

# Infectivity and pathogenicity of the oomycete *Aphanomyces invadans* in Atlantic menhaden *Brevoortia tyrannus*

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**ABSTRACT:** Atlantic menhaden *Brevoortia tyrannus* develop characteristic skin ulcers in response to infection by the oomycete *Aphanomyces invadans*. To investigate pathogenicity, we conducted a dose response study. Juvenile menhaden were inoculated subcutaneously with 0, 1, 5, 10, 100, and 500 secondary zoospores per fish and monitored for 37 d post-injection (p.i.). Survival rates declined with increasing zoospore dose, with significantly different survivorship curves for the different doses. Moribund and dead fish exhibited characteristic ulcerous lesions at the injection site starting at 13 d p.i. None of the sham-injected control fish (0 zoospore treatment) died. The LD<sub>50</sub> (lethal dose killing 50% of exposed menhaden) for inoculated fish was estimated at 9.7 zoospores; however, some fish receiving an estimated single zoospore developed infections that resulted in death. Menhaden were also challenged by aqueous exposure and confirmed that *A. invadans* was highly pathogenic by this more environmentally realistic route. Fish that were acclimated to culture conditions for 30 d, and presumably free of skin damage, then aqueously exposed to 100 zoospores ml<sup>-1</sup>, exhibited 14% lesion prevalence with 11% mortality. Net-handled fish that were similarly infected had a significantly higher lesion prevalence (64%) and mortality (64%). Control fish developed no lesions and did not die. Scanning electron microscopy of fish skin indicated that zoospores adhered to intact epidermis, germinated and penetrated the epithelium with a germ tube. Our results indicate that *A. invadans* is a primary pathogen of menhaden and is able to cause disease at very low zoospore concentrations.

**KEY WORDS:** Dose response · Epizootic ulcerative syndrome · LD<sub>50</sub> · Lesions · Mortality · Secondary zoospores · Tissue repair · Ulcerative mycosis

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## INTRODUCTION

Atlantic menhaden *Brevoortia tyrannus* with deeply penetrating ulcerous skin lesions have been observed since the mid 1980s in mid-Atlantic United States estuaries (Hargis 1985, Dykstra et al. 1989, Noga et al. 1991, Noga 1993). Histologically, these lesions are characterized by severe myonecrosis and extensive granulomatous myositis associated with penetrating fungal hyphae (Noga et al. 1988, Noga 1993). Origi-

nally, the condition, termed ulcerative mycosis (UM), was attributed to infection by oomycete water moulds of the genus *Aphanomyces* (Noga & Dykstra 1986). Massive fish kills were reported to occur in North Carolina estuaries as a result of the disease (Dykstra et al. 1986, Noga & Dykstra 1986). Environmental factors were believed to play an important role in outbreaks of the disease, with the oomycete agent likely being a secondary invader (Noga & Dykstra 1986, Noga et al. 1988, Levine et al. 1990).

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Recently, the lesionous menhaden as well as acute fish kills have been attributed to the activity of the toxic dinoflagellates *Pfiesteria piscicida* and *P. shumwayae* (Burkholder et al. 1992, 1995, 2001, Noga et al. 1996, Glasgow et al. 2001). However, investigations of menhaden from Chesapeake Bay indicate that oomycete hyphae have high host specificity to menhaden, an association typical of an aetiological agent. The agent infecting menhaden from Chesapeake Bay was identified as *Aphanomyces invadans* (Blazer et al. 1999, 2002, Kiryu et al. 2002). *A. invadans* has been implicated as the causative agent of epizootic ulcerative syndrome (EUS) in Asia (Roberts et al. 1993, Lilley & Roberts 1997, Catap & Munday 1998), red spot disease (RSD) in Australia (Fraser et al. 1992, Callinan et al. 1989, 1995), and mycotic granulomatosis (MG) in Japan (Egusa & Masuda 1971, Miyazaki & Egusa 1972, 1973a,b,c, Hatai et al. 1977, Wada et al. 1994, 1996). It causes devastating losses to cultured fishes in India, Malaysia, Indonesia and Hong Kong (reviewed by Lilley et al. 1998). Gross and histopathologies of EUS have been well described (Roberts et al. 1993, Catap & Munday 1998, Lilley et al. 1998) and are identical to that of UM in menhaden (Blazer et al. 1999, 2002, Kiryu et al. 2002).

Kiryu et al. (2002) experimentally induced the ulcerous lesions in menhaden by both inoculation and bath exposure with secondary zoospores of the endemic isolate of *Aphanomyces invadans*. They fulfilled Koch's postulates and demonstrated that *A. invadans* is the causative agent of UM in menhaden. However, in their injection study, zoospore doses were high, and not necessarily reflective of environmental exposure. The objectives of this study were: (1) to conduct a dose-response study to determine an LD<sub>50</sub> (lethal dose killing 50% of exposed menhaden) for secondary zoospores using inoculations, (2) to compare mortality of fish exposed to zoospores via inoculation or bath challenge, (3) to better assess the pathogenicity of *A. invadans* using formal survival analysis, (4) to examine the dynamics of gross alterations caused by the disease, and (5) to describe tissue repair in fish surviving exposure.

## MATERIALS AND METHODS

**Fish collection and maintenance.** Atlantic menhaden were obtained from 2 sources. Fish used in the zoospore injection trials (Age-class 1; n = 134; mean weight 13.4 g, range 3.2 to 27.8 g; mean total length 113 mm, range 71 to 144 mm) were collected from upper Chesapeake Bay tributaries. Fish used in aqueous exposures (Age-class 1; n = 78; mean weight 9.3 g, range 2.4 to 26.2 g; mean total length 97 mm, range

69 to 141 mm) were collected by cast net from Sarah Creek, York River, Virginia. Fish were held in a static recirculating system consisting of 2 × 712 l fiberglass tanks containing filtered (35 µm) York River water diluted to 12 psu (= ‰) at temperatures between 20 and 23°C (injection trials), or held under ambient conditions (salinity 20 to 24 psu; temperature 25 to 28°C) (aqueous exposures). Water changes (25%) in maintenance aquaria and experimental treatments were performed weekly to maintain water quality. Fish were fed a daily diet of algal paste, supplemented with HiPro 0.5GR Debut Corey Starter (Corey Feed Mills).

**Oomycete culture and sporulation.** *Aphanomyces invadans*, WIC strain, an endemic strain isolated from a lesionous menhaden in Wicomico River, Maryland (see Blazer et al. 1999), was used in this study. Culture characteristics and methods of sporulation have been previously described (Kiryu et al. 2002). Zoospore densities were estimated with a haemocytometer (Neubauer/Bright-Line) using samples fixed with 10% neutral-buffered formalin.

**Zoospore inoculations.** Fish were acclimated for 4 d in 10 × 76 l glass aquaria (9 to 15 fish per tank) containing 6 psu artificial seawater (ASW, Marinemix Forty Fathoms) and maintained at room temperature (daily mean 21.6 ± 0.61°C). Tanks were equipped with 2 Whisper filters (Tetra/Second Nature) containing a preconditioned filter bag filled with crushed coral. Fish were lightly anaesthetised in 75 mg l<sup>-1</sup> tricaine methanesulfonate (MS-222) and inoculated with 0.1 ml of a zoospore suspension to deliver 1, 5, 10, 100, or 500 zoospores per fish. Each fish was injected subcutaneously using a 27 gauge, 12.7 mm needle and 1 ml syringe on the left flank. Control fish were injected with 0.1 ml sterile water (1 psu). Two replicate tanks were assigned to each treatment, except for the 100 and 500 zoospores per fish treatments, which were not replicated. To confirm oomycete viability, 0.1 ml of each inoculum was plated in triplicate onto glucose peptone-penicillin-oxolinic acid agar (GP-POX agar; Willoughby & Roberts 1994, Lilley et al. 1998). Hyphal growth in all treatments indicated viable zoospores within each dose. Fish were monitored and fed daily for 37 d. Dissolved oxygen (DO), pH (Orion 290A), ammonia (Hach), and nitrite (Hach) concentrations were monitored weekly. Water quality parameters ranged from 6.4 to 7.7 mg l<sup>-1</sup> DO, pH 6.8 to 8.0, 0.2 to 8.0 mg l<sup>-1</sup> total ammonia, and 0.0 to 16.0 mg l<sup>-1</sup> nitrite. The absence of *Pfiesteria*-like dinoflagellates was confirmed by microscopic examination of 25 ml of tank water fixed with Lugol's iodine.

**Aqueous exposure.** Fish (6 to 18 per tank) were acclimated for >30 d in 6 × 76 l glass aquaria containing 20 psu ASW, each equipped with a preconditioned Whisper filter. Each tank was held at room tempera-

ture (daily mean  $19.2 \pm 1.09^\circ\text{C}$ ). Fish in the 6 aquaria were assigned to 3 treatments of 2 replicates each: (1) net-stress aqueous exposure; (2) untraumatized aqueous exposure; and (3) unexposed control. Menhaden have deciduous scales. Therefore, fish in the net-stress treatment experienced minor loss of scales with epidermal abrasion, providing a portal of entry for the water mould.

For all treatments, salinity was gradually lowered from 20 psu to 1 psu over a 30 d period. Salinities of 2 psu or less are required for sporulation, motility, and infectivity of *Aphanomyces invadans* (Blazer et al. 2002). Once acclimated, fish were held at 1 psu for 24 h before exposure. For all treatments, the volume of each tank was lowered to 30 l, and filtration was turned off during the exposure (5.5 h). For Treatment 1, fish in each tank were captured and held in a net for 30 s (net-stress), zoospores were then added to the aquaria to give an estimated concentration of 100 zoospores  $\text{ml}^{-1}$ . For Treatment 2, fish remained undisturbed (untraumatized), and zoospores were added to the aquaria to give an estimated density of 100 zoospores  $\text{ml}^{-1}$ . The control (Treatment 3) consisted of the same protocol as Treatment 2, except that sterile water (1 psu) was added instead of zoospores. Fish in Treatments 1 and 2 were exposed to zoospores for 5.5 h (with aeration only). Viability was confirmed during the exposure period by microscopically examining water samples for zoospore motility. Exposure was stopped by adding 12 psu ASW to each tank, raising the salinity to 6 psu, which inhibited zoospore motility (Blazer et al. 2002). Fish in all treatments were monitored daily for 27 d and fed as described above. Water samples were devoid of dinoflagellates.

**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 selected fish were photographed. For the injection study, the fish were cut in half transversely at the lesion or injection site to examine the gross pathology. The area of each lesion was calculated using the simple equation for a rectangle. In the inoculation experiment, all moribund fish and fish surviving to the end of the experiment were examined histologically. In the aqueous exposure, only moribund fish with ulcers and fish surviving to the end of the trial were examined histologically. Fish were killed by overdose with MS-222 and fixed in 10% neutral-buffered formalin. Tissues were decalcified with formic acid-sodium citrate solution (Luna 1968) prior to processing for histology. For fish exhibiting lesions, 2 different areas, approximately 4 to 5 mm apart within the lesion site, were examined. All slides were stained with Harris' haematoxylin and eosin (H&E; Luna 1968).

Selected slides were stained with Grocott's methenamine-silver nitrate (GMS) to demonstrate oomycetous hyphae, or Masson's trichrome (MT) to demonstrate collagen fibrils in the tissues (Luna 1968).

**Scanning electron microscopy.** Juvenile menhaden were killed by overdose with MS-222 and moved into a 50 ml culture flask containing  $1.10 \times 10^4$  cells  $\text{ml}^{-1}$  secondary zoospores. The fish were incubated with zoospores for 3 h, then removed by the caudal fin and fixed overnight at  $4^\circ\text{C}$  with modified Karnovsky solution (4% paraformaldehyde and 5% glutaraldehyde in 0.1 M sodium cacodylate buffer without  $\text{CaCl}_2$ , at pH 7.4). Samples were rinsed in 3 changes of 0.1 M sodium cacodylate buffer at room temperature, post-fixed with 1%  $\text{OsO}_4$  for 1 h, and rinsed 3 times with the buffer solution. Samples were then dehydrated through a graded ethanol series, critical point-dried (Polaron Model E3100), mounted on an aluminum stub (Ted Pella), and sputter-coated with 20 nm of Au:Pd (Anatech Hummer VII). Tissues were examined with a scanning electron microscope (Leo 435 VP).

**Statistical analyses.** The LIFEREG model (SAS Institute 1989), with the accelerated failure time model (Lee 1992, Cantor 1997) was used for censored survival analysis. The LIFEREG statistic is reported as a log-rank chi-square. Five different survival models (exponential, Weibull, Gamma, log-normal, or log-logistic) were examined for best fit, followed by testing with the model  $y = \text{dose}$  or  $y = \text{treatment}$  with the best-fitted model. Because no control fish died during the course of study in both injection and aqueous exposure, controls were excluded from the LIFEREG analyses. Lesion area was also analyzed by LIFEREG with the model  $y = \text{area}$  separated by dose with the best-fitted model. Normal maximum-likelihood estimates of  $\text{LD}_{50}$  and the 95% CIs at 37 d post-injection (p.i.) were calculated using a probit model (SAS Institute 1989),  $\Phi^{-1}(x) = \alpha + \beta x + 5$ , where  $\Phi^{-1}(x)$  = cumulative distribution of mortality for the standard normal distribution;  $\alpha$  = estimated parameter for the intercept;  $\beta$  = estimated parameter for the slope; and  $x = \log_{10}(\text{dosage})$ .

## RESULTS

### Survival

Survivorship decreased in relation to increasing zoospore dose (Table 1). In most cases, fish that developed frank ulcers died shortly thereafter. Fish that did not develop ulcers possessed numerous granulomas histologically, and often showed signs of significant tissue repair around the inoculation site. Fish began dying 13 d after inoculation with zoospores (Fig. 1). Mean mortality rates per day (%) starting after 13 d

Table 1. *Brevoortia tyrannus*. Relationships between *Aphanomyces invadans* dosage and survivorship, percentage of fish exhibiting lesions (both ulcers and tissue repair/healing), percentage of fish without lesions, and percentage of fish dying with ulcerous lesion within 37 d post injection with secondary zoospores of *A. invadans*. Granulomas and tissue repair were confirmed histologically. All fish exhibiting ulcers died or became moribund, with the exception of 1 fish that was injected with 5 zoospores per fish. All fish exhibiting tissue repair did not develop ulcers; i.e. they were surviving fish with lesions

Dose (zoospores per fish)	n	Survivorship	% fish exhibiting			Surviving fish with no lesions		Dead fish	
			Lesions	Ulcers	Tissue repair	n	% granulomas	n	% ulcer
0	18	100	0	0	0	18	–	0	–
1	29	76	31	21	10	19	21	7	86 <sup>a</sup>
5	30	57	53	47	7	14	50	13	100
10	28	43	64	57	7	10	50	16	100
100	14	36	86	64	21	2	100	9	100
500	15	13	93	80	13	0	–	13	92 <sup>b</sup>

<sup>a</sup>One fish died without developing an ulcer and without showing a granulomatous response  
<sup>b</sup>One fish died without developing an ulcer, but exhibiting a moderate granulomatous response

p.i. were 1.0% d<sup>-1</sup> (1 zoospore treatment), 1.8% d<sup>-1</sup> (5 zoospore treatment), 2.4% d<sup>-1</sup> (10 zoospore treatment), 2.7% d<sup>-1</sup> (100 zoospore treatment), and 3.6% d<sup>-1</sup> (500 zoospore treatment). The log-normal distribution was the best-fit curve for survivorships with LIFEREG analysis (log likelihood = -82.449). The shape of the survivorship curves was not different among menhaden inoculated with 5, 10, and 100 zoospores per fish (Fig. 1). The survivorship of fish inoculated with 1 zoospore was significantly higher than other treatments, except for that from fish inoculated with 5 zoospores (log-rank chi-square,  $p < 0.05$ ). The survivorship of fish inoculated with 500 zoospores was significantly ( $p < 0.05$ ) lower than that of fish injected with 1, 5, 10, and 100 zoospores (Fig. 1). None of the sham-injected control fish died during the study.

*Aphanomyces invadans* was highly pathogenic when injected into menhaden. The LD<sub>50</sub> over the 37 d period was estimated as 9.73 zoospores per fish (95% CI 3.55 to 27.86 zoospores per fish) by Probit analysis (Fig. 2).

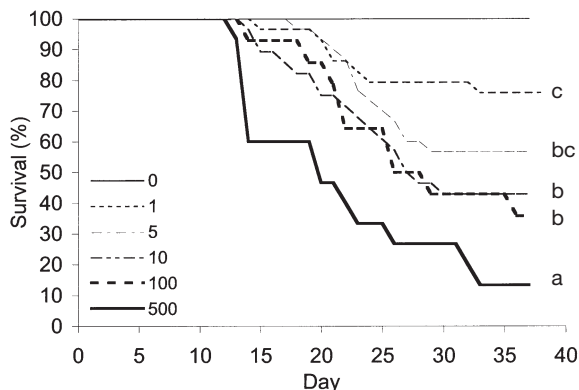


Fig. 1. *Brevoortia tyrannus*. Survival of fish inoculated with different doses of zoospores of *Aphanomyces invadans*. Significant differences ( $p < 0.05$ ) in shape of the survivorship curves (LIFEREG analysis) are indicated by the letters a, b, and c

Survivorship of fish exposed to zoospores in untraumatized and net-stress treatments was 89 and 36%, respectively (Table 2). None of the control fish died during the experiment. The mean daily mortality rate was 0.4% d<sup>-1</sup> (untraumatized) and 2.4% d<sup>-1</sup> (net-stress). Survivorship among the treatments was best fit by a log-normal distribution (log likelihood = -39.571), and the survivorship of net-handled fish was significantly lower than that of untraumatized fish (LIFEREG analysis, log-rank chi-square,  $p < 0.01$ ; Fig. 3).

## Gross pathology

### Inoculation study

Immediately after inoculation, during recovery from anaesthesia, some fish, including sham-injected controls, exhibited reddening at the injection site. In control fish, the reddening at the injection started to disappear around 2 d p.i., and was not grossly visible at 7 d p.i. For

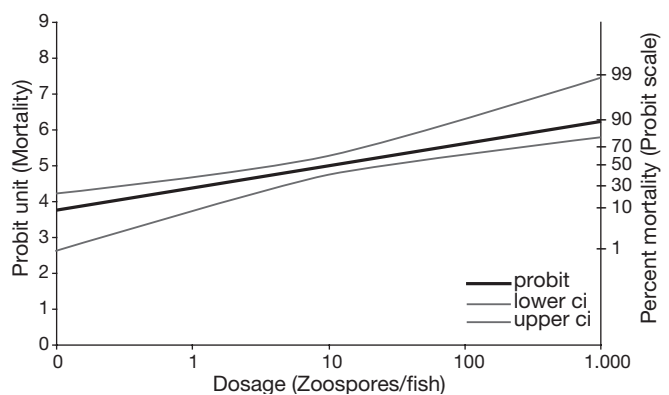


Fig. 2. *Brevoortia tyrannus*. Mortality curve (Probit), with 95% upper and lower CIs at 37 d post-injection, used to estimate the LD<sub>50</sub> of 9.7 zoospores per fish

Table 2. *Brevoortia tyrannus*. Mortality and prevalence of the lesions developing over 27 d in menhaden following aqueous exposure to secondary zoospores (100 zoospores ml<sup>-1</sup>, exposure duration 5.5 h) of *Aphanomyces invadans*

Treatment	n	% fish exhibiting		
		Survivorship (%)	Diffuse reddening lesions	Focal ulcerous lesions
Net-stress	14	36	29	64
Untraumatized	36	89	6	14
Control	28	100	0	0

fish inoculated with zoospores, incipient ulcerous lesions were first visible at 5 d p.i., and became more apparent after 7 d p.i. The number of fish developing lesions increased with dosage (Table 1). All of the fish exhibiting necrotic ulcerous lesions died or became moribund, with the exception of 1 fish inoculated with 5 zoospores (exhibiting a 9 × 14 mm ulcerous lesion) which survived for the duration of the experiment (37 d).

Grossly, a single focal necrotic lesion occurred at the injection site of each fish regardless of zoospore dose. The size of the lesion (range: 3 × 4 to 14 × 29 mm; maximum elevation 6 mm; 1 to 10 mm penetrating into tissue) was not related to the dose; fish that were administered 1 zoospore developed large ulcers (Fig. 4a,b). Sloughing of necrotic tissues frequently occurred after 22 d p.i. (Fig. 4c). The area of the lesion was positively correlated with fish mortality in the 1, 5, and 10 zoospores treatments (LIFEREG,  $p < 0.01$ ). However, there were no significant associations with time nor with lesion size for fish in the 100 and 500 zoospores treatment. All dead and moribund fish exhibited necrotic ulcerous lesions, except for 2 fish; 1 fish (treatment: 1 zoospore) died without developing lesions and without showing a histologically confirmed granulomatous response; another fish (treatment: 500 zoospores) died without developing a lesion, but

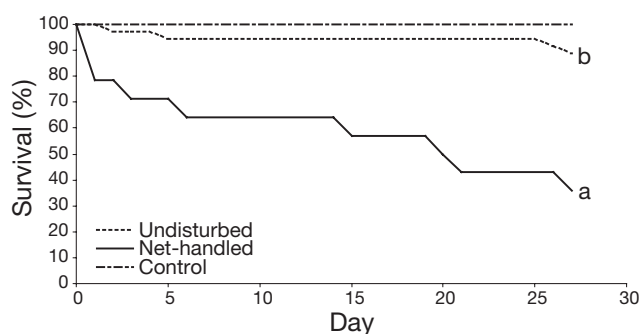


Fig. 3. *Brevoortia tyrannus*. Survival of fish exposed to zoospores of *Aphanomyces invadans* in bath challenges. Significant differences ( $p < 0.05$ ) in shape of the survivorship curves (LIFEREG analysis) are indicated by the letters a and b

with a moderate granulomatous response. Between 18 and 37 d p.i., multifocal necrotic ulcerous lesions (usually 2 to 3 areas on left side trunk including the injection site) were observed in 7% of fish administered 10 zoospores, 21% of fish administered 100 zoospores, and 20% of fish administered 500 zoospores. Two of these fish (1 each from 10 or 500 zoospore treatments) also had ulcerous lesions involving the vent area (Fig. 4c).

Lesions exhibited histological features indicative of tissue repair (for histopathology, see subsection below). Tissue repair was observed microscopically in 7 to 21% of the fish that were injected with zoospores (Table 1). At 37 d p.i., 2 stages in the tissue repair process were observed. The first, presumably early, stage was seen in 4 fish inoculated with more than 5 zoospores per fish. Grossly, the focal lesion (approximately 1 × 1 to 7 × 7 mm, 1 to 2 mm elevation) appeared red, raised, granular, shiny, and had a gritty surface (Fig. 4d). The cut appearance of the lesion was pink at the center, demarcated from normal skeletal muscle tissue and penetrated 1 to 4 mm into the tissue from the external surface. The second, presumably more advanced, stage was seen in 9 fish from all of the fish inoculated with more than 1 zoospore treatment. Grossly, a focal, concave, translucent lesion (approximately 6 × 7 mm) was observed (Fig. 4e). However, the cut appearance of the skeletal muscle was normal. Gross lesions were not seen in 45 fish (14 to 66% of fish) administered zoospores (Table 1); however, several of these fish had insidious granulomas (see 'Histopathology').

#### Aqueous exposures

At the termination of the experiment, 64% of fish in the net-stress treatment exhibited multiple ulcerous lesions. Four fish that exhibited diffuse reddening of the skin died early, within 1 to 3 d post exposure. At termination, 14% of fish in the untraumatized challenge exhibited single focal (5 × 15 mm) ulcerous lesions (Table 2), and only 11% had died. Two fish exhibiting diffuse reddening of the skin died between 2 and 5 d post-exposure, probably of unrelated causes. The other 2 fish, exhibiting focal ulcerous lesions, died at 26 and 27 d post-exposure respectively (Table 2). None of the control fish died or developed lesions during the experiment.

#### Histopathology

##### Inoculation study

The tissues from 17 moribund ulcerated fish collected between 13 and 36 d p.i. exhibited deep pene-

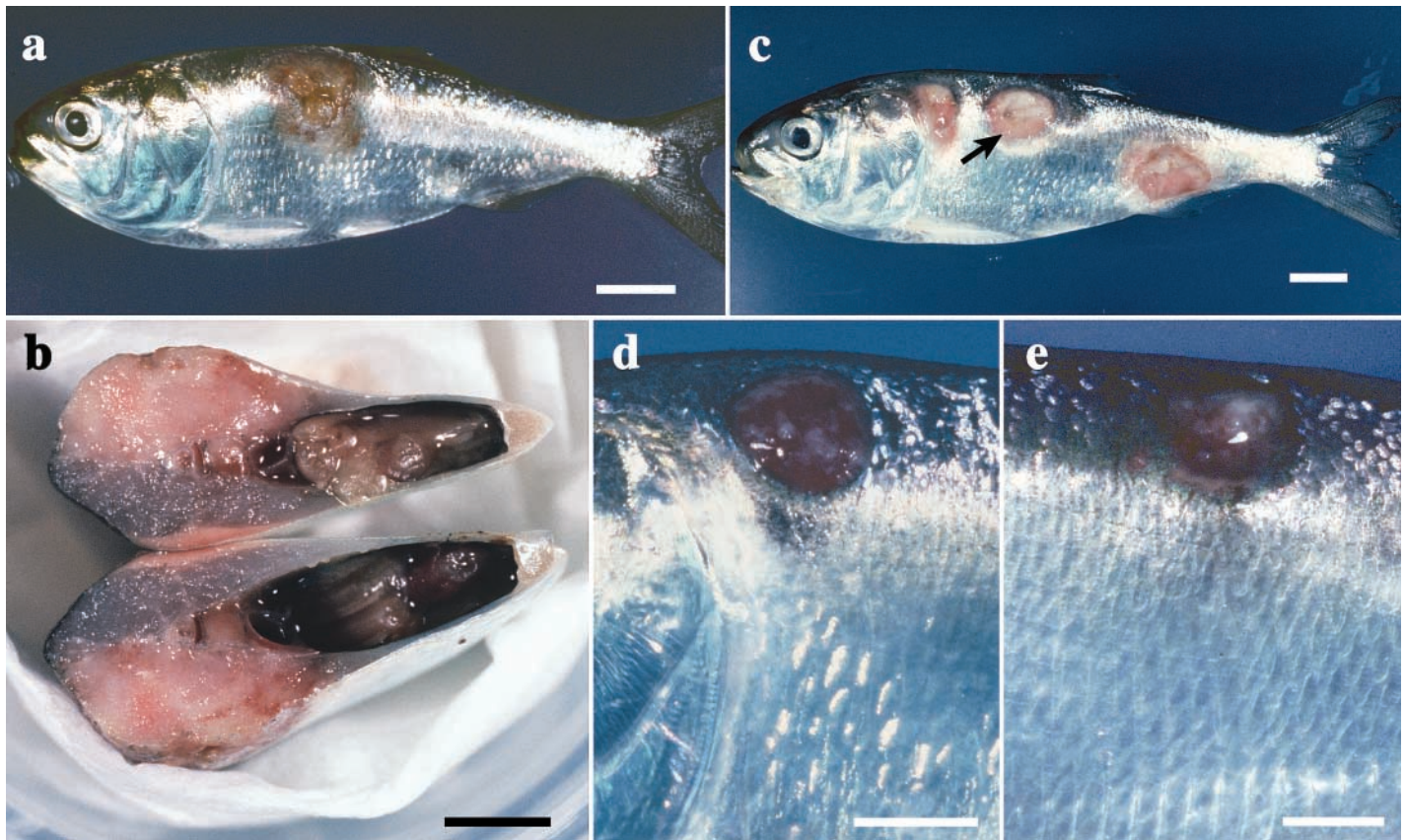


Fig. 4. *Brevoortia tyrannus*. Gross pathology: (a) Ulcerous lesion at the injection site at 14 d post-injection (p.i.) (1 zoospore per fish) (scale bar = 10 mm). (b) Cut appearance through lesion of fish from (a) (scale bar = 5 mm). (c) Multiple ulcerous lesions, 1 involving the vent (10 zoospore treatment at 26 d p.i.). Primary injection site is shown (arrow) (scale bar = 10 mm). (d) Early stage of tissue repair process exhibiting raised, shiny red, granular, and gritty appearance (10 zoospores treatment at 37 d p.i.) (scale bar = 5 mm). (e) Advanced stage of tissue repair process exhibiting concave, translucent, and smooth appearance (1 zoospore treatment at 37 d p.i.) (scale bar = 5 mm)

tration by hyphae, causing necrosis of nearly half of the left flank (Fig. 5a; Table 3). In 15 fish, the muscle tissue on the right flank opposite the injection site was invaded by hyphae. In 12 fish, the kidney was invaded, and in 1 fish the liver was affected. Granulomas were also observed in the spinal cord in 4 fish (Fig. 5b) as well as in the areolar connective tissue between the spinal cord and neural arch in 8 fish. In addition, isolated macrophages were commonly observed within the lesions (Fig. 5b). Granulation tissue, an indication of tissue repair, was evident in 3 moribund fish (2 with 100 zoospores and 1 with 5 zoospores) between 24 to 36 d p.i.

Histopathologically, 13 fish that survived inoculation with zoospores (37 d p.i.) exhibited tissue repair (Table 3). These fish exhibited relatively less hyphal invasion of the tissues (kidney, uninjected

flank, spinal cord and the areolar connective tissue) compared to those of moribund fish. In 4 fish, the surface of the lesion was characterized by a granular appearance; internally, there were prominent granu-

Table 3. *Brevoortia tyrannus*. Number of inoculated fish exhibiting hyphae of *Aphanomyces invadans* invading soft tissues. Moribund fish were collected 13 to 36 d post-injection (p.i.) and surviving fish with tissue repair or without gross lesions were collected at 37 d p.i. Right-side muscle is skeletal muscle on the right flank opposite to the injection site, spinal cord is within the parenchyma of the spinal cord, and areolar connective tissues (CT) are between spinal cord and neural arch

	n	Kidney	Right-side muscle	Spinal cord	Areolar CT	Liver
Moribund	17	12	15	4	8	1
Surviving with tissue repair	13	1	3	0	1	0
Surviving without lesions	45	2	0	0	0	0

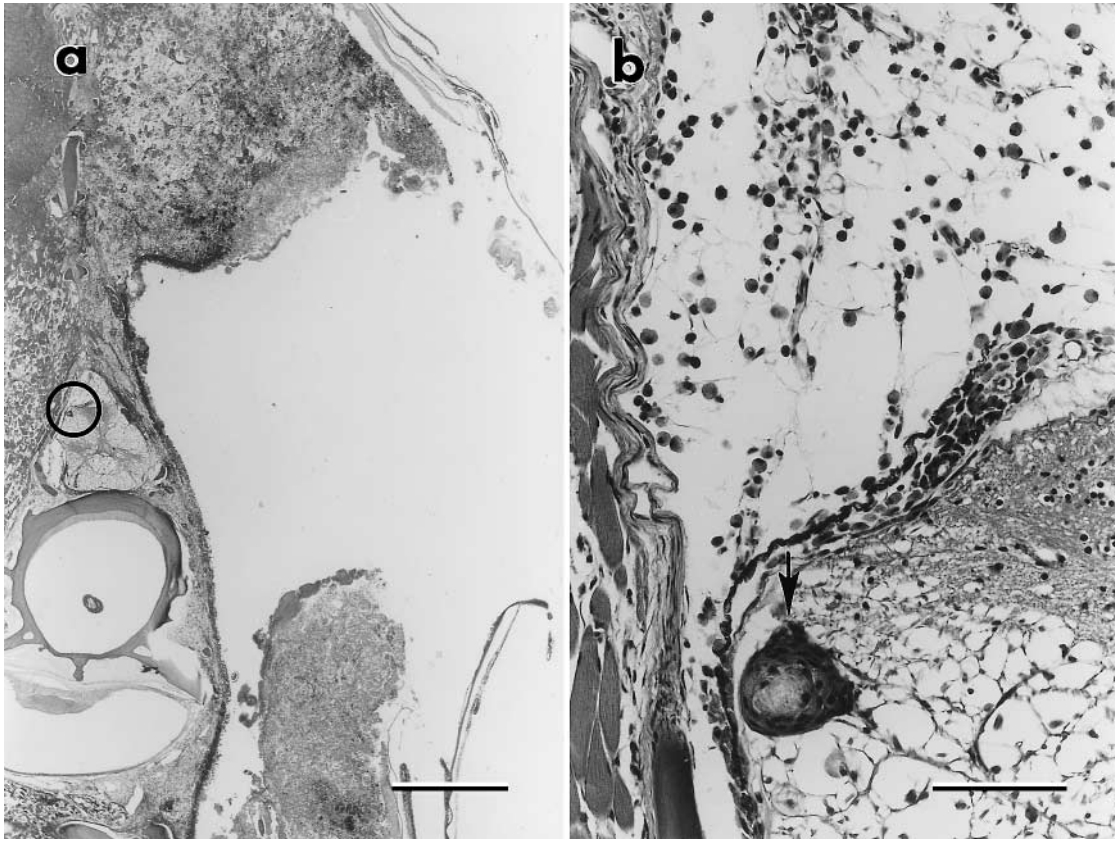


Fig. 5. *Brevoortia tyrannus*. Histological sections of menhaden injected with zoospores sampled at 22 d post-injection, moribund fish. (a) Sloughed skeletal muscle, 1 zoospore treatment (H&E; scale bar = 1500  $\mu$ m). (b) Higher magnification of (a) in circled area showing granuloma (arrow) in the spinal cord and isolated macrophages with pseudopodia along with eosinophilic granular cells infiltrating between the spinal cord and neural arch (H&E; scale bar = 200  $\mu$ m)

lomas composed of 4 to 5 layers of epithelioid cells (Fig. 6a). The innermost epithelioid cells had strongly eosinophilic cytoplasm and nuclear pycnosis. Degrading oomycetous hyphae were detectable as basophilic bodies within the core of the granulomas. This stage of tissue repair exhibited well-organized scaffolding of vascularization (Fig. 6b), a replacement of lost muscle tissues with granulation tissues, and a proliferation of fibrocytes (Fig. 6c). Within the lesion, leucocytic infiltration, including eosinophilic granular cells (EGCs; Fig. 6c), macrophages and lymphoid cells, was prominent.

A possibly more advanced stage of tissue repair was observed in 9 fish and was characterized by a thickening of epithelial and dermal cells of the skin (Fig. 6d) with scale regeneration (Fig. 6e). Remodeling of the skeletal muscles with regenerating myofibrils was apparent by the basophilic appearance of the cytoplasm (Fig. 6d). The lesion was also characterized by the lack of overt necrotic space, but elongate, ovoid granulomas (2 to 3 layers of epithelioid cells) were dis-

tributed sporadically along myosepta or among skeletal muscles with few to no leucocytic infiltrations, and there were few vascularized areas (Fig. 6f). Muscle tissues adjacent to the myosepta were occasionally accompanied by collagen fibrils (MT). Macrophage aggregates were also sporadically observed in these lesions.

Thirty-nine percent of the fish that were inoculated with *Aphanomyces invadans* exhibited no gross lesions (Table 3). Histological samples were taken from 2 anterior and 2 posterior portions around the inoculation site of these 45 fish (39%). Granulomas were detected in the skeletal muscle of 18 fish (Table 1). Granulomatous inflammation was similar to that observed in the lesions undergoing the more advanced stage of tissue repair. The granulomas were most often detected in the anterior portion of the inoculated area (67%), with their detection decreasing in the 3 posterior sections (50, 17, and 6% respectively), which was a result of the inoculation. Invasiveness to other organs was limited in this group; only 2 fish exhibited minor

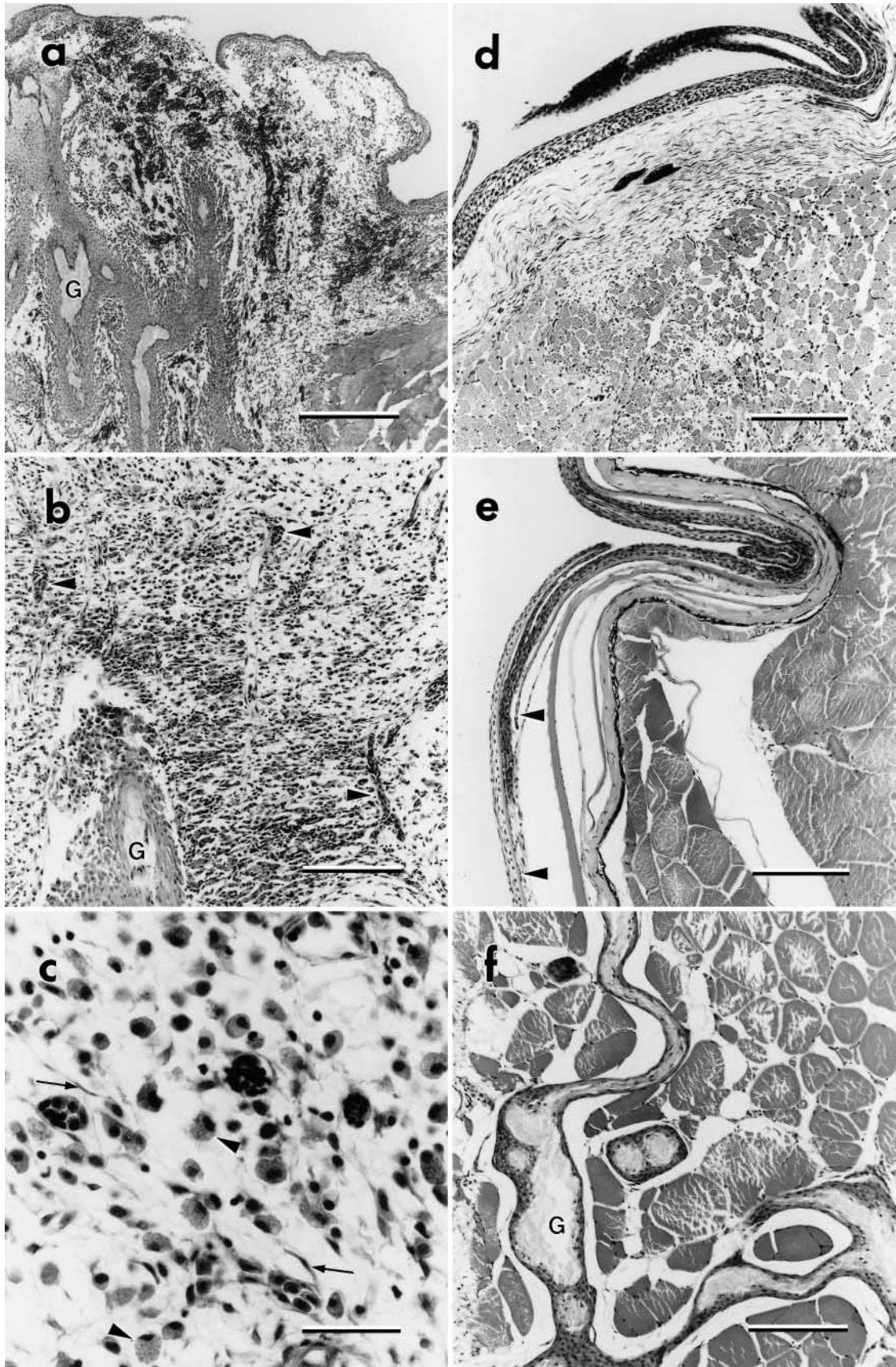




Fig. 6. *Brevoortia tyrannus*. Histological sections of menhaden injected with zoospores sampled at 37 d post-injection exhibiting tissue repair at early (a–c) and late (d–f) stages, 100 zoospores treatment. (a) Granular appearance at the skin surface and well-developed granulomas (G) in a confined area (H&E; scale bar = 500  $\mu\text{m}$ ). (b) Well-organized capillary vascularization (arrowheads) and granular tissues (H&E; scale bar = 100  $\mu\text{m}$ ). (c) Higher magnification showing eosinophilic granular cells (EGCs; arrowheads) and fibrocytes (arrows) filling the lesion area H&E; scale bar = 50  $\mu\text{m}$ ). (d) Thickening of epithelial cells and subdermal layer of the skin tissue and remodeling of the skeletal muscle with regenerated myocytes (H&E; scale bar = 200  $\mu\text{m}$ ). (e) Scale regeneration (arrowheads) (H&E; scale bar = 100  $\mu\text{m}$ ). (f) Elongated granuloma in the deep skeletal muscle, not exhibiting surrounding inflammatory cells (scale bar = 100  $\mu\text{m}$ )

granuloma formation in the kidney (Table 3). After 37 d p.i., the sham-injected controls exhibited clinically healthy skeletal muscle tissues with no obvious pathological findings at the site of inoculation.

#### Aqueous exposures

The pathology arising from aqueous exposures has been described for the early (5 d post-exposure) and middle (21 and 27 d post-exposure) stages of infection (Kiryu et al. 2002). Ulcerous fish collected at 27 d post-exposure in the present aqueous exposure exhibited identical histopathological findings to those described by Kiryu et al. (2002).

Scanning electron microscopy of menhaden exposed to secondary zoospores indicated that the oomycete could encyst on the intact epidermis of the fish (Fig. 7). Germination tubes were evident and apparently penetrated through the epidermal tissues. Adjacent intact epidermal cells appeared normal, but infected cells may have lost their surficial integrity, presumably as a result of penetration of the oomycete hyphae.

#### DISCUSSION

*Aphanomyces invadans* was highly pathogenic to Atlantic menhaden in this study. The calculated  $\text{LD}_{50}$  was 9.7 zoospores per fish, and a single zoospore was capable of initiating an ulcer that could lead to fish mortality. All but one of the menhaden that developed ulcers died in the inoculation experiment. Further, multi-focal lesions developed within fish inoculated with an estimated 1 zoospore, indicating that 1 zoospore can potentially give rise to multiple lesions, thus

confirming the observation that the hyphae travel along the myosepta (Kiryu et al. 2002).

The marked pathogenicity of *Aphanomyces invadans* may result from its highly invasive nature. Hyphae were observed penetrating into the spinal cord, kidney tissues, liver, and uninoculated quadrant of infected menhaden. This invasiveness into the soft tissues has been described from both laboratory infections with ayu *Plecoglossus altivelis* (Wada et al. 1996), and in field-caught menhaden (Noga & Dykstra 1986,

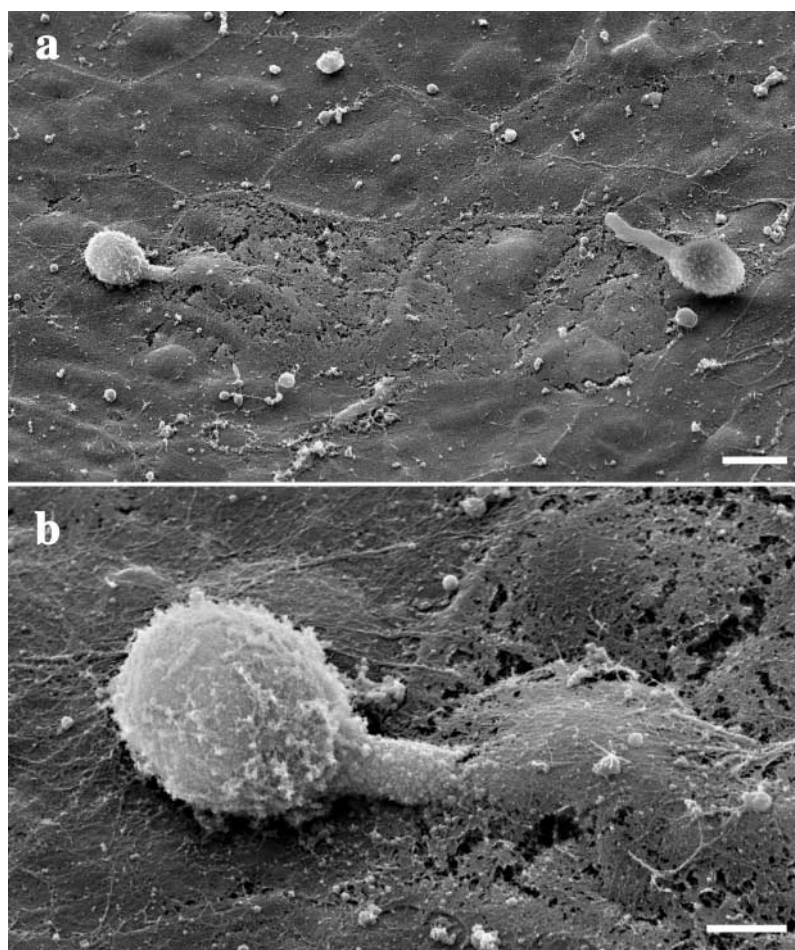


Fig. 7. *Aphanomyces invadans*. (a) Scanning electron micrograph of secondary zoospore with protruding germination tube starting to invade deeper to the menhaden skin (scale bar = 6  $\mu\text{m}$ ). (b) Detail of secondary zoospore with penetration of germination tube firmly into the skin epithelium (scale bar = 1.5  $\mu\text{m}$ )

Noga et al. 1988, Blazer et al. 1999). In experimentally infected ayu, hyphal growth and invasion into the trunk muscles and soft tissues was rapid, with the hyphal mass in the trunk muscle doubling every 10 d (Wada et al. 1996). Frank ulcers in naturally infected menhaden from the field exhibited inflammation and necrosis of the visceral organs into which the hyphae had penetrated (Blazer et al. 1999). In naturally infected goldfish *Carassius auratus* Miyazaki & Egusa (1972) found that if the initial infection was at the operculum (head) or caudal (trunk), then hyphae invaded the brain and spinal cord, and the size of the granuloma was usually smaller than that observed in other organs.

In North Carolina, fish kills involving hundreds of thousands of menhaden have reported prevalences of ulcerative lesions of up to 90% (Burkholder et al. 1992, 1995). Given (1) that *Aphanomyces invadans* is highly pathogenic in laboratory exposures (Kiryu et al. 2002, present study), (2) that it is capable of acting as a primary pathogen (see below), (3) that the water mould causes major mortalities in SE Asia as EUS (Lilley et al. 1998), and (4) that stressed (net-handled) fish are more susceptible to the development of lesions and mortalities (Kiryu et al. 2002, present study), we speculate that *A. invadans* is a significant causal or contributory factor in the large fish kills of menhaden observed in estuaries along the eastern US coast. Further, we speculate that the presence of lesions on dead and dying menhaden involved in large-scale fish kills implicates *A. invadans* as the causal agent, and as such, these kills may be inadvertently correlated with confounding factors such as *Pfiesteria piscicida* (Burkholder et al. 1995, 2001, Burkholder & Glasgow 1997) or hypoxia (Paerl et al. 1998). Thus, a school of menhaden exposed to a highly invasive, highly pathogenic water mould with a high morbidity rate, further compromised by adverse and long-term environmental stressors (i.e. extensive and long-term hypoxia; Paerl et al. 1998, Glasgow et al. 2001), may become infected and succumb to the ulcerative disease.

Menhaden inoculated with *Aphanomyces invadans* experienced 3 outcomes. Fish developed either (1) ulcers and died, (2) internal granulomas without frank ulcers and survived, or (3) clinical signs of tissue repair and survived. Many menhaden inoculated with the oomycete did not develop ulcers; rather, they possessed single or multifocal granulomas (21 to 100% of fish in various treatments). The presence of internal granulomas without overt lesions probably resulted from the artificial nature of exposure (inoculation). Subcutaneous inoculation as the route of infection was necessary to achieve controlled, environmentally realistic doses. However, ulcers more likely develop from the 'outside-in' process of infection (Fig. 7) as opposed

to lesions artificially induced via the 'inside-out' process of inoculation (see Law 2001).

High prevalences of infection with *Aphanomyces invadans* reportedly occur in years with high rainfall, or in regions with low salinities (Callinan et al. 1989, Levine et al. 1990, Noga 1993, Lilley et al. 1998). *A. invadans* has a low salinity tolerance and will not grow well in salinities above 15 psu (Dykstra et al. 1986, Levine et al. 1990, Blazer et al. 2002). The secondary zoospores of *A. invadans* lose their infectivity above 4 to 6 psu (Fraser et al. 1992, Lilley et al. 1998, Kiryu et al. 2002), indicating that infections must occur below 4 psu (Kiryu et al. unpubl. data). Rainfall is thought to contribute to epizootics of EUS by (1) reducing salinity to allow the oomycete to sporulate, (2) inducing sublethal exposure to acidified runoff, resulting in epidermal necrosis and exposure of the dermis (Sammur et al. 1996), and (3) reducing dissolved oxygen to <1 mg l<sup>-1</sup> with concomitant damage to the epidermis (Plumb et al. 1976). However, few of these environmental factors have been explored in relation to the development of UM in menhaden.

We have demonstrated that the WIC isolate of *Aphanomyces invadans* is a primary pathogen of menhaden. That is, the oomycete can attach to, germinate on, and invade the intact epidermis of the fish. By contrast, outbreaks of red spot disease (RSD) in cultured fishes in Australia have been shown to occur only following skin injury (Callinan 1995). Previous challenge experiments with strains of *A. invadans* from SE Asia have indicated that zoospores attach to the dermis, not the epidermis, before invasion; and, therefore, the oomycete is a secondary invader requiring a portal of entry (Lilley et al. 1998). In salmonids, *Saprolegnia* spp. are often considered secondary invaders and have been correlated with predisposing factors such as induced stress (e.g. Pottinger & Day 1999), handling stress or trauma to the skin, but primary cases of saprolegniasis have been reported (Roberts 2001). While the WIC strain of *A. invadans* is highly infectious given a portal of entry, bath challenges on untraumatized fish indicate that it may not require a portal for infection to occur. Clearly, the relationships between integumental damage, exposure, development of lesions, and fish mortality require further study.

Menhaden have a strong cellular response to infection by *Aphanomyces invadans*. Granuloma formation can occur in as little as 5 d after administration of zoospores in both bath exposure and subcutaneous injection (Kiryu et al. 2002). In the present study, a subset of infected animals did not develop ulcers; rather, they had internal granulomas with adjacent tissues undergoing classical signs of tissue repair (e.g. Roberts 1975, 2001, Iger & Abraham 1990). In comparison, Callinan et al. (1989) observed wound healing in sea

mullet *Mugil cephalus* naturally infected with *A. invadans*, as did Chinabut et al. (1995) in snakehead *Channa striatus* after challenge with *A. invadans* zoospores. However, we did not find evidence of tissue repair in fish exposed to zoospores in bath challenges. The bath-exposed fish, especially those in the net-stress treatment, exhibited multi-focal infection patterns along the myosepta. It is possible that these fish were less likely to recover from multiple lesions than those with single localized lesions.

In conclusion, we have shown that *Aphanomyces invadans* is a highly infectious, highly pathogenic, highly invasive primary pathogen in menhaden. Whereas the pathogen is facilitated by a portal of entry, such a portal is not required for infection to occur.

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