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DIFFERENTIAL SURVIVAL OF *ICHTHYOPHONUS* ISOLATES INDICATES PARASITE ADAPTATION TO ITS HOST ENVIRONMENT

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ABSTRACT: In vitro viability of *Ichthyophonus* spp. spores in seawater and freshwater corresponded with the water type of the host from which the spores were isolated. Among *Ichthyophonus* spp. spores from both marine and freshwater fish hosts (Pacific herring, *Clupea pallasii*, and rainbow trout, *Oncorhynchus mykiss*, respectively), viability was significantly greater ($P < 0.05$) after incubation in seawater than in freshwater at all time points from 1 to 60 min after immersion; however, magnitude of the spore tolerances to water type differed with host origin. *Ichthyophonus* sp. adaptation to its host environment was indicated by greater seawater tolerance of spores from the marine host and greater freshwater tolerance of spores from the freshwater host. Prolonged aqueous survival of *Ichthyophonus* spp. spores in the absence of a host provides insight into routes of transmission, particularly among planktivorous fishes, and should be considered when designing strategies to dispose of infected fish carcasses and tissues.

Ichthyophonus hoferi, a cosmopolitan protozoan parasite primarily of marine fishes, has been associated with repeated epizootics of wild fishes for >100 yr (Sindermann, 1990; McVicar, 1999; Kocan et al., 2004). Previous taxonomic groupings incorrectly positioned the organism with fungi, based on observed life history stages that superficially resemble fungal spores and hyphae (reviewed in McVicar, 1999); however, recent molecular phylogenetic assessments resulted in reclassification of the organism within the Mesomycetozoea, a novel clade of protozoans positioned near the animal–fungal divergence (Mendoza et al., 2002). Although its geographical distribution is extremely broad (reviewed in McVicar, 1999), only 2 species are currently recognized (Rand et al., 2000); presumably, other species have been incorrectly grouped with *I. hoferi* based on similar gross morphologies (Fish, 1934; McVicar, 1999). Therefore, the organism will hereafter be referred to generically as *Ichthyophonus*.

Ichthyophonus currently occurs in high prevalences and intensities among populations of wild Pacific herring (*Clupea pallasii*) throughout the northeastern Pacific (Marty et al., 1998; Hershberger et al., 2002; Jones and Dawe, 2002; Marty et al., 2003) and among populations of cultured, freshwater rainbow trout in southern Idaho. Temporal emergence of *Ichthyophonus* in the Pacific remains unknown because of a paucity of historical fish health surveys involving wild marine fish. The parasite was first reported in the northeastern Pacific from a single Pacific staghorn sculpin (*Leptocottus armatus*) collected from the coast of Oregon in 1983 (Olson, 1986); however, subsequent histological examination of archived fish tissues indicates that the parasite was also present in Pacific staghorn sculpin and Pacific herring from Puget Sound, Washington during the late 1970s and early 1980s (P. Hershberger and M. Myers, unpubl. obs.). *Ichthyophonus* was first reported in southern Idaho (Rucker and Gustafson, 1953) during an epizootic of cultured rainbow trout in 1952 and was presumably introduced by feeding raw, infected carp viscera to the larger fish in the raceways (Erickson, 1965). Since its introduction into the Idaho rainbow trout industry, effective disease management strategies have resulted in minimal fish health or economic impacts.

Although piscivorous fishes can become infected with *Ichthyophonus* by consuming infected prey (Kocan et al., 1999), the route of transmission to planktivorous fishes such as Pacific herring remains poorly understood. It is likely that either an intermediate host is involved or that a free-living, water-borne stage exists. Therefore, the ability of *Ichthyophonus* to survive prolonged periods in the absence of a fish host may be crucial for its ability to complete its life cycle. The objectives of the present study were to compare the freshwater and seawater tolerances of *Ichthyophonus* isolates that originated from freshwater and marine fish hosts (rainbow trout and Pacific herring, respectively) and to compare the genetic relatedness of the isolates from both hosts.

MATERIALS AND METHODS

Primary *Ichthyophonus* spores were isolated from tissues of wild Pacific herring, *Clupea pallasii* (collected in Case Inlet and Skagit Bay, Puget Sound, Washington) and cultured freshwater rainbow trout, *Oncorhynchus mykiss* (collected from Clear Springs Foods, Buhl, Idaho). Heart tissue was aseptically removed and transferred to *Ichthyophonus* isolation medium, consisting of Eagle's minimum essential medium supplemented with 5% fetal bovine serum, 100 international units (IU) ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 100 $\mu g\ ml^{-1}$ gentamicin, and buffered to pH 7.8 with 1 M Tris (MEM-5T+A, pH 7.8). Explant cultures were incubated at 15 C for 14 days and examined with an inverted microscope at ×40 magnification for proliferation of primary *Ichthyophonus* spores.

To obtain adequate quantities of *Ichthyophonus* spores, and standardize the parasite life history stage throughout all experiments, isolated primary spores were cultivated in vitro through a high-low-high pH cycle to stimulate hyphenation and resporulation (Spanggaard et al., 1994). Briefly, hyphenation was stimulated by transferring primary *Ichthyophonus* spores from the isolation medium (MEM-5T+A, pH 7.8) to hyphenation medium, consisting of HEPES-buffered Eagle's minimum essential medium supplemented with 5% fetal bovine serum, 100 IU ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 100 $\mu g\ ml^{-1}$ gentamyicin, and 1% glucose; pH was adjusted to 3.5 with HCl (MEM-5H+A+G, pH 3.5). Hyphenation proceeded for 3–5 days at 15 C, after which resporulation was stimulated by transferring the hyphal stages to fresh isolation medium (MEM5-T+A, pH 7.8). The resulting secondary spores were vortex mixed to disaggregate clusters and used for experimental trials.

Genetic relatedness among the rainbow trout and Pacific herring isolates was compared by amplifying segments of the highly conserved, 18S ribosomal DNA (rDNA) gene and comparing the aligned nucleotide sequences. DNA extraction, polymerase chain reaction (PCR), and sequencing were performed as described previously (Halos et al., 2005).

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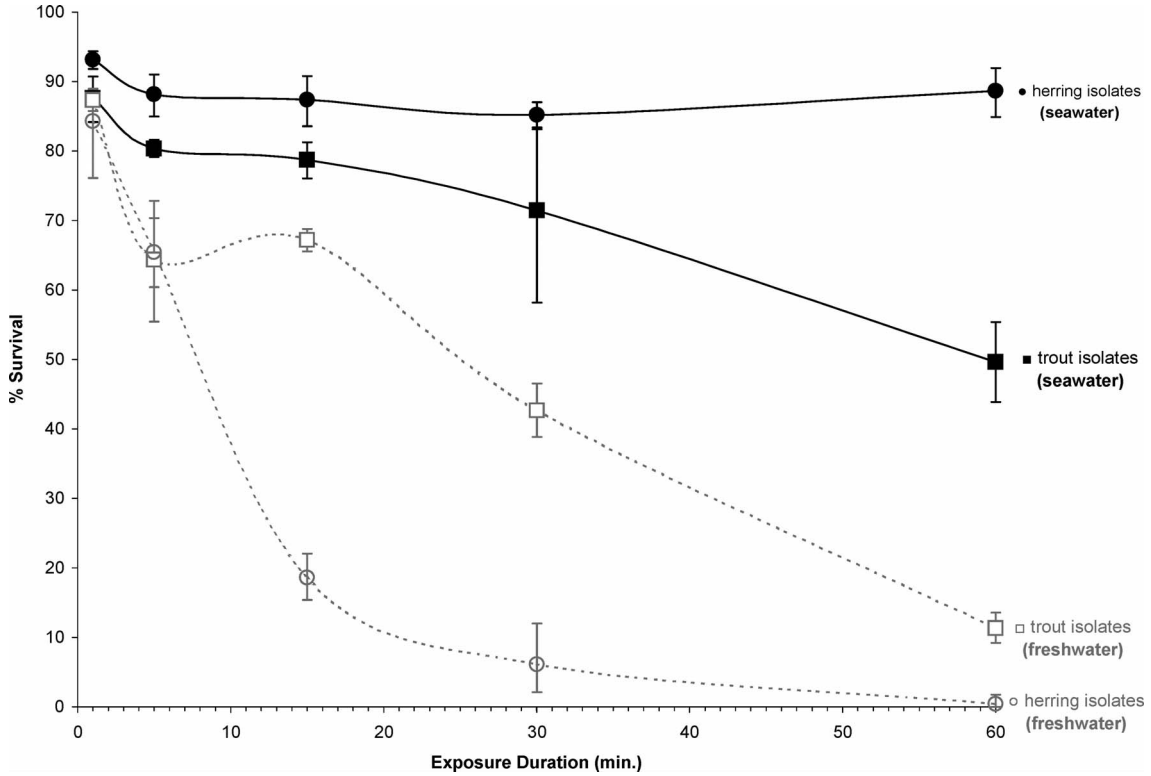

FIGURE 1. Survival of *Ichthyophonus* spores in seawater and freshwater from trial 1. Data points indicate the percentages corresponding to the means of arcsine-transformed proportions for the replicates (n = 3); 87–501 spores from each replicate were analyzed each sampling day. Error bars indicate 2 SD from the mean. Mean survival in duplicate control groups (not exposed to seawater or freshwater) was 87.6% (trout isolates) and 93.3% (herring isolates) at the completion of the trials.

Briefly, primers specific for *Ichthyophonus* (Criscione et al., 2002) were used to amplify a 640-base pair (bp) fragment (region A) or a 673-bp fragment (region B). The PCR fragments were direct sequenced in the forward and reverse directions. Sequence was aligned using the Sequencher software (Gene Codes Corporation, Ann Arbor, Michigan) and compared visually to each other and published sequences.

Three exposure trials were performed, each comparing the tolerance of *Ichthyophonus* spores from rainbow trout and Pacific herring to autoclaved freshwater (municipal tap water) and autoclaved seawater (Puget Sound ambient water collected from 20 m in depth and then sand filtered and UV irradiated); salinities were 0 and 30‰, respectively. To avoid potential biases resulting from pseudoreplication, different *Ichthyophonus* isolates from each host species were pooled and used during each trial. Pooled isolates from each host species were subdivided into 8–9 test tubes/trial. Upon initiation of each trial, isolation medium in the tubes was decanted and replaced with 4-ml aliquots of sterile freshwater (n = 3 tubes/trial), seawater (n = 3 tubes/trial), or fresh isolation medium (controls, n = 2–3 tubes/trial). Subsamples of spores from each tube (n = 42–676 spores) were transferred to wells on 24-well plates after predetermined exposure durations (1–180 min.) and stimulated by incubation in hyphenation medium (MEM-5H+A+G, pH 3.5) at 15 C for 24 hr. Stimulated spores were then evaluated at ×40 magnification; those that failed to hyphenate were considered inactivated, or killed. Proportions of hyphenating (live) spores in the treatment replicates were arcsine transformed, and groups within each trial were compared using the 2-factor analysis of variance (ANOVA), or Student's *t*-test, or both. Statistical significance was assigned to comparisons with $P \leq 0.05$. Spore survival in each treatment group was reported as the back-transformed percentage corresponding to the mean arcsine-transformed proportion among the replicates.

RESULTS

The Pacific herring and rainbow trout *Ichthyophonus* isolates used in this study had 100% nucleotide identity across regions A and B of the 18s rDNA (deposited as GenBank EU332787 and EU332790). These sequences were also 100% identical to sequences derived previously from Pacific herring and Chinook salmon (Criscione et al., 2002; typified by GenBank records AF467795 and AF467796) and from Puget Sound rockfish (Halos et al., 2005).

Among *Ichthyophonus* spores isolated from both Pacific herring and rainbow trout, viability was significantly greater ($P < 0.05$, 2-factor ANOVA) after incubation in seawater than in freshwater at all time points from 1 to 60 min after immersion; however, magnitude of the spore tolerances to water type differed with host origin (Figs. 1–3). After 60-min exposures, survival of spores from herring ranged from 87 to 93% in seawater versus 0–2% in freshwater, and survival of spores from trout ranged from 47 to 50% in seawater versus 1–11% in freshwater ($P < 0.0005$; Figs. 1–3). Mortality of spores from herring was negligible throughout the 60- to 180-min seawater incubations, with only 4.6–10.0% reductions in viability compared with control spores in culture medium; however, viability of spores from trout declined 33.2–55.7% compared with control spores. As a result, the survival differences between the spore types became more pronounced with seawater incubation time (Figs. 1–3). Conversely, although 88.7–99.6% of *Ichthyophonus* spores from both rainbow trout and Pacific herring were no longer viable after incubation in freshwater for 60 min, inactivation occurred more quickly among spores from herring than among those from rainbow trout. Freshwater survival of spores from herring was significantly less than that of spores from trout after

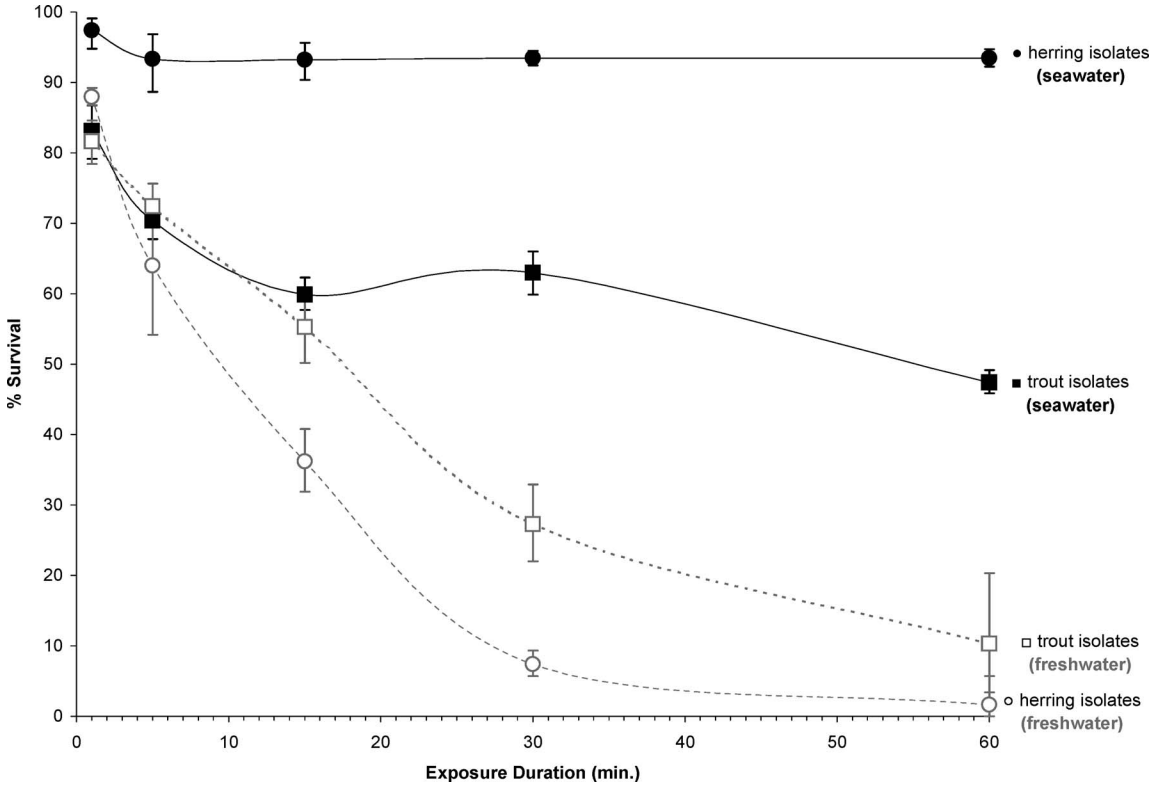

FIGURE 2. Survival of *Ichthyophonus* spores in seawater and freshwater from trial 2. Data points indicate the percentages corresponding to the means of arcsine-transformed proportions for the replicates (n = 3); 101–676 spores from each replicate were analyzed each sampling day. Error bars indicate 2 SD from the mean. Mean survival in duplicate control groups (not exposed to seawater or freshwater) was 80.56% (trout isolates) and 99.98% (herring isolates) at the completion of the trials.

15 min (18.6 vs. 67.2%, $P = 2 \times 10^{-5}$), 30 min (6.1 vs. 42.7%, $P = 0.001$) and 60 min (0.5 vs. 11.3%, $P = 0.001$) in trial 1 (Fig. 1); after 15 min (33.2 vs. 55.2%, $P = 0.004$) and 30 min (7.4 vs. 27.3%, $P = 0.001$) in trial 2 (Fig. 2); and after 30 min (8.1 vs. 17.9%, $P = 0.01$) in trial 3 (Fig. 3).

DISCUSSION

In vitro viability of *Ichthyophonus* spores in seawater and freshwater corresponded with the water type of the host from which the spores were initially isolated. Mortality of *Ichthyophonus* spores isolated from a marine host (Pacific herring) was negligible (4.6–10.0%) after 60–180 incubations in seawater, and these data support previous reports of prolonged survival of *Ichthyophonus* spores from another marine host (Atlantic herring) that remain viable in seawater for 6 mo (Sindermann and Scattergood, 1954) to 2 yr (Spanggaard and Huss, 1996). However, significantly reduced seawater viability of *Ichthyophonus* spores isolated from rainbow trout emphasizes our inability to ascribe generalized life history characteristics and behaviors to a cosmopolitan parasite such as *Ichthyophonus*, especially considering the current lack of comprehensive molecular phylogenetic analyses, resulting in our ignorance of *Ichthyophonus* phylogeny.

Adaptation of *Ichthyophonus* spores to their host environment was indicated by greater freshwater tolerance among spores cultured from the freshwater host (rainbow trout) than among those cultured from the seawater host (Pacific herring). *Ichthyophonus* is generally accepted to be a parasite of marine origin, and most isolations are reported from marine fishes (reviewed in McVicar, 1999 and Sindermann, 1990); however, the parasite became established in cultured freshwater rainbow trout in the Hagermann Valley, Idaho, after its likely introduction via infected, raw fish tissue that was used as feed in the 1940s–1950s (Rucker and Gustafson, 1953; Erickson, 1965). Since its freshwater introduction in the Hagermann Valley, *Ichthyophonus* has not been eradicated, but sound fish husbandry practices have resulted in negligible impacts to fish health or product quality. Increased freshwater survival of spores from rainbow trout strain relative to those from herring likely resulted from novel selective pressures occurring in the intensive freshwater culture environment, a process that would have occurred during the decades after its introduction. This proposed mechanism of freshwater adaptation is likely in a pleomorphic species, such as *Ichthyophonus*, where numerous life history stages occur in the parasitized host and can be stimulated in vitro (Franco-Sierra and Alvarez-Pellitero, 1999). The ecological and pathological significance of these various forms has yet to be determined conclusively, but they likely provide plasticity in life history strategies that enable relatively rapid adjustments to novel hosts and environments. This hypothesis is supported by infectivity studies that indicate rapid transmission of the rainbow trout strain to naïve cohorts via cohabitation with infected lots, but lack of similar transmission in Pacific herring (data not shown).

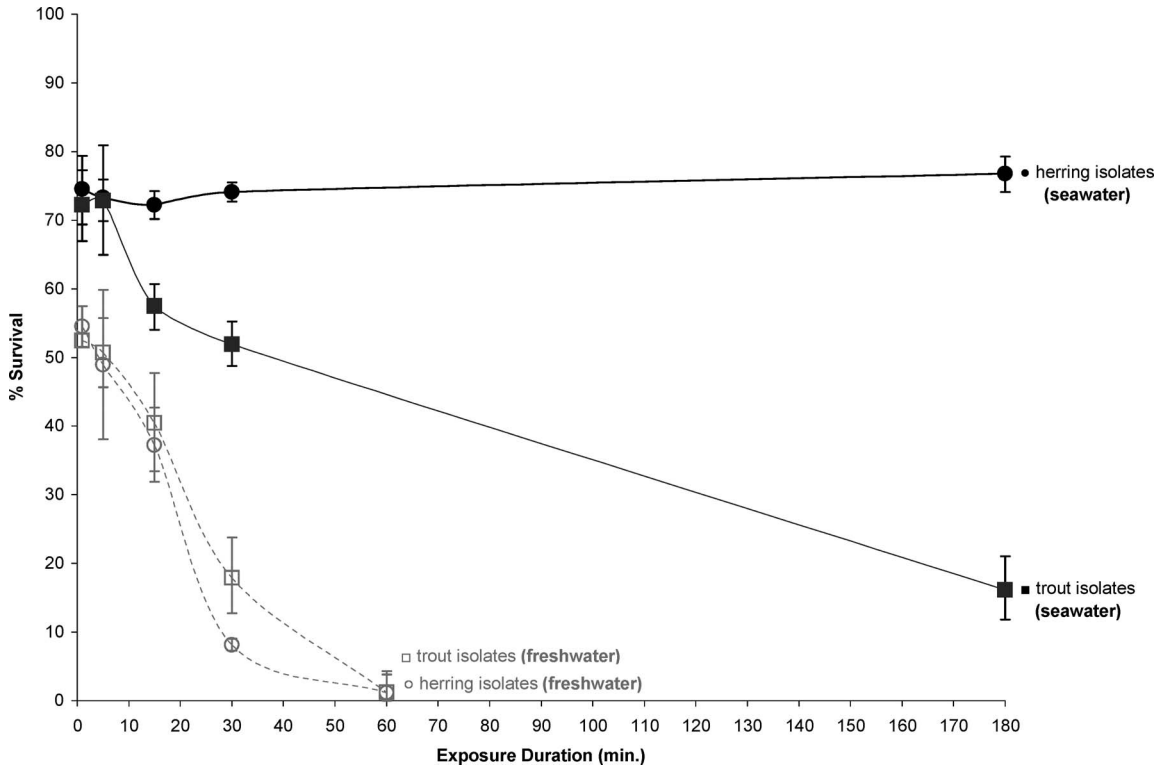

FIGURE 3. Survival of *Ichthyophonus* spores in seawater and freshwater from trial 3. Data points indicate the percentages corresponding to the means of arcsine-transformed proportions for the replicates (n = 3); 42–286 spores from each replicate were analyzed each sampling day. Error bars indicate 2 SD from the mean. Mean survival in triplicate control groups (not exposed to seawater or freshwater) was 71.92% (trout isolates) and 86.8% (herring isolates) at the completion of the trials.

Alternatively, differences in *Ichthyophonus* tolerances to water types may result from the effects of parasite speciation. Currently, only 2 species of *Ichthyophonus* are recognized, *I. hoferi* and *I. irregularis* (Rand et al., 2000), and the majority of reports in the scientific literature have been ascribed to the former species. Based on reports of extremely broad host and geographical distributions of *I. hoferi* that include >80 species of freshwater and marine fishes throughout the world (Spanggaard et al., 1994), the likelihood is minimal that all reports and isolations are representatives of the same species (McVicar, 1999). Criteria for inclusion within the species *I. hoferi*, i.e., spore size and morphology, histological staining characteristics (periodic acid-Schiff-positive), and in vitro culture characteristics, are currently loosely defined and are likely inadequate at discerning morphologically indistinguishable species (reviewed in Sindermann, 1990). Although sequencing of the highly conserved 18S rDNA region indicated that *Ichthyophonus* isolates from rainbow trout and Pacific herring were members of the same haplotype as isolates from Yukon River Chinook salmon (Criscione et al., 2002), Puget Sound rockfish (Halos et al., 2005), and Columbia River American shad (data not shown), variable tolerances to water types and host/geographical separation indicate the presence of cryptic species. Phylogenetic analysis using a more variable gene is necessary to provide further phylogenetic resolution of *Ichthyophonus*.

Prolonged in vitro survival of *Ichthyophonus* in its host's water type has ecological implications involving parasite life history and transmission. Although piscivorous fishes, such as salmonids and sculpins, can become infected by consuming *Ichthyophonus*-infected tissues (McVicar and McLay, 1985; Kocan et al., 1999), the natural route of transmission for planktivorous fishes, such as Pacific herring, remains unknown. Prolonged seawater survival of *Ichthyophonus* spores from Pacific herring (Figs. 1–3) and Atlantic herring (Sindermann and Scattergood, 1954; Spanggaard and Huss, 1996) provides support for a free-living infectious stage or a stage occurring in an intermediate, or paratenic invertebrate, host in the marine environment. For example, late-stage external signs of ichthyophoniasis in Atlantic and Pacific herring include the appearance of pigmented ulcers along the flank of host, which result from *Ichthyophonus* dissemination from the primary site of infection (typically the heart) to all other internal organs and into the skeletal muscle where it ultimately ruptures through the skin. The expelled 50- to 200-μm spores are likely very stable in seawater and may complete a direct life history pattern via ingestion by another herring (Fish, 1934). Similarly, an indirect life history pattern may occur if the released spores are assimilated by an intermediate zooplankter or detritivore that is later consumed by the definitive, planktivorous host. Regardless of the current uncertainty involving *Ichthyophonus* life history strategies, responsible handling practices should be used to prevent inadvertent amplification of the parasite. In particular, disposal of *Ichthyophonus*-infected carcasses and tissues into adjacent water bodies is not recommended. Rather, disposal of infected tissues should involve some combination of halogen exposure (Hershberger et al., in press), heat treatment, and disposal in a sanitary landfill.

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