by proliferative stages and spores occupy the myocyte, with
131 inflammatory changes occurring after infections become so severe that the myocytes rupture.
132 Spores of this parasite can also be observed in the kidney, spleen, intestine, and ovaries in
133 heavier infections (Sanders et al. 2010).

134 Molecular Diagnostics

135 Conventional PCR (Whipps and Kent 2006; Murray et al. 2011) and qPCR-based (Sanders and
136 Kent 2011) assays targeting unique portions of the small subunit ribosomal DNA (ssrDNA) gene
137 are available for testing of zebrafish tissues for *P. neurophilia*. The qPCR assay of Sanders and
138 Kent, in combination with sonication, has also been applied to detect *P. neurophilia* ssrDNA in
139 water, sperm, and eggs, providing a potential non-lethal assay for screening populations of fish
140 for this parasite. As with most PCR-based assays, these tests are very sensitive and provide a
141 relatively fast method of screening for the presence of *P. neurophilia* in zebrafish. No PCR-

142 based assays currently exist for the specific detection of *P. hyphessobryconis,* but this is a
143 potential target for future studies.

144 Transmission

145 In order to control the spread of a pathogen in a population, it is important to understand its
146 mode or modes of transmission. In general, microsporidia infecting fish are transmitted directly,
147 presumably *per os* via ingestion of infected tissues or spores present in the water (Dyková and
148 Woo 1995; Shaw and Kent 1999). The two microsporidia thus far described in zebrafish, *P.*
149 *neurophilia* and *P. hyphessobryconis,* have been shown to infect fish by this method by
150 experimental exposure (Kent and Bishop-Stewart 2003; Sanders et al. 2010). Thus removal of
151 dead and moribund fish would be expected to limit the potential exposure of tank mates to these
152 two parasites.
153 Murray et al. (2011) reported spread of the parasite within a tank from < 6% to 77%
154 prevalence over one year. They also showed that detritus from positive tanks place in tanks
155 containing parasite-free fish could spread the infection. We have found that live, infected fish
156 transmit *P. neurophilia* by shedding it in the water, infecting recipient fish held in the same water
157 but separated from each other by a screen cage. Five flow through cohabitation tanks were set up
158 using infected "donor" fish segregated within a suspended breeding cage with a screen bottom
159 placed in the same tank with uninfected recipient zebrafish obtained from the *P. neurophilia*
160 specific pathogen free colony housed at the Sinnhuber Aquatic Research Laboratory (SARL) at
161 Oregon State University (Kent et al. 2011). One control tank was set up which consisted of both
162 recipient and donor fish from the negative fish stock. After 2 months of cohabitation, donor fish
163 were removed and posterior brains and spinal cords were examined by wet mount for the
164 presence of *P. neurophilia.* The overall prevalence of *P. neurophilia* in the donor fish was 81%

165 with no spores detected in the 10 negative control donor fish. Histological examination of the
166 donor fish revealed that 3 experimental tanks, and the negative control tank, contained both male
167 and female donor fish, while a fourth experimental tank contained all males except one
168 immature female. After an additional 2 months, the recipient fish were euthanized and examined
169 by histology to determine infection status. Recipient fish from all positive tanks were infected,
170 with an overall incidence of 66%. No infection was detected in the negative controls. Tank 4,
171 which contained no sexually mature female donor fish, showed 57% incidence of infection.

172 These results provide evidence that *P. neurophilia* is shed by live infected fish, and
173 illustrate the route by which the parasite can spread throughout a population of fish in a single
174 tank. This finding is consistent with other reports that *Loma salmonae*, a microsporidian parasite
175 of salmonids, is similarly transmitted to tank mates by cohabitation (Shaw et al. 1998, Ramsey et
176 al. 2003). The potential routes by which *P. neurophilia* may be transmitted by live, infected fish
177 become apparent by observing the tissue distribution of the parasite. While *P. neurophilia*
178 primarily targets neural and muscle tissue, we occasionally observe spores in the gut epithelium
179 (Figure 2f) and in the kidney tubules (Figure 2g), each of these tissues providing a portal through
180 which infectious spores can be shed into the water through feces or urine. Additionally, Kent and
181 Bishop-Stewart (2003) reported the frequent occurrence of spores in the ovarian stroma (Figure
182 2b) and since that report we have also detected spores within developing follicles (Figures 2a, 2c,
183 2d, 2e), supporting maternal transmission during spawning as another likely route of infection.

184 It is difficult to quantify microsporidian spores in histological sections, and thus entire
185 ovaries from females from nine separate infected populations were surveyed to more precisely
186 determine the concentration of *P. neurophilia* (unpublished observations). Ovaries of 10 fish
187 from each population were pooled, homogenized, and a sample of spores counted by

188 hemocytometer. The average number of *P. neurophilia* spores seen was 44,000 per fish (range
189 12,000 – 88,000). Zebrafish frequently spawn spontaneously in aquaria, and hence release of
190 eggs, ovarian fluids, and tissues at spawning provides an important potential route of horizontal
191 transmission. However, the fact that recipient fish were positive from the tank in which donor
192 fish had no sexually mature females suggests that spores are also released from infected fish by
193 routes other than spawning. Observation of spores in the renal tubules and the intestinal
194 epithelium (Figure 2f, 2g) supports this hypothesis.

195 Sex products not only provide an important source of infection to tank mates of the same
196 age cohort, but also a source of infection to progeny by maternal transmission. Indeed, this route
197 of infection has been reported for other microsporidia of fishes. The potential for maternal
198 transmission, either transovum or transovarial, has been reported for *Loma salmonae* (Docker et
199 al. 1997), and *Ovipleistophora ovariae* (Phelps & Goodwin 2008). Phelps & Goodwin (2008)
200 provided the most conclusive evidence for vertical transmission of fish microsporidia, showing
201 the presence of the DNA from *Ovipleistophora ovariae* within spawned eggs of the golden
202 shiner *Notropis chrysoleucas* by qPCR. Further evidence for the maternal transmission of *P.*
203 *neurophilia* was observed in the experiment described by Sanders and Kent (2011), where
204 parasite DNA was detected in the eggs and water from a group spawn of infected zebrafish. We
205 have tested the spawn water and eggs of several other groups of fish, and consistently found PCR
206 positive water and eggs (unpublished observations).

207 There are other experimental and observational lines of evidence that suggest maternal
208 transmission of *P. neurophilia*, either transovarial (pseudovertical, outside of the egg or sperm)
209 or transova (true vertical, within the egg or sperm). Evidence of true vertical transmission of *P.*
210 *neurophilia* was observed in a follow-up experiment performed from a laboratory study

211 described by Ramsey et al. (2009). Six week old zebrafish (AB strain), obtained from the ZIRC
212 were experimentally exposed to *P. neurophilia* spores at 10,000 spores/fish. At 8 weeks post-
213 exposure, six pairs of fish were separately spawned and the embryos reared in individual covered
214 beakers in sterile water. Three pairs of unexposed fish were spawned separately as a negative
215 control with the progeny reared under identical conditions. After spawning, all adult fish were
216 processed for histology and slides were stained using the Kinyoun acid fast method to determine
217 infection status and tissue distribution of the parasite (Ramsay et al. 2009). At 8 weeks post-
218 hatch, juvenile fish were euthanized, viscera removed, and the remaining tissues (spinal cord,
219 somatic muscle, head) were placed in pools of five fish and DNA extracted for PCR analysis
220 using the method of Whipps and Kent (2006). *Pseudoloma neurophilia* was detected in 2 of 3
221 pools of fry from one spawning pair. Histological analysis of the adult pairs showed the presence
222 of microsporidian spores in the spinal cord, ovary, and most importantly in developing follicles
223 of the spawning female (Fig 2e). As these fry were raised in isolation from the original spawning
224 pair and the parasite was seen developing in eggs from the female, there is evidence that the
225 infection was transmitted vertically, either by infection of the eggs prior to fertilization or by the
226 exposure of the larval fish to spores present in high numbers in eggs which did not develop
227 further. However, as *P. neurophilia* spores were also observed in the ovarian stroma, transovarial
228 transmission (i.e. via spores outside of eggs) cannot be excluded.

229 There is limited evidence currently available for the potential for maternal transmission
230 of *Pleistophora hyphessobryconis*. Schäperclaus (1941) found infections in 8 day old neon tetras
231 which had been derived from infected parents, suggesting the possibility of maternal
232 transmission. We observed spores of this microsporidium in the ovarian tissue of infected
233 females (Sanders et al. 2010), but no spores were seen in developing follicles in this study. The

234 low prevalence of this parasite in laboratory zebrafish colonies would seem to minimize the
235 importance of this mode of transmission for *P. hyphessobryconis*.

236 Parasite Surveillance

237 Routine monitoring

238 Routine disease and pathogen monitoring is important not only in the control of microsporidian
239 parasites, but also for the detection of other pathogens as well as in the monitoring of the overall
240 health of the colony (Kent et al. 2009). It is only through routine monitoring of healthy, as well
241 as moribund fish, that colony managers can detect potential health problems in fish. No
242 serological tests are presently available for zebrafish. Histological analysis is the best overall
243 method for routine health monitoring of zebrafish due to the ability to assess all tissues and to
244 detect novel pathogens which would not be detected by specific PCR-based assays. Screening of
245 fish in specific tanks by PCR to determine the presence or prevalence of *P. neurophilia* is also
246 recommended, however, careful consideration of sample size is required to ensure the statistical
247 relevance of these data (Kent et al. 2009, 2011).

248 Sentinel program

249 The use of a sentinel program is a very effective means to monitor microsporidian infections in
250 laboratory colonies. Exposing a population of known uninfected fish to the untreated effluent
251 from other tanks on the system allows facility managers to assess the infection status of fish in
252 the system on a large scale. For the monitoring of chronic microsporidian infections such as
253 *Pseudoloma neurophilia* it is recommended that sentinel fish be held at least 3 months prior to
254 sampling (Kent et al. 2009). The presence of *P. neurophilia* or other microsporidian parasites in

255 the sentinel fish is an indication that infected fish are present somewhere in the facility.
256 Ultraviolet sterilization is a common feature in recirculating water systems. It is useful to hold a
257 sentinel population exposed to effluent post UV treatment in order to assess the efficacy of the
258 filtration and disinfection of effluent water.

259 Facility Design Considerations

260 Receiving fish into the facility/quarantine

261 The practice of "eggs only" movement of fish between facilities has been successfully used for
262 years in salmonid aquaculture to exclude pathogens from salmon facilities (Kent and Kieser
263 2003). It is recommended that fish received in a facility as embryos be held in quarantine
264 isolation and a subset examined before introduction into the main facility. Also, if possible, the
265 parents of these fish should be examined for pathogens that may be maternally transmitted (e.g.,
266 *P. neurophilia*). It is recommended that the quarantine area be physically separated from the
267 main housing area, with restrictions on staff entering the main facility from the quarantine area.
268 After determining that the brood stock is not infected with a microsporidian parasite, the progeny
269 may be moved into the main facility. The short generation time of zebrafish facilitates this
270 process greatly, allowing managers to bring adults into quarantine, spawn them, and then move
271 only the progeny of those adults which are screened and determined to be microsporidian free
272 into the main facility. This approach was used to establish a specific pathogen free (SPF) for *P.*
273 *neurophilia* zebrafish laboratory at Oregon State University (Kent et al. 2011). Now two wild
274 type lines of these fish are available to the research community through the Sinhuber Aquatic
275 Research Laboratory at Oregon State University (spf fish order@zebrafish.org).

276 Separation of tanks within the main facility

277 The separation of tanks in the main facility is very important in the control of microsporidia. As
278 microsporidian spores are transmitted by water and horizontally by infected fish, splashes and
279 mixing of fish in tanks may result in the spread of these parasites throughout the facility. In fact,
280 we have observed the spread of *P. neurophilia* from a single tank of infected fish to other fish in
281 separate tanks housed in the same unit in which the effluent water was discharged into an open
282 tray and frequently splashed (unpublished observations). We have also seen *P. hyphessobryconis*
283 transmitted in a similar way to fish housed on the same rack as infected fish (Sanders et al.
284 2010). The transmission of another aquatic parasite, *Ichthyophthirius multifiliis,* between tanks via
285 aerosolization of water in a laboratory has also been demonstrated (Wooster et al. 2001). Thus,
286 covering of tanks and minimizing splashing of effluent is key to controlling the spread of
287 microsporidiosis as is the isolation of tanks with known infected fish from those which are
288 microsporidian free or of unknown infection status.

289 UV sterilization of water in recirculating systems

290 Ultraviolet (UV) sterilization of municipal drinking water has been used for several years to
291 inactivate protozoan pathogens such as *Cryptosporidium* and *Giardia*. These systems have also
292 been shown to be effective in the inactivation of microsporidian parasites of human health
293 concern such as *Encephalitozoon intestinalis* at a dose of 6 mJ/cm^2 (Huffman et al. 2002). The
294 effectiveness of UV sterilization is highly dependent upon proper prefiltration of incoming water
295 to remove particulates,cleaning the quartz sheath that the UV bulb is inserted into, and the
296 replacement of UV bulbs at regular intervals. As stated previously, it is important to maintain a
297 group of sentinel fish downstream of the UV treatment in order to assess its efficacy.

298 Husbandry Considerations

299 ## Egg disinfection

300 The purpose of egg disinfection is to kill pathogens which are present on the surface of the eggs,
301 preventing their spread to progeny and potentially other fish in the facility. This method had been
302 successful in the control of many pathogens in salmon aquaculture (Kent and Kieser 2003). For
303 zebrafish eggs, bath treatment with 25 to 50 ppm sodium hyphochlorite for 10 min is generally
304 the method recommended for disinfection (Harper and Lawrence 2010). Unfortunately, this level
305 of bleach is ineffective at killing *P. neurophilia* (Ferguson et al. 2007). A similar situation can be
306 seen with the disinfection procedures for salmonid eggs in which, the iodine treatments used
307 were shown to be ineffective at eliminating 100% of spores of *Loma salmonae*, even at very high
308 levels of iodine (Shaw et al. 1999). Therefore, microsporidian spores are highly resistant to
309 current methods of surface sterilization of eggs and these methods cannot be relied upon to
310 eliminate *P. neurophilia* or other microsporidia from a population, nor can it be relied upon to
311 effectively prevent the spread of microsporidian parasites between fish colonies. Further
312 compounding this problem is the potential for transmission of the parasite within eggs.
313 Transovum (true vertical transmission) of this parasite would prevent the efficacy of any surface
314 decontamination of eggs for *P. neurophilia,* thus requiring careful screening of fish and the use
315 of SPF fish stocks to prevent the spread or introduction of the parasite.

316 ## Screening of sperm, eggs, larval fish

317 Current molecular diagnostic methods can easily be applied to the testing of eggs, sperm and
318 larval fish. In fact, the method of Whipps and Kent (2008) was used to screen eggs and larval
319 fish in the development of a *P. neurophilia* specific-pathogen free zebrafish colony at Oregon
320 State University (Kent et al. 2011). The qPCR method of Sanders and Kent (2011) was shown to

321 be effective in testing sperm and eggs with a sensitivity of 10 spores per μl and 2 spores per egg,
322 respectively. The cryopreservation of zebrafish sperm presents a special problem for preventing
323 the spread of microsporidians. While *P. neurophilia* has not been seen in the testes of fish
324 (Murray et al. 2011), there is the potential for contamination of sperm from the kidneys or gut of
325 the fish during manual stripping. Further compounding this problem is the potential for survival
326 of the parasite during cryopreservation. While the ability of *P. neurophilia* to survive during
327 cryopreservation is unknown, *Nucleospora salmonis*, a microsporidian parasite infecting
328 salmonids, is maintained for long periods by cryopreservation in tissue culture (Wongtavatchai et
329 al. 1994). Also, cryopreserved spores of mammalian microsporidia, which are viable, are readily
330 available from the American Type Culture Collection, Manassas, VA.

331 ## Disinfection of equipment

332 The resistance of infectious microsporidian spores to environmental conditions requires the use
333 of appropriate disinfection procedures to control the spread of these pathogens. Chlorine is
334 commonly used to disinfect tanks and other equipment in zebrafish facilities. Ferguson et al
335 (2007) found that 100 ppm chlorine (pH 7) effectively kills > 95% of *P. neurophilia* spores.
336 Unfortunately, this is lethal for embryos and this is not suitable for egg disinfection. We are not
337 aware of any studies which specifically test the efficacy of chlorine on *Pleistophora*
338 *hyphessobryconis*, but it is likely that it would be killed at similar concentrations.

339 # Other Considerations

340 Several zebrafish lines which are specific pathogen free (SPF) for *Pseudoloma neurophilia* have
341 been developed at the colony housed at the Sinnhuber Aquatic Research Laboratory (SARL)
342 (Kent et al. 2011). The development of these SPF lines was facilitated by the construction of a

343 new fish facility which enabled the introduction of fish only after they were determined to be
344 free of *P. neurophilia*. These fish are rigorously screened in order to maintain their SPF status.
345 Obviously, the control of this parasite in existing facilities is much more complex and requires
346 systematic screening and isolation of zebrafish with known infections in order to eliminate or
347 reduce the presence of *P. neurophilia* infections in the colony (Murray et al. 2011).

348 There are currently no known treatments for microsporidiosis in zebrafish. However,
349 Fumigillin DCH, an agent used to treat the microsporidium *Nosema apis,* in honey bees, has
350 been shown to be effective for several microsporidia infecting fishes (Shaw and Kent 1999).
351 Albendazole and monensin also have some efficacy in the treatment of salmonids for infections
352 by *Loma salmonae* (Speare et al. 1999; 2000). The use of these drugs on experimental fish, while
353 potentially eliminating the pathogen, could also introduce other changes in the host, confounding
354 research (Baker 2003). Toxic effects of Fumigillin DCH have been observed in salmonids
355 (Laurén et al. 1989), thus its utility would be limited to the treatment of fish not used as
356 experimental animals (e.g., brood stock). Ultimately, the elimination of *P. neurophilia* from
357 existing lines of zebrafish may require rederivation of those lines using the methods described by
358 Kent et al. (2011).

359 Conclusion and Recommendations

360 The chronic and often subclinical nature of *P. neurophilia* infections in zebrafish requires the use
361 of rigorous screening methodologies in order to ascertain the true prevalence of this parasite in
362 laboratory zebrafish colonies. Its continued presence in laboratory zebrafish facilities highlights
363 the need for increased surveillance, implementation of biosecurity protocols, and further research
364 into the transmission and control of these pathogens. Future studies to determine the efficacy of
365 decontamination protocols, such as the dosage of UV required to inactivate spores of *P.*

366 *neurophilia* in water and the survivability of the parasite during cryopreservation are needed.
367 Additionally, the potential for introduction of novel microsporidia to zebrafish facilities
368 underscores the need to obtain fish from reputable suppliers who are able to provide a health
369 history of the fish. We also strongly recommend that zebrafish be obtained from suppliers who
370 do not maintain zebrafish with other aquarium fish species. As the treatment of zebrafish with
371 antimicrosporidial drugs may exacerbate impacts on research outcomes, the only effective
372 method of controlling *P. neurophilia* infections in zebrafish is identification and removal of
373 infected fish and avoiding introduction of the parasite by proper quarantine and screening of
374 incoming fish.

375 Whereas methods to avoid the infection and SPF zebrafish are now available, we have
376 seen little enthusiasm for using parasite-free zebrafish by some researchers. This is often due to
377 the perception that subclinical infections have little or no impact on research endpoints (see Kent
378 et al. 2011 this issue). Therefore, another research need is the demonstration of the specific
379 physiological, immunological, molecular, behavioral, etc. changes associated with subclinical
380 infections by this extremely common parasite of zebrafish.

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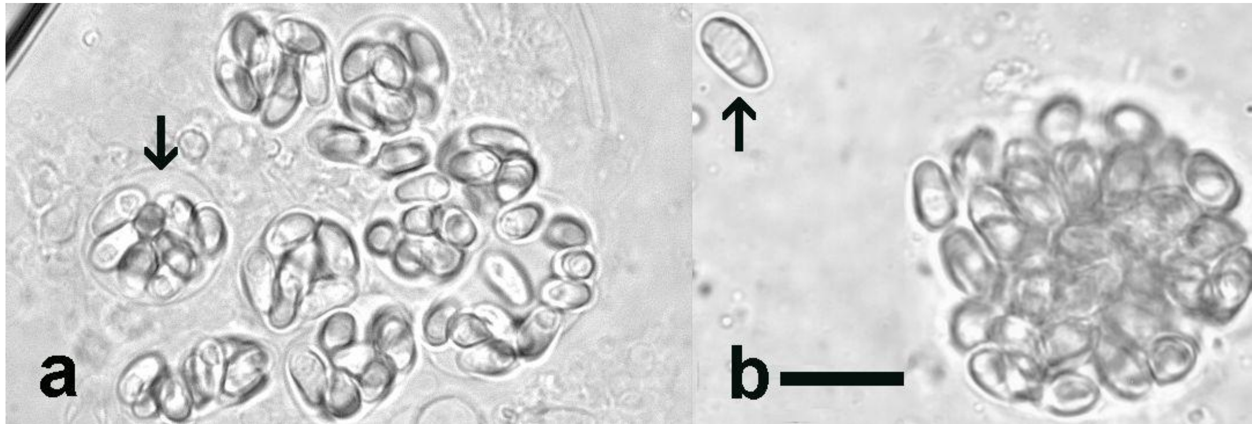
470 **Figures**

471 Figure 1. Wet mounts of microsporidian spores from zebrafish. A) Aggregates of spores of
472 *Pseudoloma neurophilia*, contained within sporophorous vesicles (arrow). B) *Pleistophora*
473 *hyphessobryconis* from the skeletal muscle. Note prominent posterior vacuole in spores (arrow).
474 Bar = 10 µm.



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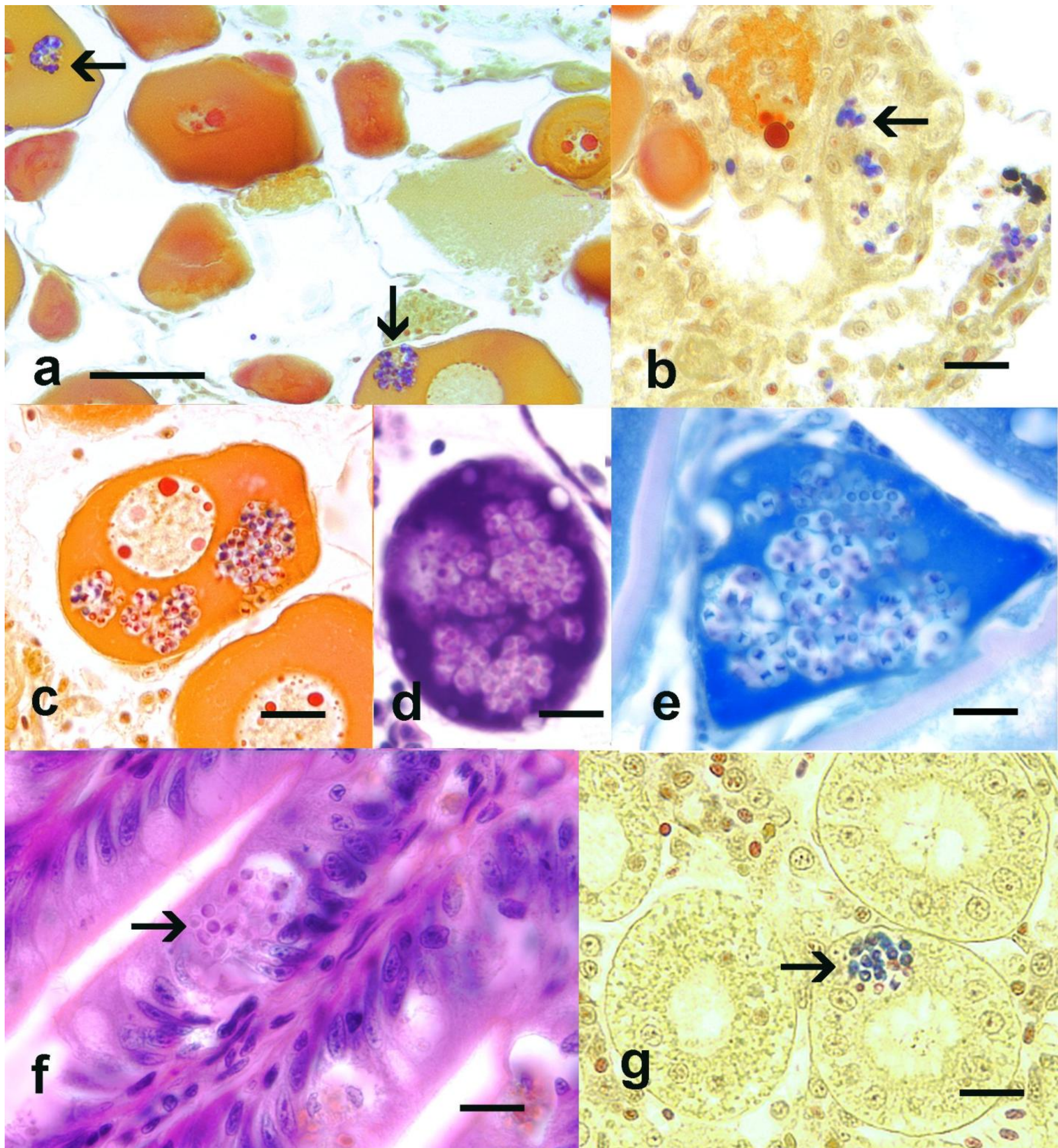
491 Figure 2. Histological sections of ovarian, intestinal and kidney infections of *Pseudoloma*
492 *neurophilia* from zebrafish. Bar = 10 µm unless otherwise indicated. A) Gram-positive (blue)
493 staining spores in follicles (arrows). Bar = 50 µm. B) Gram-positive spores (arrows) in stroma of
494 ovary. C). Numerous, Gram positive spores in developing follicle. D) Developing follicle replete
495 with spores. H&E. E) Spores within a developing follicle. Kinyoun acid fast stain. Note the faint
496 acid-fast appearance of spores due to overdecolorization. F) Spores (arrow) in intestinal
497 epithelium. H&E. Spores in renal tubule. Gram stain.



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