

Induction of Skin Ulcers in Atlantic Menhaden by Injection and Aqueous Exposure to the Zoospores of *Aphanomyces invadans*

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Abstract.—The infectivity and role of *Aphanomyces invadans* in the etiology of skin ulcers in Atlantic menhaden *Brevoortia tyrannus* were investigated with two laboratory challenges. In the first experiment, Atlantic menhaden received subcutaneous injections with secondary zoospores from one of three cultures of *Aphanomyces*: WIC (an endemic isolate of *A. invadans* in Atlantic menhaden from the Wicomico River, Maryland), PA7 (an isolate of *A. invadans* from striped snakehead *Channa striata* (also known as chevron snakehead), infected with epizootic ulcerative syndrome from Thailand), and ATCC-62427 (an isolate from Atlantic menhaden from North Carolina). Fish were injected with 1.9×10^2 (WIC-low), 1.9×10^3 (WIC-high), 5.2×10^2 (PA7), or 6.0×10^2 (ATCC-62427) zoospores and held in static water at 23.5°C (6‰ salinity) for 21 d. Both low and high doses of WIC caused incipient, granulomatous lesions after 5 d. Fish injected with the high-dose WIC died within 7 d. All fish injected with the low-dose WIC were dead after 10 d. Fish injected with zoospores of PA7 developed lesions after 9 d. Fish injected with the ATCC-62427 isolate or those that received subcutaneous injections of sterile water (controls) did not develop lesions. In the second experiment, fish were bath-exposed with zoospores of the WIC isolate after various trauma-inducing treatments. These treatments consisted of handling fish with a net (net stress, exposed for 2 h to either 70 or 700 zoospores/mL), physically removing a few scales (trauma, exposed for 1 h to 700 zoospores/mL), or acclimating fish with less handling (acclimated, untraumatized, exposed for 5.5 h to 110 zoospores/mL). Unexposed fish served as controls. Mortality ranged from 94% to 100% for net-handled and traumatized fish, with the prevalence of ulcerous lesions ranging from 70% to 79% in net-handled fish. However, mortality was 24% for the “untraumatized” fish and the prevalence of lesions was 32%. Fish injected with or exposed to bath challenges of zoospores developed lesions that were grossly and histologically identical to those observed in naturally infected Atlantic menhaden from several estuaries and rivers along the mid-Atlantic coast of the USA. The deeply penetrating ulcers were characterized by dermatitis, myofibrillar degeneration, and deep, necrotizing granulomatous myositis. Experimentally induced lesions, however, exhibited invasiveness, often involving the kidney. Injected or bath-exposed fish developed incipient granulomas after 5 d, which progressed to overt lesions over 7–9 d. We have here demonstrated that ulcerative skin lesions can be experimentally induced in Atlantic menhaden after exposure to oomycete zoospores of an endemic strain of *A. invadans*.

Since the mid-1980s, high prevalences of skin ulcers have been reported in Atlantic menhaden *Brevoortia tyrannus* from coastal waters and estuaries of the eastern USA (Hargis 1985; Noga 1993; Blazer et al. 1999). These ulcers are circular, often located near the anus, and deeply penetrating, with peripheral hemorrhage, extensive necro-

sis, and tissue loss (Noga and Dykstra 1986; Noga 1993). Microscopically, these ulcers are characterized by extensive myonecrosis and granulomatous myositis. This condition was initially called ulcerative mycosis (UM) by Noga and Dykstra (1986) because highly invasive, deeply penetrating oomycete hyphae were consistently observed in lesions present in menhaden (Noga et al. 1988; Noga 1993; Blazer et al. 1999; Dykstra and Kane 2000). *Aphanomyces* spp. were isolated in the 1980s from menhaden with UM (Dykstra et al.

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1986; Noga and Dykstra 1986); however, experimental transmission studies with those isolates were inconclusive (Noga 1993).

Recently much scientific and public attention has focused on the association of ulcerative lesions in Atlantic menhaden with exposure to *Pfiesteria piscicida*, a toxin-producing, bloom-forming, heterotrophic dinoflagellate (Burkholder et al. 1992, 1995, 2001). *Pfiesteria piscicida* and related *Pfiesteria*-like organisms have been implicated as causative agents of acute fish kills involving Atlantic menhaden in estuaries of North Carolina and other U.S. coastal areas (Burkholder et al. 1992, 1995, 2001). *Pfiesteria* toxin or toxins are the reported causes of the lesions, and the oomycetes *Aphanomyces* spp. are considered to be opportunistic invaders that colonize the open lesions (Burkholder and Glasgow 1997). Although superficial loss of epidermis has been reported in laboratory exposure of fishes to *P. piscicida*, the deeply penetrating granulomatous ulcers commonly observed in wild Atlantic menhaden have not been reproduced by experimental exposure to these dinoflagellates (Noga et al. 1996; Vogelbein et al. 2001).

The microscopic pathology of UM in Atlantic menhaden is identical to that described for epizootic ulcerative syndrome (EUS) of Indo-Pacific fishes, now known to be caused by *Aphanomyces invadans* (Miyazaki and Egusa 1972; Fraser et al. 1992; Wada et al. 1994; Lilley et al. 1998). The EUS lesions have been successfully reproduced in the laboratory by injection of fishes with hyphae (Hatai et al. 1977; Roberts et al. 1993; Blazer et al. 2002, this issue) and secondary zoospores (Wada et al. 1996; Lilley and Roberts 1997; Catap and Munday 1998; Khan et al. 1998), cohabitation with infected fish (Cruz-Lacierda and Shariff 1995), and bath exposures to secondary zoospores after exposure to acidified water (Callinan et al. 1996). Low salinities (2–6‰) were correlated with the occurrence of UM in Atlantic menhaden (Dykstra et al. 1986), and the associated *Aphanomyces* sp. isolated from the ulcers grew best in low-salinity agars (Hearth and Padgett 1990; Blazer et al. 2002). Key factors in outbreaks of EUS in Southeast Asia are warm temperatures (>19°C) and low salinity (Chinabut et al. 1995; Lilley et al. 1998), conditions that appear to be necessary to the transmission of both UM and EUS and that may facilitate the virulence of these agents, as observed by deaths in the field.

Blazer et al. (2002) have recently isolated and identified a strain of *A. invadans* from Chesapeake Bay Atlantic menhaden. Further they have shown

that this isolate of *A. invadans* is widespread along the East Coast and is probably infective to menhaden. We therefore conducted laboratory challenges of Atlantic menhaden to (1) fulfill Koch's postulates (Evans 1976) by experimentally reproducing the gross and microscopic lesions associated with UM in Atlantic menhaden; (2) examine infectivity to Atlantic menhaden of three different isolates of *Aphanomyces* spp. by injection and bath challenge studies; and (3) describe the development of the characteristic ulcerative lesions in menhaden experimentally exposed to a pathogenic isolate of *A. invadans* endemic to Chesapeake Bay.

Methods

Fish collection and maintenance.—Atlantic menhaden used in laboratory exposure studies were derived from two sources. Fish used in the zoospore injection trials (age-class 1; $N = 46$; mean weight = 14.6 g, range = 8.6–27.3 g; mean total length = 123 mm, range = 106–147 mm) were collected from upper Chesapeake Bay tributaries and kindly provided to us by John Jacob at the University of Maryland. Fish were kept in a static water-recirculating system consisting of two 712-L fiberglass troughs containing 35- μ m-pore-filtered York River water diluted to 12‰ salinity with well water. Temperature was maintained between 20°C and 23°C with a heater, and 25% of the water was changed weekly to maintain water quality.

Fish used in the bath challenge experiments (age-class 1; $N = 82$; mean weight = 4.9 g, range = 0.7–14.9 g; mean total length = 80 mm, range = 44–117 mm) were collected by cast net from Sarahs Creek, York River, Virginia. Fish were held as above in flow-through filtered (35 μ m) York River water with a salinity of 20–24‰ and a temperature of 22–28°C. Holding Atlantic menhaden at the higher salinity had the advantage of avoiding infection by *A. invadans* because secondary zoospores sporulate only at low salinities (0–2‰; Blazer et al. 2002).

Fish were fed approximately 10 g of algal paste daily, supplemented with several grams of ground Tetra Marine Flakes (Tetra Werke, Melle, Germany) or HiPro 0.5GR Debut Corey Starter (Corey Feed Mills Ltd., New Brunswick, Canada).

Oomycete culture and sporulation.—Three isolates of *Aphanomyces* spp. were used in this study: (1) WIC, an endemic isolate of *A. invadans* from a lesioned Atlantic menhaden in Maryland (U.S. Geological Survey, Leetown, West Virginia; Blazer et al. 1999); (2) PA7, an isolate of *A. invadans*

from striped snakehead *Channa striata* (also known as chevron snakehead), from Nonthaburi, Thailand (Lilley and Roberts 1997); and (3) *Aphanomyces* sp. (ATCC-62427), an isolate from Atlantic menhaden from North Carolina (American Type Culture Collection, Rockville, Maryland; Dykstra et al. 1989). Isolates were routinely maintained on glucose peptone–penicillin–oxolinic acid agar (GP-POX agar; Willoughby and Roberts 1994; Lilley et al. 1998) for 5 d, sub-cultured into GP-POX broth, and held for 3–4 weeks at room temperature.

For zoospore production, a piece of agar containing hyphae from the growing edge of a colony was excised and placed in 25 mL of GP-POX broth in a 25-mL tissue culture flask (Becton Dickinson Labware, Franklin Lakes, New Jersey). For large-scale sporulation, 20 pieces of agar containing hyphae were aseptically excised and placed in 500 mL of GP-POX broth in a 600-mL tissue culture flask (Becton Dickinson Labware). Cultures were allowed to grow for 7 d and washed twice with filter-sterilized (Whatman 54; Whatman International Ltd., Maidstone, England) estuarine water from the Great Wicomico River, Virginia, adjusted to 1‰ salinity. To induce sporulation, cultures were resuspended and incubated in sterilized water (Great Wicomico River, adjusted to 1‰ salinity) for 12–24 h at room temperature. Total zoospore densities were estimated by removing an aliquot of culture medium that was preserved in 10% neutral-buffered formalin (4:1), centrifuging for 10 min at $3,500 \times$ gravity, and counting the zoospores in a 10- μ L aliquot with a hemacytometer (Neubauer/Bright-Line, Buffalo, New York).

Zoospore injection study.—Fish were acclimated for 7 d in five 76-L glass aquaria (8–10 fish/tank) containing 6‰ artificial seawater (Marinemix Forty Fathoms; Marine Enterprises International, Inc., Baltimore, Maryland) maintained at room temperature (daily mean $23.5^{\circ}\text{C} \pm 0.57^{\circ}\text{C}$ SE). Tanks were equipped with Whisper filters (Size C; Tetra/Second Nature, Tetra Sales USA, Blacksburg, Virginia) containing a chemically preconditioned filter bag filled with crushed coral (biological filtration). Zoospore dosages for subcutaneous injections were adjusted as follows (zoospores/mL): 1.9×10^4 (high-dose WIC), 1.9×10^3 (low-dose WIC), 5.2×10^3 (PA7), or 6.0×10^3 (ATCC-62427). Fish were lightly anesthetized in 75 mg/L tricaine methanesulfonate (MS-222) and then injected with 0.1 mL of the appropriate suspension by using a 27-gauge, 12.7-mm needle and a 1-mL syringe. Each fish was injected on the left flank,

just below the dorsal fin and above the lateral line. Control fish were treated in the same manner but received 0.1-mL injections of sterile water (1% saline).

To confirm oomycete viability, 0.1 mL of each inoculum was plated in triplicate onto GP-POX agar. After 4 d of incubation of the plates at room temperature, 7–11 mycelial colonies were detected in low-dose WIC; 30–80% of the plate was covered with mycelia in high-dose WIC; 13–18 mycelia colonies were detected in the PA7 dose; and the entire plate was covered by mycelia in the ATCC dose. No growth was observed in controls.

Fish were monitored and fed daily for 21 d. Tissues from one or two live fish selected at 5, 9, and 14 d postinjection; all of the moribund fish; and all surviving fish at 21 d postinjection were examined histologically. Dissolved oxygen, pH (Orion model 290A; Orion Research Inc., Boston), ammonium, and nitrite concentrations (Hach, Loveland, Colorado) were recorded at the middle and end of the study. Water quality characteristics ranged from 6.7 to 7.0 mg/L for dissolved oxygen, 7.1–7.9 for pH, 0.1–0.7 mg/L for total ammonium, and 0–3.0 mg/L for nitrite. At the same time, the absence of presumptive *Pfiesteria*-like dinoflagellates was confirmed by microscopic examination of 25–50 mL of tank water fixed with Lugol's iodine. At 7 d postinjection, half of the water in each tank was changed.

Zoospore bath challenges.—Fish (16–20/tank) were acclimated for 16–20 d in five 76-L glass aquaria containing 20‰ artificial seawater and equipped with preconditioned Whisper filters as described above. Each tank was held at room temperature (daily mean, $23.0^{\circ}\text{C} \pm 1.26^{\circ}\text{C}$ SE). Fish in the five aquaria were assigned to one of five treatments: (1a) net-stress, low zoospore concentration; (1b) net-stress, high zoospore concentration; (1c) scale removal, high zoospore concentration; (2) untraumatized, low zoospore concentration; and (3) unexposed control. Only the WIC isolate was used for the bath challenge trials.

For treatments 1a, 1b, and 1c, salinity was decreased from 20‰ to 6‰ by performing 25% water exchanges three times every 3–5 d over a 16-d period. Salinity adaptation was necessary to accommodate a low salinity (1‰) for zoospore viability during bath exposures (Blazer et al. 2002). After acclimation, fish in treatments 1a and 1b were transferred by dip net into two 4-L exposure vessels at 1‰ (8–10 fish each) containing either 70 (1a; low concentration) or 700 (1b; high concentration) zoospores/mL and exposed for 2 h.

Fish were then returned to the same holding tanks by using a dip net. Fish in treatment 1c were lightly anesthetized, after which each had several scales removed with forceps from an area on the left side below the dorsal fin and above the lateral line. Subsequently, 1 mL of a high-concentration zoospore suspension (700/mL) was gently sprayed directly onto the traumatized area. The fish were then placed for 1 h in a 4-L vessel containing the same high concentration of zoospores for additional exposure and recovery from the anesthetic. Fish were then returned to the same holding tank.

For treatments 2 and 3, the salinity adjustment was conducted in the same manner as above but further lowered from 6‰ to 1‰ by using daily 25% water exchanges over an additional 4-d period. Acclimated fish were held at 1‰ for 24 h before the trials began. For treatment 2, the volume of each tank was decreased to 30 L, filtration was discontinued, and zoospores were added to a concentration of 110/mL. The control, treatment 3, consisted of the same protocol as treatment 2, except that 1 L of sterile water (1‰) was added instead of zoospores. Fish in treatments 2 and 3 were exposed for 5.5 h under aeration. Oomycete exposure was stopped by adding 12‰ artificial seawater to increase the salinity of each tank to 6‰, which inhibited secondary zoospore production and motility (Y. Kiryu, unpublished data). Zoospore viability was confirmed during the exposure period by examining water samples from each treatment microscopically. Fish in all treatments were monitored daily for 21 d and fed as described above. We histologically processed tissues from two fish per tank at 5 d postexposure, from all of the moribund fish, and from all surviving fish at 21 d postexposure.

Gross examination and histological processing.—Aquaria were monitored at least twice daily, and dead and moribund fish were removed. All of the fish were weighed, measured (total length), examined for gross pathology, and photographed (for fish with lesions only). Live and moribund fish were killed by overdose with 100 mg/L MS-222, after which the injection site and lesions were excised and fixed in 10% neutral-buffered formalin. Tissues were decalcified with formic acid–sodium citrate solution (Luna 1968), dehydrated with ethanol, and embedded in paraffin, and the paraffin blocks were sectioned in 5- μ m-thick slices with a rotary microtome. All slides were stained with Harris' hematoxylin and eosin. Subsamples of selected slides were stained with Grocott's meth-

amine–silver nitrate (GMS) to demonstrate oomycete hyphae in the tissues (Luna 1968).

Results

Injection Study

Grossly visible lesions developed rapidly in fish injected subcutaneously with secondary zoospores of the WIC isolate. Initial clinical signs in moribund fish appeared at 4 d postinjection in the WIC–high-dose exposure and 5 d postinjection (Figure 1a) in the WIC low-dose exposure (lesion size, 4 mm \times 6 mm) and consisted of reddened, slightly raised focal lesions (2 mm \times 5 mm) at the injection site. Lesions increased in size over time (Figure 1b) and were generally larger (14 mm \times 15 mm) in the WIC low-dose treatment than those (10 mm \times 15 mm) in the WIC high-dose exposure. All fish injected with the high dose (1,900 zoospores/fish), and 8 of 10 fish injected with the low dose (190 zoospore/fish) developed characteristic lesions. The two fish from the WIC low dose that did not develop external lesions died at 3 d postinjection. All fish injected with WIC zoospores died within 4–7 d (WIC high dose) or 3–10 d (WIC low dose) postinjection (Table 1).

Fish injected with secondary zoospores of the PA7 isolate developed typical skin lesions more slowly and survived longer than fish injected with the WIC isolate, despite receiving a higher dose of zoospores (WIC low dose = 190 zoospores/fish versus PA7 low dose = 520 zoospores/fish; Table 1). Typical incipient lesions (10 mm \times 15 mm) had developed at the injection site by 9 d postinjection. Fish mortality was 100%, with most suc-

TABLE 1.—Mortality and prevalence of lesions on Atlantic menhaden at 21 d after inoculation with secondary zoospores of *Aphanomyces* spp.; WIC-High = isolates from Wicomico River, Maryland, high dose; WIC-Low = WIC isolate, low dose; PA7-Low = isolate from Thailand, low dose; and ATCC-Low = ATCC-62427 isolate, low dose.

Isolate and treatment dose	Exposure level (zoospores/fish)	Dead/total ^a	Focal ulcerous lesions/total	
			Live ^b	Dead ^c
WIC-High	1.9×10^3	8/8	2/2	8/8
WIC-Low	1.9×10^2	7/7	3/3	5/7
PA7-Low	5.2×10^2	5/5	2/4	4/5
ATCC-Low	6.0×10^2	2/5	0/7	0/2
Control		0/4	0/8	

^a Some fish were censored for histology

^b One or two live fish were sampled for histology on days 5, 9, and 14 after inoculation.

^c Includes moribund fish.

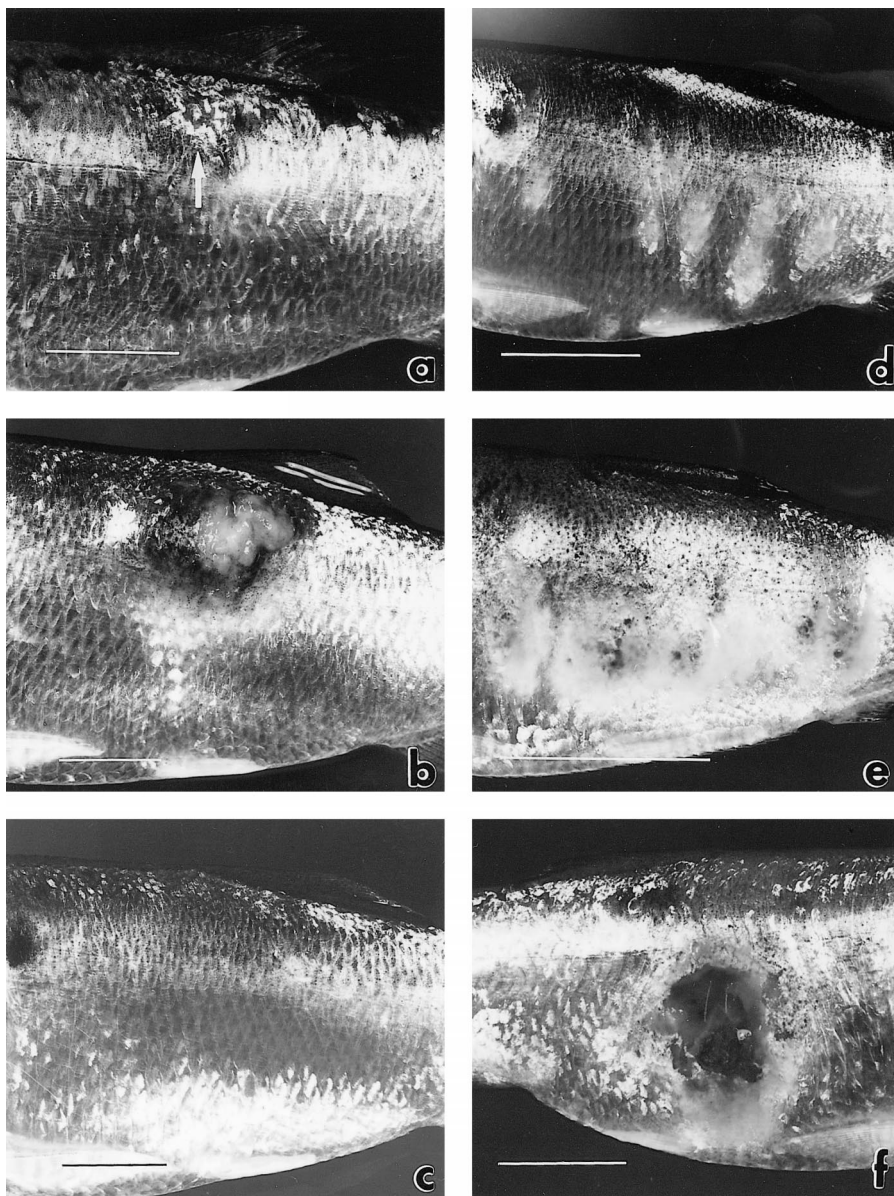


FIGURE 1.—Gross pathology of Atlantic menhaden subcutaneously injected (a–c) or bath-exposed (d–f) to secondary zoospores of *A. invadans* (WIC isolate). Bar = 1 cm. (a) Incipient lesion (arrow) at the injection site (5 d postinjection); (b) Advanced lesion exhibiting central necrosis at the injection site (9 d postinjection); (c) Control fish sham-injected with 1‰ saline water (5 d postinjection); (d) Multifocal early lesions in bath-exposed fish exhibiting reddening and striated pattern (5 d postbath exposure; net-stress high dose, 700 zoospores/mL); (e) Multiple pale, striated lesions (7 d postbath exposure; net-stress high dose, 700 zoospores/mL); (f) Focal advanced, deeply penetrating ulcer exposing underlying muscle (17 d postexposure; treatment 2, 110 zoospores/mL).

cumbing within 3 to 21 d postinjection. One fish that died 3 d postinjection did not exhibit lesions. Moreover, gross, externally visible signs were not observed in two fish killed for histological examination at 5 d postinjection; however, these fish

had microscopic granulomatous lesions, indicative of the presence of the oomycete at the injection site.

Fish injected with ATCC-62427 or the 1‰ salinity water (controls) did not develop gross le-

TABLE 2.—Mortality and prevalence of lesions on Atlantic menhaden following treatments that induced trauma, handling with net (net stress) or scale removal (trauma), and on an untraumatized group at 21 d after exposure to a bath containing secondary zoospores of *Aphanomyces invadans* isolates from the Wicomico River, Maryland.

Treatment and number	Exposure time (h)	Exposure concentration (zoospores/mL)	Dead/total ^a	Diffuse reddening lesions ^b /total	Focal ulcerous lesions/total	
					Live ^c	Dead ^d
1a net stress	2.0	70	17/18	6/20	5/5	9/15
1b net stress	2.0	700	17/17	4/19	2/2	13/17
1c trauma	1.0	700	16/16	12/16		4/16
2 untraumatized	5.5	110	4/17	0/19	4/15	2/4
3 control	5.5	0	1/13	0/14	0/13	0/1

^a Some fish were censored for histology.

^b Lesions found on dead fish only. Total includes fish censored for histology.

^c Live fish were sampled for histology at 5 d postexposure.

^d Includes moribund fish.

sions during the course of the study (Figure 1c). None of the fish from the control treatment died, but two of five fish injected with ATCC-62427 died after 12 and 14 d postinjection (Table 1). No dinoflagellates were observed in any of the treatments throughout the study.

There were no gross behavioral changes evident between fish given different treatments. All fish, including those exhibiting ulcerous lesions at the injection site and those in bath exposures, fed well until they became moribund. Morbidity entailed fish swimming less rapidly, often in sideways or in otherwise altered orientation, and often showing erratic movements. No moribund fish recovered. They usually died within a few hours.

Bath Challenges

Fish rapidly developed skin ulcers when challenged with WIC zoospores in bath exposures after net stress (treatment 1a: low dose; treatment 1b: high dose). The first visible lesions were multifocal, hyperemic, slightly raised areas on the trunk. Initial lesions were arranged in a striated pattern (Figure 1d) and were first detected 5 d after exposure to the zoospores. The striated pattern remained, but reddening was reduced at 7 d postinjection (Figure 1e). Mortality was high in the net-stressed fish, reaching 100% after 9 d for fish exposed to the high concentration of WIC and 21 d for those exposed to the low concentration. However, 4 of 19 fish exposed to the high concentration of WIC, and 6 of 20 fish exposed to the low concentration, developed diffuse reddening of the trunk and rapidly died (1–3 d postexposure) without developing the typical ulcerous lesions (Table 2). Similarly, in Treatment 1c (traumatized fish), 12 of 16 fish died rapidly by 3 d postexposure after developing diffuse reddening of the trunk. The re-

maining four fish died at 8–9 d postexposure with severe ulceration that exposed underlying musculature at the site where scales had been removed.

In treatment 2 (untraumatized, low dose), the first incipient ulcers, usually single and focal, occurred notably on the trunk at 5 d postexposure. Over time, these lesions developed into deeply penetrating focal ulcers that exposed the underlying musculature (Figure 1f). The prevalence of focal ulcers was moderate, with 6 of 19 fish exhibiting lesions (Table 2). Mortalities were low; only 4 of 17 fish died during the course of the study. None of the fish from the control treatment (treatment 3) showed lesions, and only one fish died at 17 d postexposure from unexplained causes. None of the fish in treatments 2 and 3 exhibited the diffuse reddened lesions that we observed in the net-stressed and traumatized fish (Table 2).

Because of constraints on aquarium space, we did not include an unexposed control group for the net-stress treatment. The presence of lesions on the untraumatized fish and the lack of lesions on the unexposed controls, combined with the increased prevalence of lesions on the traumatized fish, indicated that the oomycete was more infectious to those fish that had a preexisting portal of entry.

Koch's postulates.—During the bath experiment (high-dose WIC), we successfully reisolated *A. invadans* from two freshly dead fish after 7 d postexposure, using methods described by Lilley et al. (1998). Before the reisolation, the presence of hyphae was confirmed microscopically from skin scrapings. The morphology and growth patterns of the reisolated oomycete were identical to those of the WIC isolate. Note that the WIC strain was originally isolated from Atlantic menhaden. Atlantic menhaden have now been challenged with

it via inoculation and batch exposures, and it has been reisolated on several occasions, thus fulfilling Koch's postulates. No dinoflagellates were observed in the tanks throughout the experiments.

Histopathology

Injection study.—Fish injected with both high and low doses of the WIC zoospores developed similar microscopic pathological changes. In the high-dose-treated fish at 4 d postinjection, skeletal muscle, especially along the myosepta, exhibited severe vacuolar and granular degeneration and necrosis associated with growth and invasion of hyphae (Figure 2a). Areas of myodegeneration were characterized by the presence of hemorrhage; mild, unorganized, mixed leukocytic infiltrates; and necrotic muscle tissue debris (Figure 2a). Hyphae demonstrated by GMS stain (Figure 2b) were invading along the myosepta. Large areas deep in the muscle at the injection site exhibited vacuolation, granular and hyaline degeneration, and myofiber lysis. At 5 d postinjection, the injection site was characterized by intense leukocytic infiltration, hemorrhage, granulomatous inflammation, and myodegeneration (Figure 2c). Early granulomas (usually consisting of only one or two layers of macrophages surrounding the hypha); well-developed, more mature granulomas (usually four to five layers of epithelioid cells surrounding the hypha; Figure 2d); and multifocally distributed accumulations of unencapsulated hyphae, macrophages, other inflammatory cells, and necrotic tissue debris characterized this area. Extensive granular and vacuolar degeneration without inflammation was observed in the deep muscle subjacent to the injection site. Some myocyte nuclei were centrally displaced within myofibers. At 6–7 d postinjection, the overlying epidermis had sloughed off and exposed the dermis and underlying muscle tissues. A few granulomas were even observed in deep muscle on the side of the body opposite the injection site. Large areas of surrounding skeletal muscle were undergoing vacuolation and granular degeneration. Unencapsulated hyphae as well as leukocytic infiltration were observed along the myosepta. At 7 d postinjection, the kidney was not affected, but oomycete hyphae had penetrated into the areolar connective tissues between the spinal cord and neural arch. At 9–10 d postinjection, lesions were advanced, the necrotic center of the lesion had increased in size, and hyphae were penetrating deeply into the muscle tissue; intense granulomatous inflammation surrounding the invading hyphae was accompa-

nied by adjacent peripheral myonecrosis and loss of skin over the injection site. A surficial zone of necrotic inflammatory tissue characterized the outer surface of the developing ulcer, which had been colonized by opportunistic bacteria.

Fish injected with PA7 zoospores did not exhibit gross lesions at 5 d postexposure. Internally, the dermis and skeletal muscle immediately underlying the epidermis at the injection site were intact. However, leukocytic infiltrates were observed tracking along the myosepta. Multifocal accumulations of unencapsulated hyphae, hyphae surrounded by a single layer of macrophages, and granulomas consisting of four to five layers of epithelioid cells were observed in the deeper muscle layers. Hyphae alone or hyphae encapsulated by a layer of macrophages were growing deeper into the muscle. At 9 d postexposure, the skin and skeletal muscle immediately overlying the injection site had sloughed, exposing the deep musculature. At this stage, granulomas were widely distributed within skeletal muscle, especially along the myosepta. Muscle damage in this area, however, was less extensive than that observed in fish injected with WIC zoospores and was limited to granular degeneration (Figure 2e). Granulomas often exhibited central necrosis, the central cavity being filled with eosinophilic proteinaceous droplets, debris, and hyphae. At 14 d postexposure, large areas of skeletal muscle within the expanding ulcer started to degenerate, creating spaces filled with cellular debris.

Fish injected with only 1‰ water (controls) as well as those injected with ATCC-62427 exhibited only mild degeneration of skeletal muscle at 5 d postinjection, as evidenced by small, hyalinized myocytes containing centrally displaced nuclei. However, no necrosis, granulomatous inflammation, or oomycete hyphae were observed (Figure 2f). At 9 d postinjection, regenerating skeletal muscle cells were observed in control fish.

Bath challenges.—Net-stressed fish exhibited superficial lesions within 5 d postexposure. Histologically, free hyphae, hemorrhage, and mixed leukocytic infiltration were observed in the skin and superficial muscle tissues (Figures 3a and 3b). In some fish, by 5 d postexposure, hyphae had begun to penetrate deeper into the skeletal muscle. Multifocally distributed hyphae as well as two types of granulomas were seen in the skeletal muscle: (1) very early stages of granuloma formation with one to two macrophage layers surrounding hyphae; and (2) granulomas with four to five layers of epithelioid cells surrounding hyphae, some-

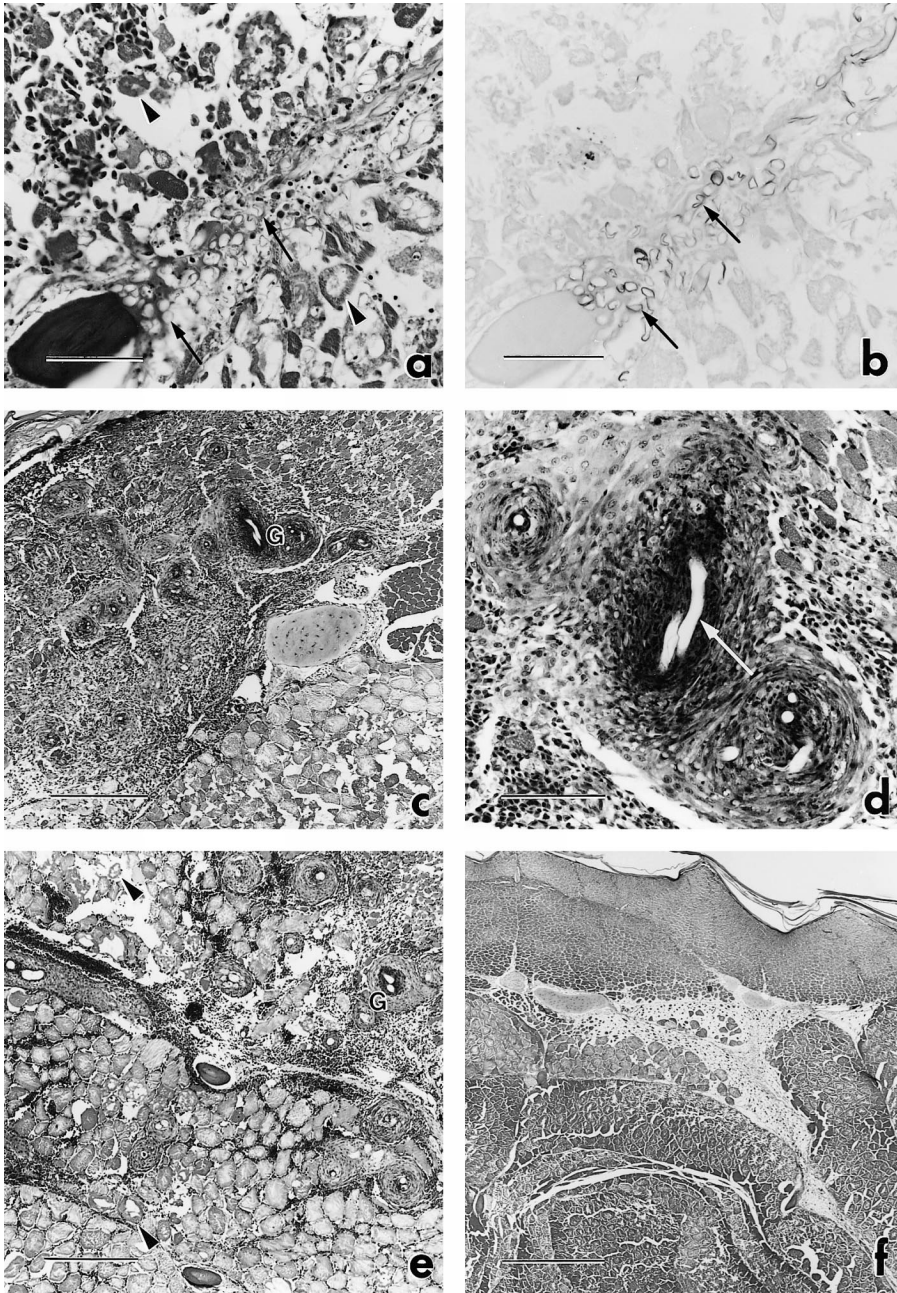


FIGURE 2.—Histopathology of Atlantic menhaden subcutaneously injected with secondary zoospores of *A. invadans* (WIC and PA7 isolates). (a) Moribund fish injected with high-dose WIC (1,900 zoospores/mL) at 4 d postinjection. Shown are oomycete hyphae (arrows) prominent along the myosepta and myodegenerative changes (arrowheads; Harris' hematoxylin and eosin [H&E] staining; bar = 50 μ m). (b) Parallel section of (a), stained with Grocott's methenamine-silver nitrate. Hyphae stained positively (arrows; bar = 50 μ m). (c) Fish injected with low-dose WIC (190 zoospores/fish) that exhibited 4 \times 6 mm ulcer at 5 d postinjection. H&E staining shows well-developed granulomas (G) and intense leukocytic infiltration at injection site and extensive myodegeneration in deep tissue (bar = 200 μ m). (d) Higher magnification of (c), showing mature granulomas sequestering hyphae (arrow; H&E stained; bar = 50 μ m). (e) Fish injected with low-dose PA7 (520 zoospores/fish) that exhibited 10 \times 15 mm ulcerous lesions at 9 d postinjection. H&E staining shows granulomas (G) widely distributed within the

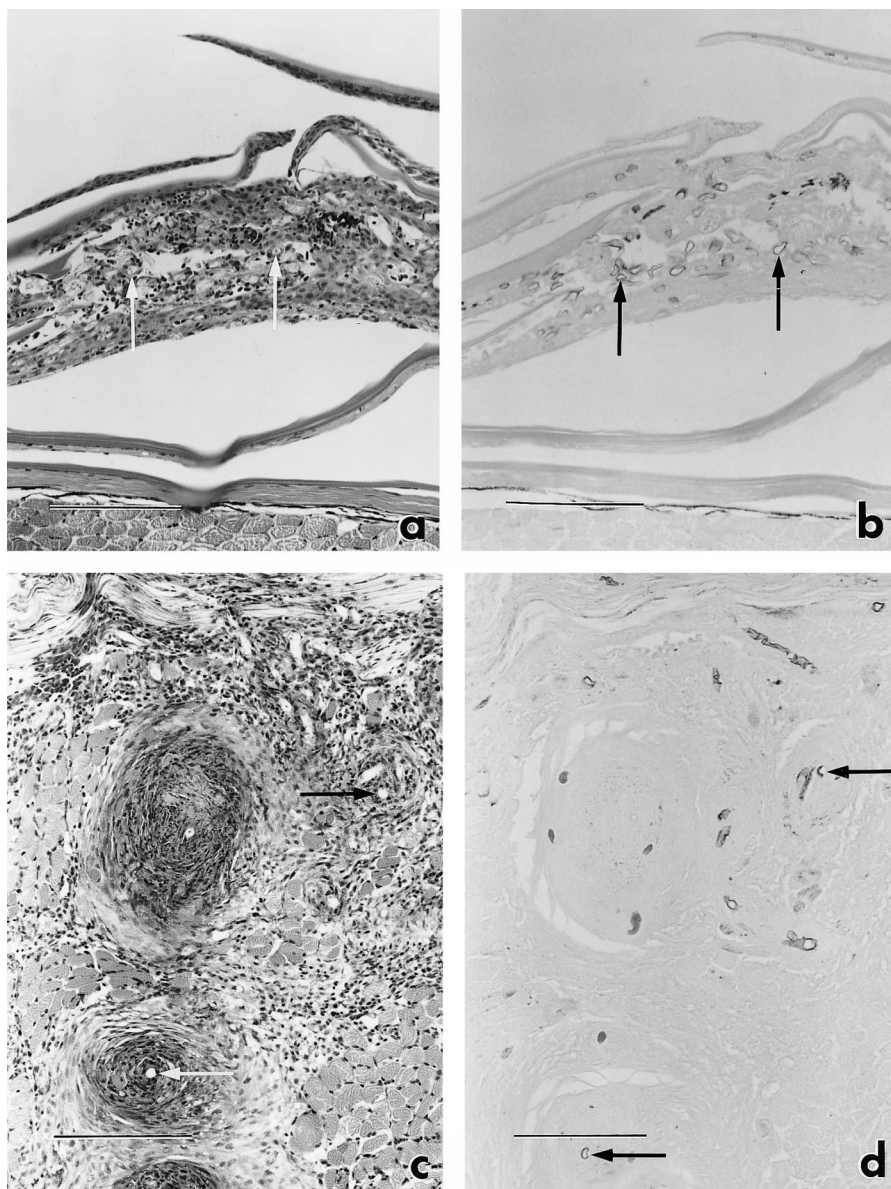


FIGURE 3.—Histopathology of Atlantic menhaden bath-exposed to secondary zoospores of *A. invadans* (high-concentration WIC, 700 zoospores/mL, net-stressed). Fish were killed 5 d postexposure. Bar = 100 μm . (a) Unencapsulated hyphae (arrows) in the skin (hematoxylin and eosin [H&E] stain). (b) Parallel section of (a), stained with Grocott's methenamine–silver nitrate (GMS). Hyphae (arrows) stained positively. (c) Well-developed granulomas surrounding hyphae (arrows), accompanied by necrotizing cores with strongly basophilic staining in the skeletal muscle immediately beneath the dermis (H&E stain). (d) Parallel section of (c), stained with GMS. Hyphae (arrows) stained positively.

←

skeletal muscle, immediately overlying the injection site, with adjacent myodegeneration (arrowheads; bar = 500 μm). (f) Control fish, sham-injected with 1% saline water, exhibiting resolving injection site at 5 d postinjection. Mild degeneration of skeletal muscle is observed in the absence of necrosis, granulomatous inflammation, or hyphae (H&E staining; bar = 500 μm).

times with necrotizing centers showing strongly basophilic staining (Figure 3c). The presence of oomycete hyphal invasion was confirmed by GMS staining (Figure 3d).

At 7–11 d postexposure, the epidermis and in some places the dermis had sloughed, exposing underlying tissues, which exhibited surficial bacterial colonization (Figure 4a). Underlying skeletal muscle exhibited degeneration and coagulative necrosis, abundant oomycete hyphae, granulomas, leukocytic infiltrates (macrophages, lymphocytes, granulocytes, and fibrocytes), and hemorrhage. In severe cases, hyphae had invaded the kidney, penetrating into the hematopoietic tissues (Figures 4a, 4b, 4c) and resulting in tissue necrosis.

Fish in the untraumatized treatment (treatment 2) exhibited a progression of lesion development identical to that described for the fish in treatment 1 that received the WIC zoospores. Sloughing of the skin with exposure of the underlying skeletal muscle was observed histologically as early as 5 d postexposure.

Discussion

This study provides the first direct experimental evidence that *A. invadans*, a fungus-like oomycete, is the primary etiologic agent of UM in the Atlantic menhaden. We demonstrated conclusively that this oomycete agent readily infects menhaden and elicits the characteristic skin ulcers commonly observed in wild populations from eastern U.S. estuaries (Noga et al. 1988; Blazer et al. 1999). Most significantly, this agent does so in the absence of *P. piscicida*, a toxic dinoflagellate recently implicated by others as the etiologic agent of this disease (Burkholder et al. 1992, 1995, 2001). Using two routes of exposure with an endemic isolate of the oomycete agent, we have fulfilled Koch's postulates (Evans 1976). When secondary zoospores were administered to fish by subcutaneous injection or bath exposure, this strain elicited ulcerous skin lesions identical to those observed in wild menhaden. Subsequent reisolation and identification of this organism (WIC) from lesions on the laboratory-challenged menhaden was successful. We obtained similar results in Atlantic menhaden experimentally injected with PA7, a strain of *A. invadans* isolated from snakehead from Thailand (Lilley et al. 1998). Interestingly, the third isolate, ATCC-62427, initially suggested as a causative agent for UM (Dykstra et al. 1986), was not pathogenic to Atlantic menhaden. Furthermore, ATCC-62427 is probably not *A. invadans* but rather a rapidly growing saprophytic species, as has been

suggested previously (Lilley and Roberts 1997; Blazer et al. 1999, 2002). Alternatively, perhaps the culture has lost its pathogenicity over the long time in laboratory culture.

The infectivity of *A. invadans* in Atlantic menhaden was enhanced by providing a portal of entry. Reproduction of EUS lesions has proved difficult in other fish species without some predisposing environmental factor that causes minor skin damage. Callinan et al. (1996) reproduced EUS lesions in estuarine fishes in Australia by physical and chemical abrasion of the skin, followed by exposure to zoospores. They were unable to induce EUS lesions in fish without first facilitating entry of the pathogen. Trauma or injury to fish, in general, provides a portal of entry for bacteria (Ventura and Grizzle 1987; Svendsen and Bøggwald 1997), fungi (Noga 1990), or even artificial microspheres (Kiryu and Wakabayashi 1999; Kiryu et al. 2000). In our study, injury from net handling or manual removal of scales dramatically enhanced transmission of the oomycete in bath exposures and resulted in a 100% lesion prevalence in exposed fish. Our experimental studies suggest that Atlantic menhaden are susceptible to infection by *A. invadans* without other predisposing factors, aside from minor issues associated with captive rearing. However, the creation of a portal of entry such as may be caused by exposure to external parasites (e.g., Turner and Roe 1967; Voorhees and Schwartz 1979;), *P. piscicida* (e.g., Noga et al. 1996), hypoxia (Plumb et al. 1976), or other environmental stressors (e.g., pH; Callinan et al. 1996) may enhance the infectivity, lesion severity, and mortality in menhaden exposed to *A. invadans*.

Regardless of the route of exposure, these pathological findings were consistent with those previously reported for EUS in Indo-Pacific fishes (Egusa and Masuda 1971; Miyazaki and Egusa 1972, 1973a, 1973b, 1973c; Roberts et al. 1993; Lilley et al. 1998) and UM of Atlantic menhaden (Noga et al. 1988; Blazer et al. 1999). The lesions in traumatized or netted fish exposed to secondary zoospores were multifocal and distributed along the trunk, usually with a striated pattern, suggesting that secondary zoospores invaded the muscle along the myosepta. This was confirmed histologically. Bath-exposed, untraumatized fish developed fewer, usually single, focal skin ulcers. Interestingly, well-developed granulomas occurred as early as 5 d after injection and bath exposure with the WIC isolate. Similarly, snakehead exposed to *A. invadans* isolate RF6 developed granulomas after 2 and 4 d at temperatures of 31°C

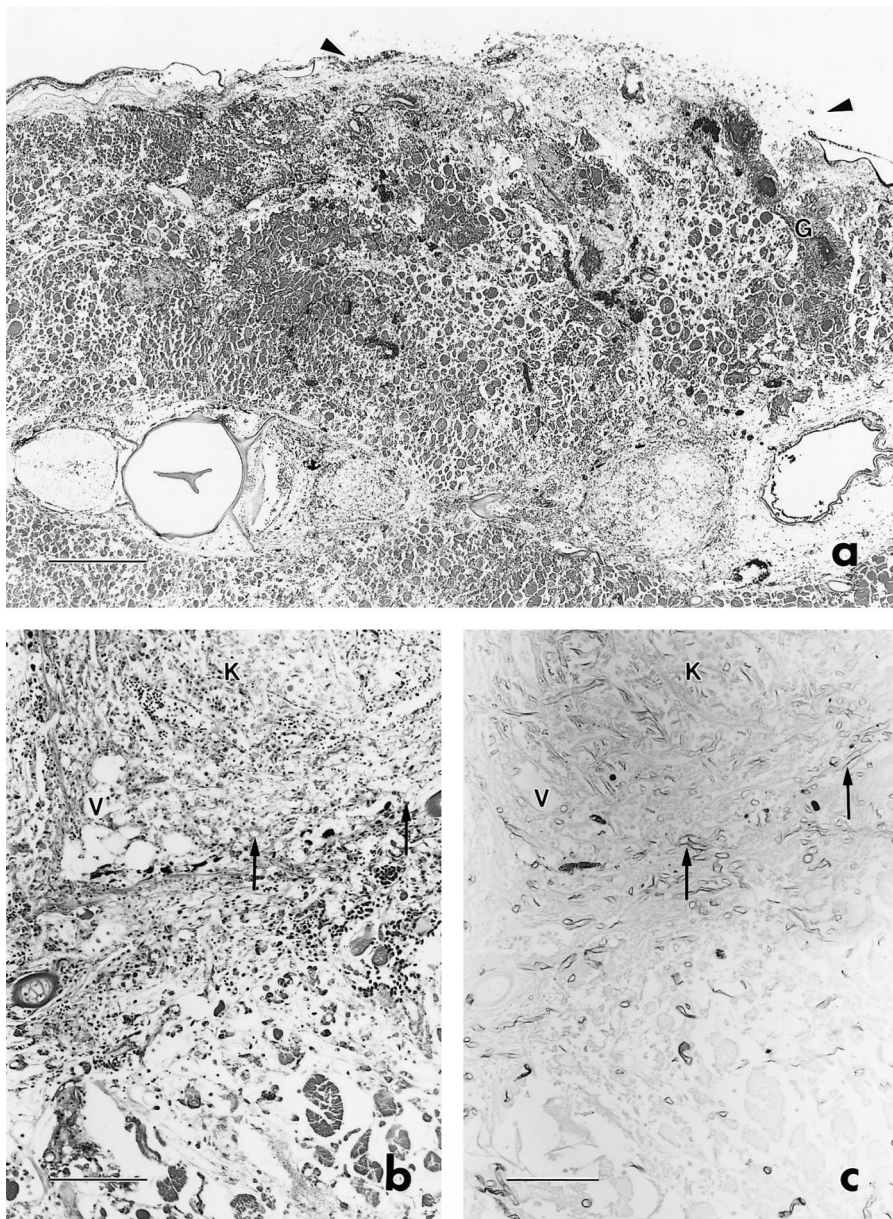


FIGURE 4.—Histopathology of Atlantic menhaden bath-exposed to *A. invadans* zoospores. (a) Moribund fish 11 d postexposure to low-concentration WIC (70 zoospores milliliter, net-stressed). Skin erosion with exposure of underlying musculature (arrowheads) is visible, as are well-developed granulomas (G) and extensive myodegeneration in deeper muscle tissue. Kidney tissue was invaded by hyphae (hematoxylin and eosin [H&E] stain; bar = 300 μ m). (b) Moribund fish at 7 d postexposure to high-concentration WIC (700 zoospores/mL, net-stressed). Shown is kidney tissue (K) invaded by hyphae (arrows) and exhibiting extensive necrosis and vacuolation (V) of renal and hematopoietic tissues. Intense myodegeneration and necrosis were observed surrounding the kidney tissue (H&E stain; bar = 100 μ m). (c) Parallel section of (b), stained with Grocott's methenamine-silver nitrate. Hyphae (arrows) stained positively (K = kidney; V = vacuoles; bar = 100 μ m).

and 26°C, respectively (Chinabut et al. 1995). Temperature is an important factor influencing the host inflammatory response, and the ambient temperature range (23–24°C) used in this study was somewhat lower than those in the snakehead study.

Atlantic menhaden appear to be highly susceptible to *A. invadans*. In both challenges, untraumatized fish experienced 24% cumulative mortality, whereas net-stressed fish exposed to moderate and high concentrations of WIC experienced 100% mortality. For the untraumatized fish, mortality was not a result of handling because controls experienced negligible mortality (8%). In India and southeast Asia, EUS has caused extensive fish mortalities under grow-out conditions in culture ponds (Lilley et al. 1998). Fish weakened by EUS frequently die from the infection or from secondary infections or after stressful events (Lilley et al. 1998). We speculate that *A. invadans* may contribute to mortalities of Atlantic menhaden in the field by compromising the energetics and survivorship of infected fish.

In this study, fish from both net-stressed and traumatized groups exhibited diffuse reddening on the trunk that resulted in acute mortalities (1–3 d postexposure), presumably from a systemic bacterial infection. Opportunistic bacteria were detected histologically on cutaneous infections in this study. Presumably the handling of these fish triggered rapid mortalities in a subset of fish. Moreover, measurement of stressors in captive fish is difficult, even in those not intentionally stressed. Thus, although Atlantic menhaden in the untraumatized, bath-challenged treatment developed lesions, we cannot say with certainty that these fish were not subject to stressors that facilitated a portal of entry for invasion by the oomycete.

The three oomycete isolates (WIC, PA7, ATCC 62427) exhibited differences in pathogenicity. The ATCC 62427 was not pathogenic to Atlantic menhaden. The PA7 isolate obtained from striped snakehead was slightly less pathogenic to menhaden than was the WIC strain isolated from Chesapeake Bay menhaden. In striped snakehead, the WIC isolate was somewhat more pathogenic than the PA7 isolate (Blazer et al. 2002). One explanation for these observations may be the amount of time the isolates have been cultured and passed in the laboratory. The PA7 isolate has been in culture for more than 5 years compared with 2 years for the WIC isolate. In addition, the WIC isolate may be better adapted to estuarine waters, whereas PA7 was isolated from fish in freshwater.

In conclusion, we successfully demonstrated the

experimental induction of lesions in Atlantic menhaden identical to those observed in naturally occurring UM. With moderate- to high-density exposures, skin ulcers characterized by myonecrosis, myositis, and granulomatous inflammation began to develop 5 d after exposure to *A. invadans* in the absence of *Pfiesteria* spp. We have fulfilled Koch's postulates for associating a pathogen with a disease, in this case *A. invadans*. In addition, we have shown that *A. invadans* induced lesions with or without fish being predisposed to injury—albeit with the caveat that captive rearing may have inherent stressors that are difficult to observe and measure.

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