Evidence for an Amoeba-Like Infectious Stage of *Ichthyophonus* sp. and Description of a Circulating Blood Stage: A Probable Mechanism for Dispersal Within the Fish Host

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EVIDENCE FOR AN AMOEBA-LIKE INFECTIOUS STAGE OF *ICHTHYOPHONUS* SP. AND DESCRIPTION OF A CIRCULATING BLOOD STAGE: A PROBABLE MECHANISM FOR DISPERSAL WITHIN THE FISH HOST

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ABSTRACT: Small amoeboid cells, believed to be the infectious stage of *Ichthyophonus* sp., were observed in the bolus (stomach contents) and tunica propria (stomach wall) of Pacific staghorn sculpins and rainbow trout shortly after they ingested *Ichthyophonus* sp.–infected tissues. By 24–48 hr post-exposure (PE) the parasite morphed from the classically reported multinucleate thick walled schizonts to 2 distinct cell types, i.e., a larger multinucleate amoeboid cell surrounded by a narrow translucent zone and a smaller spherical cell surrounded by a "halo" and resembling a small schizont. Both cell types also appeared in the tunica propria, indicating that they had recently penetrated the columnar epithelium of the stomach. No *Ichthyophonus* sp. pseudo-hyphae ("germination tubes") were observed in the bolus or penetrating the stomach wall. Simultaneously, *Ichthyophonus* sp. was isolated in vitro from aortic blood, which was consistently positive from 6 to 144 hr PE, then only intermittently for the next 4 wk. Small PAS-positive cells observed in blood cultures grew into colonies consisting of non-septate tubules (pseudo-hyphae) terminating in multinucleated knob-like apices similar to those seen in organ explant cultures. Organ explants were culture positive every day; however, typical *Ichthyophonus* sp. schizonts were not observed histologically until 20–25 days PE. From 20 to 60 days PE, schizont diameter increased from $\leq$25 μm to $\geq$82 μm. Based on the data presented herein, we are confident that we have resolved the life cycle of *Ichthyophonus* sp. within the piscivorous host.

Ichthyophonus sp. is a mesomycetozoan parasite of fishes (Mendoza et al., 2002) that has been implicated in epizootics worldwide, resulting in high mortality and economic losses to both fresh and saltwater species (reviewed in McVicar, 1999). Although the parasite was first recognized over a century ago (von Hofer, 1893), much of its life history within, and outside, the fish host remains speculative. The least understood aspects of the life history of *Ichthyophonus* sp. are those associated with the initiation of infection and the sequence of events that occur in the host immediately following exposure.

Two in vivo studies produced the most convincing evidence that the multinucleate schizonts in infected fish could produce infections following ingestion by a predator or scavenger. McVicar and McLay (1985) reported small cells (3–7 μm) in the intertrabecular spaces of the heart 26 hr after feeding schizont-infected tissue to rainbow trout. Similarly, Okamoto et al. (1987) concluded that the thick walled multinucleate spherical bodies, i.e., schizonts, obtained from infected tissues cultured at high pH (7.0–9.0) were, or contained, the infective stage for rainbow trout. The events that occurred between ingestion and infection have not been described. Spanggaard et al. (1995) proposed that *Ichthyophonus* sp. "thick walled cells," i.e., schizonts, germinated in the low pH of the stomach, and produced hyphae that, "penetrate the digestive tract and rupture when they reach a blood vessel (neutral pH)," releasing the parasite into the blood stream. McVicar (1999) offered tantalizing evidence supporting this hypothesis in the form of a micrograph of, "a germination tube penetrating through a mucosal cell." He postulated that this could result in "the spread of small infective units throughout the body via the circulatory system," but no supporting evidence of either hypothesis was offered.

In addition to the identity of the infectious agent and route of infection, the mechanism of parasite dissemination within the host remains speculative. Fish (1934) believed that the parasite was "spread via blood and lymph," but offered no supporting evidence. Likewise, Sindermann and Rosenfield (1954) stated, "Spores have been demonstrated consistently in the circulating blood," but did not describe the "spores" or explain how they accessed the blood. The latter authors concluded that, "Invasion and dissemination" (in Atlantic herring *Clupea harengus*) was completed as early as 18 days PE but presented no data to support the statement. Because they used wild-caught herring of unknown infection status and did not include control data, their observations cannot be substantiated. Based on experimental exposure of rainbow trout (*Oncorhynchus mykiss*) to infected viscera, McVicar and McLay (1985) concluded that "fungal units were disseminated free within the circulatory system," but they offered no supporting evidence such as drawings, photographs, or descriptions of the "fungal units."

Although each of the above hypotheses offers a plausible explanation for observations or experimental data, none present convincing evidence as to the nature of the infective stage, the mechanism of infection, or the mechanism of dispersal within the host. The objectives of the present study were to identify parasitic stages and describe the sequence of events occurring between oral exposure and the appearance of *Ichthyophonus* sp. schizonts in the host organs.

MATERIALS AND METHODS

Acquisition and maintenance of experimental hosts

Pacific staghorn sculpins (*Leptocottus armatus*) and rainbow trout (*O. mykiss*), both natural hosts to *Ichthyophonus* sp., were used as experimental hosts. Young-of-the-year sculpins were captured by beach seine from July through October from Port Madison (Puget Sound), Washington (47°44′5.72″N, 122°32′9.63″W) and maintained at ambient temperature in sand filtered, and ultraviolet-treated, natural seawater at the U.S. Geological Survey's Marrowstone Island Research Station (Nordland, Washington). Juvenile rainbow trout were obtained from a commercial trout farm (Buhl, Idaho) and maintained at 14.8 C in untreated flowing spring water from a deep aquifer.

Pacific staghorn sculpins

Isolation of the parasite: Laboratory-reared specific pathogen-free (SPF) Pacific herring, *Clupea pallasii* (Hershberger et al., 2010), were experimentally infected with *Ichthyophonus* sp. isolated from wild Puget

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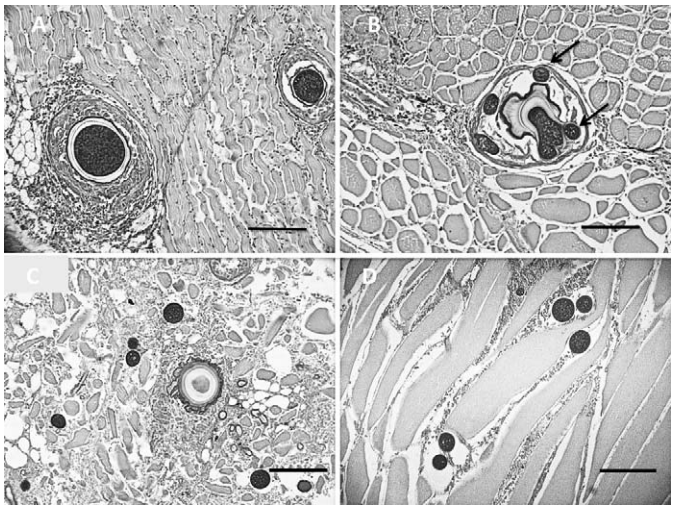

FIGURE 1. Transformation of *Ichthyophonus* sp. schizonts in the stomach (bolus) of Pacific staghorn sculpins, *Leptocottus armatus*, following ingestion of infected herring tissue. (**A**) Normal multinuclear spherical schizonts surrounded by a granuloma (0 hr–infected homogenate). (**B**) Small PAS-positive amoeboid cells (arrows) budding off of parent schizont (48 hr PE). (**C**) Empty schizont (center) surrounded by dispersing amoeboid cells (48 hr PE). (**D**) Amoeboid cells dispersed throughout digesting herring muscle (48–96 hr PE). (Bar = 50 μm) Stain, Periodic acid-Schiff (PAS) reagent.

Sound herring (Hershberger et al., 2002). When epidermal ulcers became apparent, indicating a mature infection (Kocan et al., 2010), the herring were killed with an overdose of buffered MS-222 and placed on ice. Following removal of the head, herring tissues were processed through a food grinder to produce a homogenate of uniformly distributed schizonts, which was kept chilled in an ambient seawater bath prior to being fed to sculpins.

Exposure: For this study, the term "exposure" is defined as ingestion of fresh *Ichthyophonus* sp.–infected fish tissue. Young-of-the-year sculpins measuring 10.9 ± 3.9 cm were individually exposed to *Ichthyophonus* sp. in 5 groups of 12 fish each (60 total). Prior to exposure, sculpins fasted for 48 hr, then each fish was fed a single dose of ~3 g of fresh tissue homogenate containing schizonts. When all of the infected tissue was consumed (1–4 hr), the exposed fish were placed into a community tank for the remainder of the study. Three aliquots of the exposure homogenate were cultured in MEM-5 (McVicar, 1982, 1999; Spanggaard et al., 1994) and processed by histology to verify the presence and viability of the parasite. Five additional sculpins were fed *Ichthyophonus* sp.–free herring homogenate and served as controls.

Experimental fish were killed individually by an overdose of MS-222 in buffered seawater, then immediately necropsied and examined for the presence of *Ichthyophonus* sp. using multiple diagnostic methods. Dead fish were placed on ice to impede post-mortem changes.

Stomach content: Following ingestion of *Ichthyophonus* sp.–infected herring homogenate, the bolus (stomach contents) was collected from each of 2 sculpins at 6, 24, 48, and 72 hr PE and examined for the presence of *Ichthyophonus* sp. by in vitro culture (Kocan et al., 2011) and standard single plane histology (5 μm sections). Cultures were examined for the growth of *Ichthyophonus* sp. after 10 days incubation. Duplicate histologic sections were stained with hematoxylin-eosin (H&E) and periodic acid-Schiff reagent (PAS); sections were examined microscopically to document temporal changes in parasite morphology and location.

Blood and tissue: Sub-sampling of exposed sculpins began 6 hr PE and continued for 32 days. The presence of *Ichthyophonus* sp. was determined by (1) visual examination for gross (visible) lesions; (2) parasite growth in culture; and (3) histological examination of stained tissues. Blood collected from the dorsal aorta (100–200 μl) was cultured in MEM-5 (pH 7.2) and used to make blood smears, which were air dried, fixed in absolute methanol, and processed with Giemsa and PAS stain. Explant cultures of heart, liver, spleen, and kidney were examined daily for 14 days (40× magnification) for the growth of *Ichthyophonus* sp. Tissues for histology were fixed in 10% neutral buffered formalin and stained with H&E and PAS.

Controls: To ensure that experimental sculpins were free of *Ichthyophonus* sp., >100 juveniles were sampled by explant culture over a 3-yr period from the Port Madison sample population. During the course of the study, each exposure group also included unexposed fish to ensure that the population remained free of *Ichthyophonus* sp.

Rainbow trout

Portions of the sculpin study (above) were replicated to determine whether the host species or genetic strain of the pathogen influenced the course of *Ichthyophonus* sp. infections.

Forty laboratory-reared juvenile female rainbow trout (11.8 ± 0.49 cm) were exposed to *Ichthyophonus* sp. via feeding of heavily infected homogenized trout viscera over a 6-hr period using an *Ichthyophonus* sp. type that is genetically distinct from the Pacific herring type (Rasmussen et al., 2010). Groups of 4 fish were sampled at 24, 48, 72, and 96 hr PE, then evaluated by visual examination for gross lesions and explant culture of heart, liver, kidney, and gill, as well as blood from the dorsal aorta; all tissues were also processed for histology. After 48, 72, and 96 hr PE, the bolus was removed and processed similar to the sculpin samples.

Five trout from the same exposure group were necropsied and checked for *Ichthyophonus* sp. every 10 days from 20 through 60 days PE by visual examination, growth in culture, and histology. Schizont growth was determined by measuring cell diameter in histological sections over time.

Statistical analysis

A 2 × 2 chi-square distribution was used to compare prevalence differences, and a 1-tailed paired Student's *t*-test was used to compare schizont size between groups.

RESULTS

Stomach content (bolus)

The herring tissue homogenate used to initiate infections in sculpins was *Ichthyophonus* sp.–positive prior to feeding (0 hr); following ingestion the partially digested bolus was also *Ichthyophonus* sp.–positive from 6 hr through 96 hr PE. Schizonts in the bolus underwent morphologic changes beginning with the typical thick walled multinucleate cells surrounded by a bilaminate layer and granuloma, to small amoeboid cells exiting the schizont (Fig. 1). No "germination tubes" or pseudo-hyphae were observed in the bolus, but numerous rodlet cells were present in the stomach epithelium of several exposed fish, but not control fish, indicating a local immune response.

The trout tissue homogenate used to initiate infections in experimental rainbow trout was also *Ichthyophonus* sp.–positive prior to feeding, as was the partially digested bolus at 48, 72, and 96 hr PE. In addition to changes seen in the schizonts, 2 morphologically distinct cells were observed, i.e., a small PAS-positive cell with a distinct clear "halo" and a larger PAS-positive amoeboid cell (Fig. 2A).

Stomach contents of all 5 control fish were negative for *Ichthyophonus* sp. by culture and histology.

Stomach wall

Two PAS-positive cell types resembling those seen in the digesting bolus were also observed in the tunica propria of both experimental host species; these occurred just below the columnar epithelial cells of the stomach wall (Fig. 2B–D). The smaller cell was spherical, surrounded by a clear "halo," while the larger cell was an amoeboid syncytium surrounded by a thin translucent zone. It is not clear if these represent 2 different cell types or are

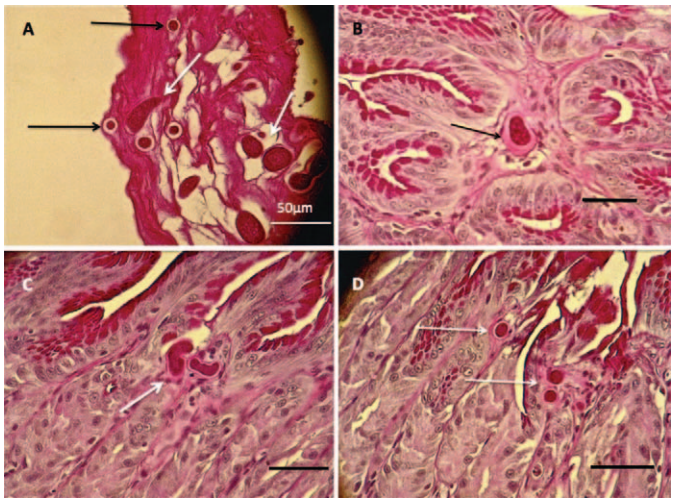

FIGURE 2. (**A**) Large amoeboid PAS-positive cells (white arrows) and small spherical PAS-positive cells with "halos" (black arrows) in digesting bolus. (**B, C**) Large amoeboid cells surrounded by a translucent zone (arrows) in the tunica propria. (**D**) Small spherical cells with "halo" in tunica propria. All photos 48 hr PE; species, rainbow trout; stain, periodic acid-Schiff reagent (PAS).

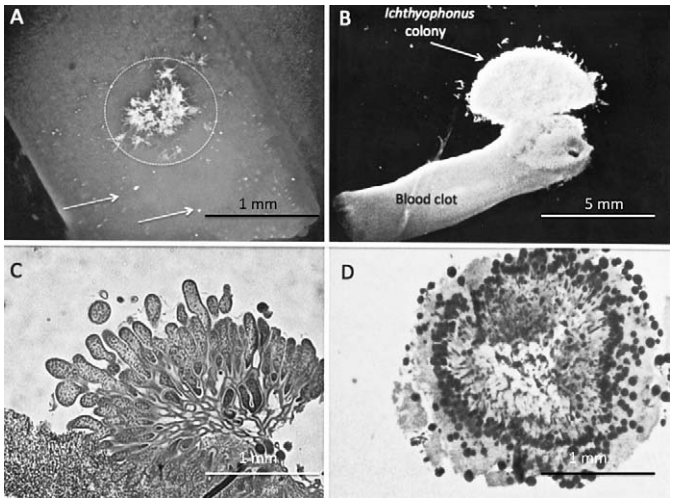

FIGURE 3. (**A**) *Ichthyophonus* sp. colonies (circle and arrows) growing from rainbow trout blood clot after 7 days in vitro. (**B**) *Ichthyophonus* sp. colony growing on a blood clot from a Pacific staghorn sculpin after 10 days in vitro. (**C**) Histologic section of B showing *Ichthyophonus* sp. colony on surface of clot (H&E). (**D**) Cross-section through sculpin blood clot showing PAS-positive *Ichthyophonus* sp. (spheres) radiating from center of clot. Trout blood collected 48 hr PE; Sculpin blood collected 24 hr PE. Culture medium, MEM-5.

different sized individuals of the same cell. We believe these cells are the initial infective stage(s) of *Ichthyophonus* sp. There was no evidence of "germination tubes" or pseudo-hyphae in the bolus or penetrating the stomach epithelium in either of the exposed host species.

Blood

Aortic blood cultured positive for *Ichthyophonus* sp. in 30% (14/46) of sculpins sampled during the first 6 days PE. The earliest positive blood was observed at 6 hr PE then detected in only 2 additional fish on days 8 and 15 PE (Table I). Blood cultured positive from 31% (5/16) of the trout sampled between 24 and 96 hr PE.

Various sized colonies of *Ichthyophonus* sp. appeared on the surface of blood clots in both sculpins and trout after 7 to 10 days in culture (Fig. 3). Histologic sections of the clots revealed that the colonies consisted of numerous non-septate hyphae terminating in multinucleate PAS-positive knob-like apices. The colonies appeared identical to those seen in previously reported tissue explant cultures and ultimately produced all currently recognized morphologic stages of *Ichthyophonus* sp. seen in vitro (Spanggaard et al., 1995). Also present in the clot were various sized small PAS-positive cells, presumably precursors to mature schizonts (Fig. 4). The mean diameter of these cells was 8.5 µm ± 2.92 with a range of 5.6 µm to 13.9 µm (n = 20).

Tissue explants

Cardiac muscle was culture positive in 52% (25/48) of sculpins sampled during the first week PE, beginning as early as 6 hr PE. For the remainder of the study (8–32 days), 83% (10/12) of the remaining fish cultured positive (Table I). *Ichthyophonus* sp. was most frequently detected in sculpin heart tissue (58%), followed

TABLE I. Detection of *Ichthyophonus* in Pacific staghorn sculpin blood and organs following a single experimental oral exposure to infected herring tissue.

	Days post-exposure														
	1	2	3	4	5	6	7	8	15	18	22	25	28	32	Total
n =	15	10	9	4	3	5	2	2	2	2	2	1	2	1	60
% Positive*	20	90	67	75	33	40	50	50	100	100	100	100	50	100	
Blood culture†	2	6	2	2	1	1	0	1	0	0	1	0	0	0	16
Explants															
Heart†	3	9	6	3	1	2	1	1	2	2	2	1	1	1	35
Spleen	1	6	4	3	1	2	1	1	1	2	0	1	1	1	25
Kidney	0	5	6	3	1	2	1	1	0	2	2	1	1	1	26
Liver	0	2	4	3	1	0	1	1	0	0	2	1	1	1	17
Histology	0	0	0	0	0	0	0	0	0	0	0	1	1	1	3
Visible lesions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Blood (films)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Based on heart explants

† Blood and heart positive by in vitro culture as early as 6 hr post-exposure.

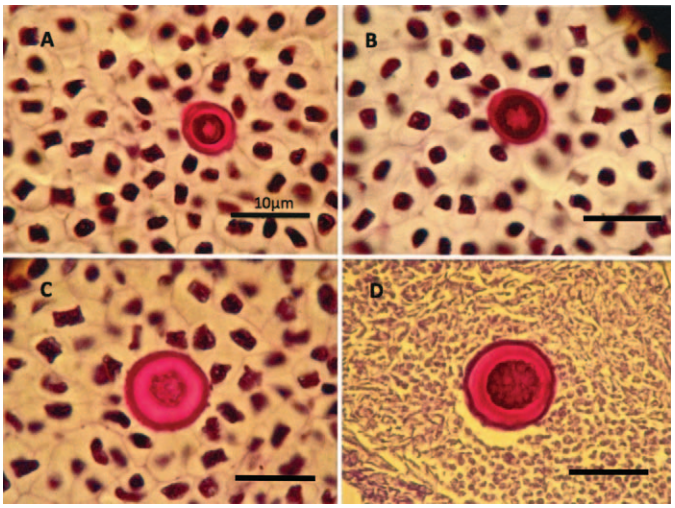

FIGURE 4. (**A–C**) Sequence of progressive growth and differentiation of *Ichthyophonus* sp. schizont in histologic section of cultured rainbow trout blood clot. (**D**) Cells forming in small schizont in tissue explant after 10 days incubation. Bar = 10 µm; Culture medium, MEM-5; stain, Periodic acid-Schiff (PAS).

by kidney, spleen, liver, and blood (43%, 41%, 28%, and 27%, respectively). The probability of detecting *Ichthyophonus* sp. in sculpins by cardiac explant was significantly greater from 2 to 4 wk PE than during the first wk; P ($\bar{X}^2 = 5.25$) = 0.022, n = 60.

Cardiac tissue cultured positive in 56% (9/16) of trout sampled during the first 96 hr PE. Of the 16 fish sampled, none was positive at 24 hr, 4 were positive at 48 hr, 4 at 72 hr, and 1 at 96 hr PE. Liver, kidney, and gill were culture positive (50%, 44%, and 31%, respectively) during the first 96 hr PE, but were not histologically positive. The remaining 25 trout from the same exposure group were sampled at 10-day intervals from 20 to 60 days PE and were all culture and histologically positive. The probability of detecting a positive trout was significantly greater from 20 to 60 days PE than during the first 96 hr; P ($\bar{X}^2 = 16.46$) < 0.001, n = 41.

Histology and gross lesions

Gross (visible) lesions were not observed at any time during the study and *Ichthyophonus* sp. was not histologically evident in freshly harvested viscera until 25 days PE. Trout sampled from 24 to 96 hr PE were histology-negative, but those sampled from 20 to 60 days PE were all histologically positive.

Schizont growth

Mean schizont diameter was consistent within individual fish and appeared to increase over time. Schizonts observed in the livers from 2 of 5 trout sampled 20 days PE were significantly smaller (25.3 µm ± 10.70) than schizonts in the remaining 3 fish (65.4 µm ± 12.03); 1-tail, paired t-test, $P < 0.001$ (n = 40) (Fig. 5). The larger schizonts in the 3 fish from day 20 did not differ in size (65.4 µm ± 12.03) from schizonts observed in 5 fish sampled on day 30 (67.9 ± 9.93). However, schizonts from fish sampled on days 40–60 were significantly larger (81.9 µm ± 15.54) than schizonts from days 20 and 30, but not different from each other; 1-tail, paired t-test, $P < 0.001$ (n = 43).

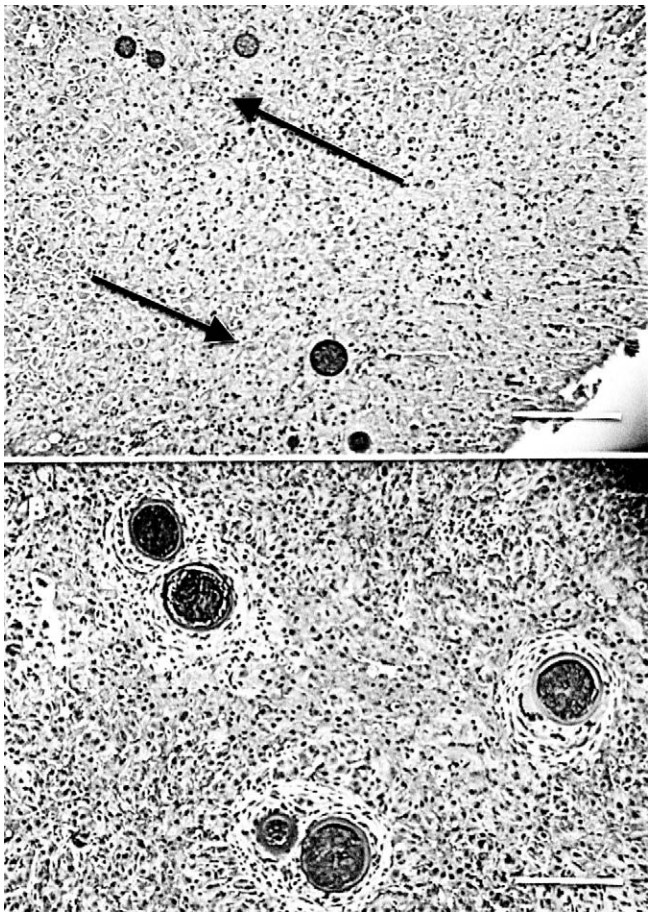

FIGURE 5. (**A**) Small schizonts (arrows) and (**B**) large schizonts in the livers of 2 *Ichthyophonus* sp.–infected rainbow trout 20 days PE. Bars = 100 µm; stain, Periodic acid-Schiff (PAS) reagent.

DISCUSSION

Ichthyophonus sp. schizonts were observed to undergo a series of morphologic changes in the partially digested bolus in both sculpins and trout. These changes consisted of a transition from the large multinucleate bi-laminate schizont in the infected host tissues to smaller cells that appear to be the infective stage. Two cell types, similar to those described in vivo (Daniel, 1933) and in vitro (Okamoto et al., 1985; Spanggaard et al., 1995), were observed in the digesting bolus and the tunica propria, just below the columnar epithelium of the stomach. The relationship of these 2 cell types is currently unknown, but we believe 1, or both, of these cells to be the initial infective stage(s) of *Ichthyophonus* sp.

Ichthyophonus sp. was regularly detected in circulating blood and organs in both host species as early as 6 hr and up to 6 days PE of infected tissues. After 6 days, the parasite was only sporadically observed in the blood, suggesting that the circulating cells entered the blood from the stomach over several days, then were sequestered in the organs where they grew to maturity. These events would explain the small cells (3–7 µm) previously reported in heart muscle of trout 26 hr PE of infected tissue (McVicar and McLay, 1985), and the short residency of a circulating stage would explain why *Ichthyophonus* sp. was only detectable by PCR in the blood of a relatively few Yukon River Chinook salmon that

were confirmed *Ichthyophonus* sp.–positive (Whipps et al., 2006). The identity of the elusive circulating cell is still not known, but it must be small enough to pass through the capillary beds in the gills before reaching the dorsal aorta, making the cells in Figures 1 and 2 likely candidates; conversely, these cells may be precursors to the small PAS-positive cells seen in cultured blood (Fig. 3).

Because the parasite is readily cultured from most organs during the first 6 days PE but not readily detectable by histology, these early positive cultures likely result from the presence of the infectious stage in blood circulating through the organs. Once the circulating cells become too large to pass through capillary beds, they become lodged in various organs and grow to the typical large schizont depicted in the *Ichthyophonus* sp. literature. If this scenario is correct, then a population of small schizonts similar in size to those observed in cultured blood should precede the large mature schizonts and, as predicted, this situation was observed (Fig. 5).

Contrary to the hypotheses proposed in several earlier studies, there was no evidence of "germination tubes" or pseudo-hyphae penetrating the gastric mucosa; rather, amoeboid cells derived from mature schizonts were simultaneously present in the digesting bolus and tunica propria. If germination tubes were the mechanism for infection as suggested by Spanggaard et al. (1994) and McVicar (1999), these structures should be readily evident in the digesting bolus. However, after examining the stomach content of infected sculpins and trout, we observed no evidence of germination tubes penetrating, or contacting, the stomach wall. Although germination tubes occur in infected fish post mortem (Franco-Sierra et al., 1997) and can be induced in low pH culture medium (Okamoto et al., 1985; Spanggaard et al., 1994), we believe these may be a mechanism for dispersal of infectious cells into the environment following death of the host, or possibly a means of infecting a necrophagic intermediate host.

Under the exposure conditions of this study, *Ichthyophonus* sp. exhibited a prepatent period of ~30 days as determined by histology (defined in Roberts and Janovy, 2005). In separate unrelated studies (data not shown), we observed the prepatent period to be <10 days when fish were exposed to high doses of *Ichthyophonus* sp., while in low-dose studies the parasite was not detected for ≥60 days and, in some cases, not at all. This is in agreement with previous reports. McVicar and McLay (1985) observed schizonts by 3 days PE, Rand and Cone (1990) reported a prepatent period of 7–14 days, and Gustafson and Rucker (1956) did not see patent infections for >100 days. The latter authors also observed that some exposed fish never converted to patency, similar to what we have seen in several low, and intermediate, level exposures.

The successful establishment of a parasite within the host's body is dependent on the ability of the infective stage to penetrate the host's first line of defense(s) and invade target tissues before being neutralized by the host's humoral and/or cellular immune defenses. The infective cell is often small, cryptic, and difficult to detect until it proliferates or grows and produces the final mature stage. *Ichthyophonus* sp. appears to follow a precise sequence of events. First, a cryptic amoeboid cell invades the host's stomach mucosa following ingestion of infected tissue. Second, a dose-dependant prepatent period follows invasion, during which the parasite is transported throughout the host's body via circulating blood but is difficult to visualize. Third, the organism increases in size by asexual nuclear division, forming a schizont in the host's organs. Fourth, a fully developed multinucleate schizont is contained by a granuloma that prevents further spread of the parasite. Finally, the schizont remains encapsulated in the granuloma until ingested by a carnivore, or following the death of the host, by a scavenger, thus beginning a new cycle.

Because the sequence of events described here is similar in 2 host species infected with 2 distinct *Ichthyophonus* sp. genotypes, it likely represents a universal response of piscivorous fish to *Ichthyophonus* sp. infection.

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LITERATURE CITED

Daniel, G. E. 1933. Studies on *Ichthyophonus hoferi*. A parasitic fungus of the herring, *Clupea harengus*. I. The parasite as it is found in the herring. American Journal of Hygiene **17:** 262–276.

Fish, F. F. 1934. A fungus disease in fishes of the Gulf of Maine. Parasitology **26:** 1–15.

Franco-Sierra, A., A. Sitja-Bobadilla, and P. Alvarez-Pellitero. 1997. *Ichthyophonus* infections in cultured fish from Spain. Journal of Fish Biology **51:** 830–839.

Gustafson, P. V., and R. R. Rucker. 1956. Studies on an *Ichthyosporidium* infection in fish: Transmission and host specificity. Special Scientific Report, U.S. Fish and Wildlife Service, Fisheries **166:** 1–7.

Hershberger, P. K., J. L. Gregg, C. A. Grady, R. M. Collins, and J. R. Winton. 2010. Susceptibility of three stocks of Pacific herring to viral hemorrhagic septicemia. Journal of Aquatic Animal Health **22:** 1–7.

———, K. Stick, B. Bui, C. Carroll, B. Fall, C. Mork, J. A. Perry, E. Sweeney, J. Wittouck, and R. M. Kocan. 2002. Incidence of *Ichthyophonus hoferi* in Puget Sound fishes and its increase with age of adult Pacific herring. Journal of Aquatic Animal Health **14:** 50–56.

Kocan, R. M., H. Dolan, and P. Hershberger. 2011. Diagnostic methodology is critical for accurately determining the prevalence of *Ichthyophonus* infections in wild fish populations. Journal of Parasitology **97:** 344–348.

———, J. L. Gregg, and P. K. Hershberger. 2010. Release of infectious cells from epidermal ulcers in *Ichthyophonus* sp.-infected Pacific herring (*Clupea pallasii*): Evidence for multiple mechanisms of transmission. Journal of Parasitology **96:** 348–352.

McVicar, A. H. 1982. *Ichthyophonus* infections in fish. *In* Microbial diseases of fish, R. J. Roberts (ed.). Academic Press, London, U.K., p. 243–269.

———. 1999. *Ichthyophonus* and related organisms. *In* Fish diseases and disorders, Vol. 3. Viral, bacterial and fungal infections, P. T. K. Woo and D. W.Bruno (eds.). CABI Publishing, New York, New York, p. 661–687.

———, and H. A. McLay. 1985. Tissue response of plaice, haddock and rainbow trout to the systemic fungus *Ichthyophonus*. *In* Fish and shellfish pathology, A. E. Ellis (ed.). Academic Press, London, U.K., p. 329–346.

Mendoza, L., J. W. Taylor, and L. Ajello. 2002. The class Mesomycetozoea: A heterogeneous group of microorganisms at the animal-fungal boundary. Annual Review of Microbiology **56:** 315–344.

Okamoto, N., K. Nakase, H. Suzuki, Y. Nakai, K. Fujii, and T. Sano. 1985. Life history and morphology of *Ichthyophonus hoferi* in vitro. Fish Pathology **20:** 273–285.

———, ———, ———, and T. Sano. 1987. Experimental oral infection of rainbow trout with cultivated spherical bodies of *Ichthyophonus hoferi*. Nippon Suisan Gakkaishi (Japanese Society of Fishery Science) **53:** 407–409.

Rand, T. G., and D. K. Cone. 1990. Effects of *Ichthyophonus hoferi* on condition indices and blood chemistry of experimentally infected rainbow trout (*Oncorhynchus mykiss*). Journal of Wildlife Diseases **26:** 323–328.

Rasmussen, C., M. K. Purcell, J. L. Gregg, S. E. LaPatra, J. R. Winton, and P. K. Hershberger. 2010. Sequence analysis of the internal transcribed spacer (ITS) region reveals a novel clade of *Ichthyophonus* sp. from rainbow trout. Diseases of Aquatic Organisms **89:** 179–183.

Roberts, L. S., and J. Janovy Jr. 2005. Foundations of parasitology, 7th edition. McGraw Hill, Boston, Massachusetts, 702 p.

Sindermann, C., and A. Rosenfield. 1954. Diseases of fishes of the western North Atlantic. I. Diseases of the sea herring (*Clupea harengus*). Research Bulletin of the Department of Sea and Shore Fisheries, Maine **18:** 1–23.

Spanggaard, B., L. Gram, N. Okamoto, and H. H. Huss. 1994. Growth of the fish pathogenic fungus, *Ichthyophonus hoferi*, measured by conductivity and microscopy. Journal of Fish Diseases **17:** 145–153.

———, H. H. Huss, and J. Bresciani. 1995. Morphology of *Ichthyophonus hoferi* assessed by light and scanning electron microscopy. Journal of Fish Diseases **18:** 567–577.

von Hofer, B. 1893. Eine Salmonidenerkrankung. Allgemeine Fischereizeitung **18:** 169–171.

Whipps, C. M., T. Burton, V. G. Watral, S. St-Hilaire, and M. Kent. 2006. Assessing the accuracy of a polymerase chain reaction test for *Ichthyophonus hoferi* in Yukon River Chinook salmon *Oncorhynchus tshawytscha*. Diseases of Aquatic Organisms **68:** 141–147.