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Infection experiments with *Aphanomyces invadans* in four species of estuarine fish

R A Johnson[1], J Zabrecky[2], Y Kiryu[3] and J D Shields[1]

1 Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA, USA
2 Spring Hill College, Mobile, AL, USA
3 National Fish Health Research Laboratory, US Geological Survey, Kearneysville, WV, USA

Abstract

Along the eastern seaboard of the US, Atlantic menhaden, *Brevoortia tyrannus*, develop characteristic ulcerative lesions, a condition termed ulcerative mycosis. These lesions are identical to those seen across Asia in fish affected by epizootic ulcerative syndrome, a condition caused by the fungus-like oomycete *Aphanomyces invadans*. Young-of-the-year menhaden inhabiting estuarine environments are the primary species affected in the USA and little is known about the factors involved in the initiation of the lesions, or why menhaden are predominantly infected. Atlantic menhaden, hogchoker, *Trinectus maculatus*, striped killifish, *Fundulus majalis*, and mummichog, *Fundulus heteroclitus*, were inoculated with *A. invadans* (80 zoospores per fish) to explore species differences in infection and lesion development. All four species developed lesions. Killifish developed frank lesions similar to those observed in menhaden but the gross lesions occurred later, approximately 5–10 days after those on menhaden. Hogchoker and mummichog did not develop gross skin ulcers; rather, their lesions appeared as reddened areas under the epidermis. Mummichogs also showed evidence of significant healing with a well-developed granuloma and significant myocyte regeneration. These experiments show that species barriers as well as ecological barriers can explain some of the factors involved in the development of lesions in, and specificity of the water mould for, menhaden.

Correspondence *Dr Jeffrey D Shields, Department of Environmental and Aquatic Animal Health, Virginia Institute of Marine Science, The College of William and Mary, School of Marine Science, Gloucester Point, VA 23062, USA (e-mail: jeff@vims.edu)*

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Introduction

Recent fish kills along the eastern seaboard of the US involving Atlantic menhaden, *Brevoortia tyrannus* (Latrobe), have attracted intense interest (for review see Dykstra & Kane 2000). Menhaden and other fish develop ulcerous skin lesions that have previously been attributed to recent activity by the toxic dinoflagellate, *Pfiesteria piscicida* (Burkholder, Noga, Hobbs & Glasgow 1992; Burkholder, Glasgow & Hobbs 1995; Burkholder, Glasgow & Deamer-Melia 2001). The characteristic skin lesions in menhaden are often located near the anus, and appear as deeply penetrating circular lesions with extensive dermatitis, necrosis, myositis and granulomatous inflammation associated with myonecrosis. A deeply penetrating, highly invasive species of oomycete, *Aphanomyces invadans*, accompanied by an intense granulomatous inflammatory response has been consistently observed in the lesions (Dykstra, Noga, Levine & Moye 1986; Noga & Dykstra 1986; Levine, Hawkins, Dykstra, Noga, Moye & Cone 1990a; Noga, Johnson, Dickey, Daniels, Burkholder & Stanley 1993; Blazer, Lilley, Schill, Kiryu, Densmore, Panyawachira & Chinabut 2002).

The occurrence of lesions in menhaden has been termed ulcerative mycosis (UM). Such lesions have been seen in menhaden since 1984 (Levine *et al.* 1990a) and are identical to other ulcerative diseases seen around the world, which are now collectively

termed epizootic ulcerative syndrome (EUS). EUS was first recognized in the 1970s in farmed ayu, *Plecoglossus altivelis* (Temminck & Schlegel), and has since spread across Asia and Europe affecting numerous estuarine species such as snakehead, *Channa striatus* (Bloch), grey mullet, *Mugil cephalus* L., and ayu (Lilley, Callinan, Chinabut, Kanchanakhan, MacRae & Phillips 1998). The disease is caused by *A. invadans*, which invades the dermis presenting initially as petechiae but rapidly invading to cause small circular lesions that continue to develop into large necrotic ulcers (Lilley & Roberts 1997).

Epidemiological studies have shown that young-of-the-year menhaden are by far the most common estuarine species to exhibit UM. Levine, Hawkins, Dykstra, Noga, Moye & Cone (1990b) conducted pound and trawl net surveys in the Tar Pamlico Estuary from May 1985 to April 1987. Of the 31 species caught in pound nets, four species exhibited lesions: Atlantic menhaden (1.7% prevalence), silver perch, *Bairdiella chrysoura* (Lacepéde) (0.8%), weakfish, *Cynoscion regalis* (Bloch & Schneider) (1.1%), and gizzard shad, *Dorosoma cepedianum* (Lesueur) (0.8%). Of the 44 species caught in trawls seven species exhibited lesions: Atlantic menhaden (15.0% prevalence), silver perch (1.2%), weakfish (0.7%), gizzard shad (0.6%), Atlantic croaker, *Micropogonias undulatus* (L.) (0.2%), spot, *Leiostomus xanthurus* Lacepéde (0.1%), and southern flounder, *Paralichthys lethostigma* Jordan & Gilbert (0.2%). In a related study with pound nets, Noga, Wright, Levine, Dykstra & Hawkins (1991) reported several species of fish other than menhaden as having ulcerous lesions, including southern flounder, hickory shad, *Alosa mediocris* (Mitchill), striped bass, *Morone saxitilis* (Walbaum), bluefish, *Pomatomus saltatrix* (L.), Atlantic croaker, weakfish, spot, silver perch, hogchoker and pinfish, *Lagodon rhomboides* (L.). Most species had only one representative individual with a lesion but only a few fish of each species were caught. Kane, Oldach & Reimschuessel (1998) examined species in the Chicamacomico River, Maryland, using cast nets and found lesions on a majority of the menhaden caught. External lesions were also seen on one spot, one spotted sea trout, *Cynoscion nebulosus* (Cuvier), and one flounder. Neither of these studies isolated the aetiological agent from the affected fish.

Several studies have demonstrated the high prevalence of lesions on menhaden. Dykstra, Levine, Noga, Hawkins, Gerdes, Hargis, Grier & Te Strake (1989) collected menhaden from the Rappahannock River, Virginia, and found that 69% possessed external ulcerative lesions in November 1986, with 29% exhibiting lesions in January 1997. Noga, Levine, Dykstra & Hawkins (1988) collected 424 menhaden in the Pamlico River from July 1984 to July 1986 and found that all exhibited at least one lesion. Neither of these studies reported lesions on any other fish caught.

Several isolates of *Aphanomyces* spp. have been obtained from infected menhaden but their infectivity and viability in fish have not been well assessed. Recently, a strain of *A. invadans* isolated from menhaden in the 1980s and deposited in the American Type Culture Collection (ATCC-62427) was shown not to be *A. invadans* (Blazer *et al.* 2002) and not to be infectious to menhaden (Kiryu, Shields, Vogelbein, Zwerner, Kator & Blazer 2002). Indeed, another strain of *A. invadans* from the Wicomico River, Maryland (WIC), was isolated from infected menhaden and was shown to be identical to an *A. invadans* from Thailand (PA7) (Blazer *et al.* 2002); the endemic strain (WIC) was also highly infectious to menhaden (Kiryu *et al.* 2002; Kiryu, Shields, Vogelbein, Kator & Blazer 2003).

We explored the infectivity of *A. invadans* (WIC strain) when inoculated into four commonly occurring species: Atlantic menhaden, striped killifish, *Fundulus majalis* (Walbaum), mummichog *F. heteroclitus* (L.), and hogchoker, *Trinectes maculatus* (Bloch & Schneider). All four species are abundant in estuaries where UM is prevalent in menhaden; however, none of the species, other than Atlantic menhaden, has been observed with a high prevalence of lesions. The objectives of this study were to (i) determine whether menhaden are more susceptible to the oomycete than other estuarine fish, (ii) examine possible host or ecological barriers to infection that may explain differences in patterns of infection, and (iii) identify a more robust model of infection than menhaden for the laboratory setting. In addition, the histopathology of infections was examined in selected species of fish for comparison with that in infected menhaden.

Materials and methods

Fish collection and maintenance

Juvenile Atlantic menhaden (estimated fork length 90–110 mm) were collected by cast net from local tributaries of the York River and held in a flow-through system consisting of several 950-L fibreglass troughs supplied with filtered (35 μm) water from the York River (salinity 20–24‰, temperature 25–28 °C). Fish were fed daily with an algal paste (*Nannochloropsis*, ~68 million mL^{-1}, 5 mL diluted in 1 L deionized water) mixture and several grams of HiPro 0.5GR Debut Corey Starter (Corey Feed Mills Ltd, New Brunswick, Canada). For the experiments, fish were kept either in 76-L glass aquaria containing artificial sea water (Marinemix Forty Fathoms, Marine Enterprises International, Inc., Baltimore, MD, USA) at 12‰ at room temperature (~23 °C) or in 206-L glass aquaria with a flow-through system (~22‰ and 23 °C). The 76-L glass aquaria were equipped with two Whisper filters (size C, Tetra/Second Nature, Tetra Sales USA, Blacksburg, VA, USA) containing a filter filled with preconditioned crushed coral (Bed Rock, Marine Enterprises International, Inc.) (biological filtration) and activated carbon. Water quality was monitored weekly and water changes made daily. The 206-L glass aquaria received water from the York River. The water was passed through a sand filter and an activated carbon filter followed by 10- and 1-μm canister filters before being distributed to the tanks. System filters were rinsed daily and replaced every few weeks as needed.

Striped killifish (mean total length 90 mm) and mummichog (mean total length 80 mm) were collected by seine nets and minnow traps baited with crab or squid from local tributaries of the York River and held in 76-L glass aquaria containing artificial sea water (12‰) at room temperature. Each tank was equipped with two Whisper filters as described above. Fish were fed every other day with Tetra-Marine Fish Flakes. Mortalities were removed daily and water quality monitored weekly. Water changes were made weekly or more frequently as necessary.

Hogchoker (mean total length 105 mm) were collected by trawl (VIMS trawl survey) from the Chesapeake Bay and held in 76-L glass aquaria containing artificial sea water (10‰) at room temperature. The bottom of each tank was covered with a thin layer (~10 mm) of autoclaved sand collected from the VIMS beach. Each tank was equipped with two Whisper filters containing a filter filled with preconditioned crushed coral (biological filtration) and activated carbon. Fish were fed numerous food items such as squid, bloodworms, Tetra-Marine Fish Flakes, brine shrimp and bait fish; however, they did not appear to feed in captivity. Dead fish were removed daily and water quality monitored weekly. Water changes were made weekly or more frequently as necessary.

Oomycete culture and sporulation

An endemic isolate of *A. invadans*, WIC, was obtained from an Atlantic menhaden in Maryland (Blazer, Vogelbein, Densmore, May, Lilley & Zwerner 1999; US Geological Survey, Leetown, WV). The culture was routinely maintained in glucose-peptone-penicillin-oxolinic acid broth (GP-POX broth; Willoughby & Roberts 1994; Lilley *et al.* 1998) for 3–4 weeks at room temperature and sub-cultured onto GP-POX agar for 5 days.

For zoospore production, a piece of agar containing hyphae (6.0 mm diameter) was excised from the growing edge of a colony on glucose-peptone yeast agar (GPY agar; Lilley *et al.* 1998) and placed in 25 mL of GPY broth in a 12.5 cm^2 culture flask (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Cultures were grown for 5 days at 23 °C in darkness and washed three times with 0.45-μm filtered (Whatman International Ltd., Maidstone, England) and autoclaved Poropotank River water, augmented to 1‰. To induce sporulation, cultures were suspended in the water for 12–36 h at 23 °C in darkness. Zoospore densities were estimated with the aid of a haemocytometer (Neubauer/Bright-Line, Buffalo, NY, USA). Briefly, an aliquot of culture was preserved in 10% neutral-buffered formalin (1 culture:5 formalin), centrifuged for 10 min at 150 rcf, 1.8 mL of supernatant removed, the pellet resuspended and a 10-μL aliquot counted with the haemocytometer.

Zoospore injection study

Fish were removed from the tanks (one tank per treatment, sample size varied between species) using nets and anaesthetized using light doses of tricaine methanesulphate (MS-222). Each fish was injected with a 0.1 mL suspension estimated to contain 80 zoospores, using a 27-gauge, 12.7 mm needle and a

1.0-mL syringe. All fish were injected intramuscularly in the right flank just below the dorsal fin. Fish were allowed to recover from the anaesthesia in clean water before being returned to their aquaria. From 12 to 18 fish were used in each treatment with several treatments replicated twice. Control fish were treated as above but were injected with 0.1 mL of 1‰ sterile water. To confirm oomycete viability, triplicate samples of 0.1 mL of the suspension were repeatedly plated onto GP-POX agar and observed over time for the presence of fungal hyphae.

Gross examination and histological sampling

Aquaria were checked daily for 28 days and any dead and moribund fish were removed. All fish were examined for gross pathological changes and those exhibiting lesions were photographed. Live and moribund fish were killed with an overdose of MS-222, their lesions excised and fixed in 10% neutral-buffered formalin. Tissues were decalcified with formic acid-sodium citrate solution, dehydrated with ethanol, embedded in paraffin wax, and blocks sectioned transversely at 5 μm with a rotary microtome. Slides were stained with Harris' haematoxylin and eosin (H & E), Perl's Prussian blue, Sudan black B, and Grocott's methenamine silver nitrate (Luna 1968). Data on the prevalence of lesions and the percentage of mortality (frequency) were collected and analysed using chi-square tests.

Results

Lesion prevalence and mortality

Menhaden inoculated with *A. invadans* developed ulcerative lesions identical to those previously described (see Kiryu *et al.* 2002). Lesions appeared around 5 days post-injection (Fig. 1a) and by day 23 all menhaden were moribund or dead. No control menhaden developed lesions, although mortality was high (Table 1). Striped killifish developed ulcerative lesions similar to those in menhaden, but they appeared 7–10 days later (Fig. 1b,c) and at significantly higher prevalence ($\chi^2 = 26.311$, d.f. $= 3$, $P < 0.001$). Killifish experienced similar mortality to menhaden, with all but one fish either moribund or dead at the termination of the experiment. No control fish developed lesions, and the killifish serving as controls experienced negligible mortality.

Hogchokers also experienced a significantly higher prevalence of lesions than menhaden and mummichog ($\chi^2 = 26.311$, d.f. $= 3$, $P < 0.001$). In hogchokers, a reddened area around the site of injection appeared within 5 days with swelling of the area by day 7 (Fig. 1d). Hogchokers in control and experimental treatments all died on day 16, presumably from starvation or handling effects.

Mummichogs experienced a lower prevalence of lesions compared with the other species. Lesions appeared as reddened/purple areas under the skin along the dorsal surface, with some exhibiting curvature of the vertebral bone starting just behind the dorsal fin, an injection point (Fig. 1e). At no time did lesions develop into frank open ulcers, as did infections in menhaden and killifish. Mortality in mummichogs was low; less than half of those that developed lesions died (Table 1). At the end of the experiment, many of the infected mummichogs appeared to be recovering from the lesions (see histology below).

Histology

No menhaden were processed for histology because the pathology of *A. invadans* infections in menhaden has been well described (Blazer *et al.* 1999, 2002; Kiryu *et al.* 2002, 2003). Striped killifish with lesions exhibited similar histopathological changes as menhaden. By day 14 post-injection, immature, newly generated granulomas with a few layers of epithelioid cells were seen (Fig. 2a). Brown-pigmented hyphae were sporadically observed near surface skin tissue and sometimes within the core of the granulomas but were rarely seen within the deeper infected areas of skeletal muscle. A few multi-nucleate giant cells were observed within the lesion areas. At 18 days post-injection, multi-nucleate giant cells, both foreign body and Langhans' types, were more abundant (Fig. 2b), and occasionally hyphae were detected within the cell body of Langhans' giant cells. At day 20, the numbers of multi-nucleate giant cells dropped, and by day 25 they were completely absent from the observed tissues (Table 2).

Lesions in mummichogs were characterized by infiltrates of inflammatory cells including macrophages, fibrocytes and granulomas at day 20 post-injection (Table 2). The granulomas surrounded discoloured, brown hyphae that filled the necrotic spaces of the skeletal muscle. Haemorrhage and congestion were seen near the skin surface. Fungal

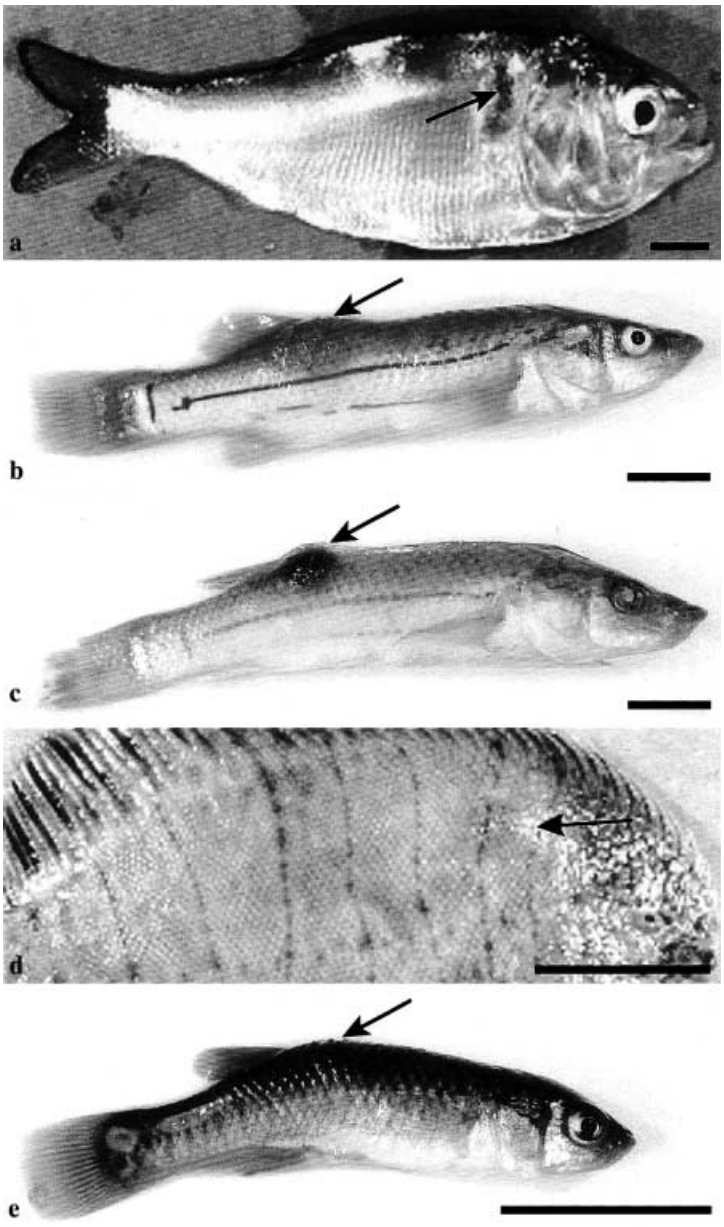

Figure 1 Gross pathology of *Aphanomyces invadans* infections. (a) Menhaden, approximately 5 days post-inoculation showing early lesion (arrow). Striped killifish, 20 days (b) and 18 days (c) post-inoculation showing ulcerative lesions similar to those in menhaden (arrows). (d) Hogchoker, 6 days post-inoculation. Reddened lesion developing at the injection site (arrow). (e) Mummichog, 20 days post-inoculation showing curvature of vertebral bones (lordosis, arrow).

invasion extended into the skeletal muscle of the side of the fish opposite from that in which zoospores were initially inoculated and intensive granulomas were detected within the areolar connective space between the spinal cord and the vertebral column (Fig. 2c). Hyphae at remote sites stained blue to black in H & E, as has been previously observed in menhaden by Kiryu *et al.* (2002).

At 27 days post-injection, the hyphae were swollen and prominently brown (Fig. 2d,e). Brown-coloured hyphae were degenerated in morphology and fragments of hyphae were observed within the cores of the granulomas. The brown-coloured pigments contained ceroid as they were positive for Sudan black and negative for Perl's Prussian blue. The hyphae were easily detected with Grocott's methenamine silver nitrate stain. Eosinophilic granular cells (EGCs) were consistently observed along with elongate-shaped granulomas surrounding the hyphae and the number of EGCs had increased in comparison with those present at day 20. Inflammatory cells, such as macrophages, were still present along the myosepta (Fig. 2d,e).

Table 1 Lesion prevalence and mortality from infection with *A. invadans* in menhaden, mummichog, killifish and hogchoker

Species/treatment	Lesions	Mortality without lesions	Mortality with lesions
Menhaden	13/24	8/24	13/24
Experimental (%)	54.2	33.3	54.2
Menhaden	0/24	12/24	0/24
Control (%)	0.0	50.0	0.0
Mummichog	22/48	2/48	10/48
Experimental (%)	45.8	4.2	20.8
Mummichog	0/30	1/30	0/30
Control (%)	0.0	3.3	0.0
Killifish	29/34*	3/34	28/34
Experimental (%)	83.5	8.8	82.4
Killifish	0/23	4/23	0/23
Control (%)	0.0	17.4	0.0
Hogchoker	28/30*	2/30	28/30
Experimental (%)	93.3	6.7	93.3
Hogchoker	0/14	14/14	0/14
Control (%)	0.0	100	0.0

*Significantly higher prevalence of lesions compared with menhaden and mummichog by chi-squared test ($P < 0.001$).

Several mummichogs exhibited signs of a healing response characterized by basophilic regenerating myocytes (Fig. 2f).

Discussion

Clearly *A. invadans* is infectious to several species of estuarine fish from the mid-Atlantic coast of the USA when inoculated as secondary zoospores. All of the fish species tested inhabit similar estuarine environments, yet Atlantic menhaden appears to be the only species that is consistently found with ulcerative lesions. This indicates that subtle host or environmental barriers to infection limit the development of *A. invadans*-induced lesions in other species. We suggest that the increased prevalence in Atlantic menhaden may be due in part to the behaviour of the species. Juvenile menhaden form large schools of thousands of fish that frequent shallow, low salinity areas. It is in these low salinity areas that zoospore attachment must occur as motility of the zoospores ceases above 2‰ (Blazer *et al.* 1999, 2002; Kiryu *et al.* 2002). Once induced, sporulation can continue for 12–60 h (Y. Kiryu & J.D. Shields, unpublished data). Thus, an entire school of menhaden that enters a low salinity region during sporulation of *A. invadans* may be exposed to secondary zoospores. This may explain the high prevalences (75–90%) of lesions reported from the Pamlico and Neuse river estuaries. In contrast, mummichogs and killifish are territorial and, at least for mummichog, maintain a home range of 36–38 m from which they rarely migrate (Abraham 1985). Schools sometimes form, but are small, numbering at most only hundreds and then only rarely. Mummichogs also prefer higher salinity areas, above 8‰, although killifish are often found in fresh waters (Abraham 1985). Thus, the low prevalence and infection by *A. invadans* in mummichogs may be ameliorated, in part, by the salinity preferences of these minnows; but salinity preferences do not explain the extremely low prevalences observed in other species.

Menhaden have an oily flesh, a thin, fragile epidermal layer, and readily lose their deciduous scales; characters that may facilitate attraction of, and infection by, zoospores of *A. invadans* (Y. Kiryu & J.D. Shields, unpublished data). Menhaden tissue has also been reported to support increased growth of hyphae of *A. invadans* when compared with agar (Dykstra *et al.* 1989). Thus, the predilection of *A. invadans* for Atlantic menhaden may also reflect the susceptibility of the host to stress, which facilitates entry, and by the nature of the oily flesh, which serves as a highly supportive nutritional source for the oomycete. Killifish showed a similar prevalence of lesions and severe pathology when inoculated with the oomycete, albeit over a longer time course. However, mummichogs were only mildly affected by the oomycete showing not only a lower prevalence of lesions but also significant healing. Mummichogs were able to eliminate the penetrating hyphae and regenerate damaged muscle tissue, in many cases showing complete healing from the infection.

The response of the mummichog was similar to that of the rosy barb, *Puntius schwanefeldi* (Bleeker) (Khan, Marshall, Thompson, Campbell & Lilley 1998). After injection with zoospores of *A. invadans*, rosy barbs developed macrophage infiltration around the injection site and by day 20 there was evidence of muscle regeneration in the tissues. However, rosy barbs experienced a 100% mortality rate after 22 days as opposed to the lower 20.8% mortality in mummichogs. Resistance to infection by *A. invadans* has been reported to occur in tilapia, stickleback and roach (Khan *et al.* 1998). Carp, *Cyprinus carpio* L., inoculated with *A. piscicida* showed no gross signs of inflammation and mycotic lesions occurred only around the injection site (Wada, Rha, Kondoh, Suda, Hatai & Ishi 1996). Thus, host resistance, as indicated by species differences, can also be a major barrier to infection.

The occurrence of multi-nucleate giant cells has been reported for fish exhibiting mycotic

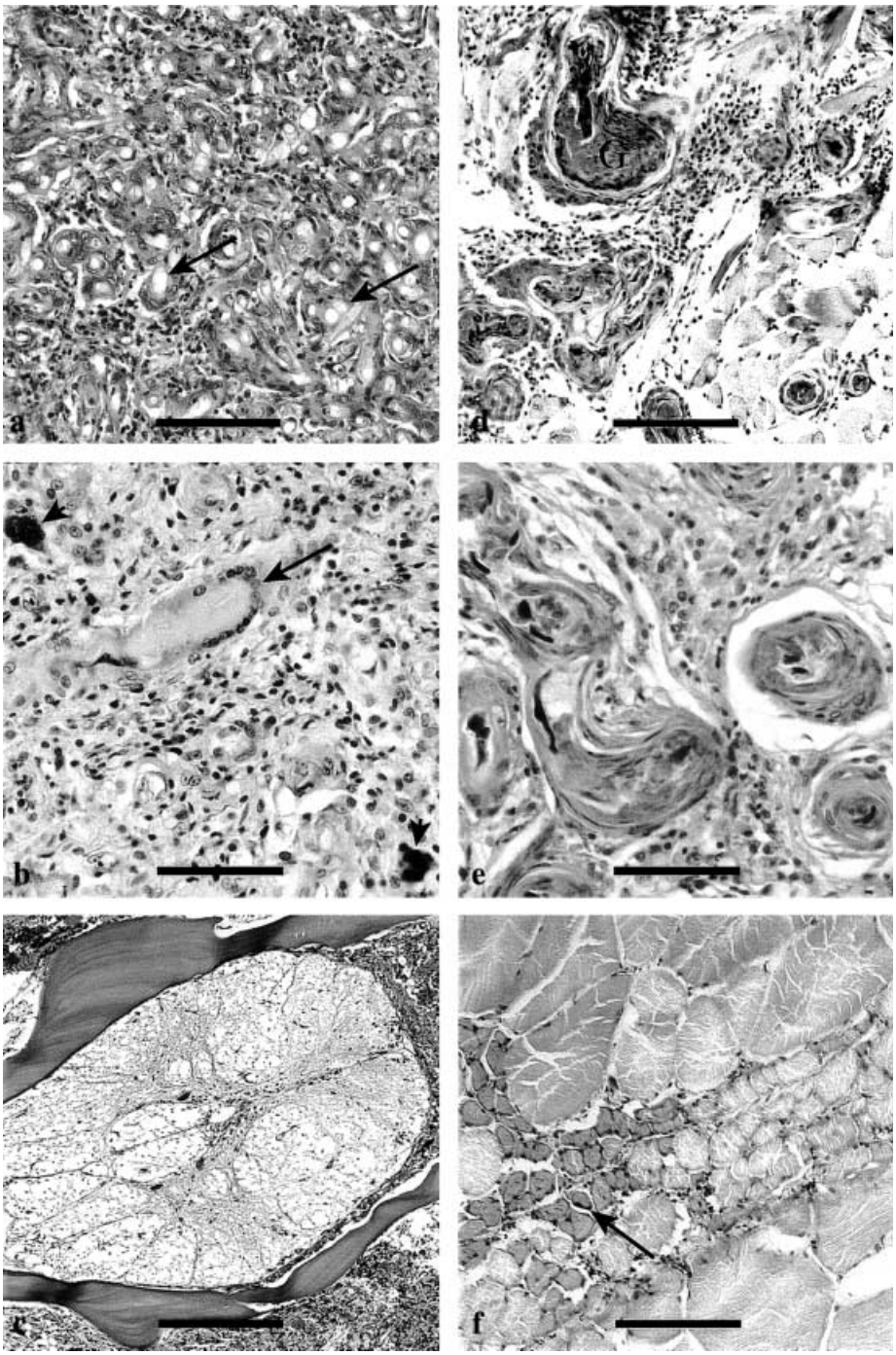

Figure 2 (a) Striped killifish, 14 days post-inoculation with *Aphanomyces invadans*. Hyphae (arrows) surrounded by a few layers of epithelioid cells, associated with myositis (H & E, bar = 100 μm). (b) Striped killifish, 18 days post-inoculation. Multi-nucleate giant cells, both foreign body type (short arrow) and Langhans' type (long arrow) engulfing hyphae (H & E, bar = 50 μm). (c) Mummichog, 20 days post-inoculation. Granuloma within the areolar connective tissues (H & E, bar = 200 μm). (d) Mummichog, 27 days post-inoculation. Brown pigmented hyphae are located in the core of the granuloma (G) (H & E, bar = 200 μm). (e) Higher magnification of (d). Note ceroid droplets in the core of the granuloma (H & E, bar = 50 μm). (f) Mummichog, 27 days post-inoculation. Remodelling of the skeletal muscle is indicated by regenerating basophilic myocytes (arrow) (H & E, bar = 100 μm).

Table 2 Characteristic histopathological findings compared among three fish species infected with *Aphanomyces invadans*

	Fish species		
Histopathological findings	Killifish	Mummichog	Menhaden[a]
Granulomas	Immature	Developed	Developed
Eosinophilic granulocytes	+	+ + +	+ + +
Multi-nucleate giant cells	+ +	–	–
Brown hyphae	+	+ + +	–
Wound healing	–	+ + +	+ +

[a] Data from Kiryu *et al.* (2002, 2003). Note that salinity during tests was 6‰.
Severity code: –, absent; +, mild; + +, moderate; + + +, severe.

granulomatosis (MG), which is now considered to be synonymous with EUS (Lilley *et al.* 1998). In Japan, Miyazaki & Egusa (1972, 1973) classified the cellular defence to MG into two types of responses by the multi-nucleate giant cells. One type is characterized by the emergence of multi-nucleate giant cells that engulf hyphae at the site of granulation tissues. In the other type of response, the multi-nucleate giant cell does not engulf the hyphae. Wada *et al.* (1996) also observed MG in artificially infected ayu and carp. Both possessed multi-nucleate giant cells that engulfed the hyphae; and especially in the carp, hyphae appeared to be successfully eliminated by the giant cells. Both types of multi-nucleate giant cells have been found in crucian carp, *Carassius auratus* (L.), and trident goby, *Tridentiger obscurus* (Temminck & Schlegel), but neither type has been reported from snakehead, *Channa argus* (Cantor), or grey mullet (Miyazaki & Egusa 1973). Khan *et al.* (1998) suggested that giant cells do not play a major role in eliminating hyphae in rainbow trout infected with EUS. In our studies, Atlantic menhaden and mummichog had no multi-nucleate giant cells, whereas striped killifish possessed multi-nucleate giant cells that engulfed the hyphae. Thus, the relevance of these types of responses to the outcome of the infection is unclear.

Lastly, menhaden can be difficult to maintain. They have deciduous scales and are easily injured during capture and subsequent handling. Being pelagic, they also require large tank space with rigorous attention to water quality. In our inoculation trials, killifish developed lesions similar to those observed in menhaden. As a pilot study, killifish were exposed in an aqueous challenge to 330 zoospores mL^{-1} for 5.5 h. After 28 days at 12‰, one of eight fish developed a lesion (R.A. Johnson & J.D. Shields, unpublished data). That is, killifish were susceptible to infection via inoculation and via bath exposure. Therefore, killifish will be a more robust laboratory model for future studies with *A. invadans* in the mid-Atlantic USA.

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