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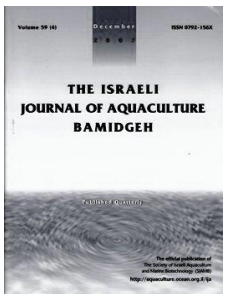

Inter-Species Transmission of the Epizootic Ulcerative Syndrome (EUS) Pathogen, *Aphanomyces invadans*, and Associated Physiological Responses

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Key words: epizootic ulcerative syndrome, intramuscular injection, cohabitation, immune response, hematology, biochemical parameters

Abstract

The epizootic ulcerative syndrome (EUS) pathogen was transmitted to catla (*Catla catla*) using two experimental infection models: intramuscular injection and cohabitation. Oomycetes, recovered from naturally infected ulcerated bata (*Labeo bata*), were identified as *Aphanomyces invadans* based on morphology and histopathology. Lesions typical of EUS were reproduced in the catla using an intramuscular injection of 1×10^5 zoospores/ml autoclaved water from a EUS-affected pond. Lesions were first visible six days after injection; all lesions were swollen and ulcerative 10 days after injection. In the cohabitation experiment with EUS-affected bata, apparently healthy catla exhibited lesions eight days after infection. Histopathology of the muscle and liver from experimentally-infected catla showed the presence of hyphae and granuloma. Twelve days after infection, immunological parameters (superoxide anion and nitric oxide production, leukocyte proliferation, lysozyme activity) of experimentally-infected catla were significantly higher ($p<0.05$) than in the control. Among the hematological parameters, red and white blood cell counts were significantly altered ($p<0.05$) in infected groups whereas differences in hemoglobin content and packed cell volume were not statistically significant ($p>0.05$). Biochemical parameters (total serum protein, serum glutamate pyruvate transaminase, serum glutamate oxaloacetate transaminase, and serum alkaline phosphatase were significantly higher ($p<0.05$) in intramuscularly-injected catla than in apparently healthy fish, however, in catla infected by cohabitation, only total serum protein significantly differed from the control ($p<0.05$).

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Introduction

The epizootic ulcerative syndrome (EUS), endemic to south and southeast Asia, is a serious disease of freshwater and estuarine finfish (Lilley et al., 1997). It is a seasonal epizootic condition characterized by infection of the oomycete *Aphanomyces invadans* and large hemorrhagic necrotizing ulcers typically producing a granulomatous response (Chinabut et al., 1995; Wada et al., 1996; Lilley et al., 1998; Vishwanath et al., 1998). EUS affects over 100 fish species although some commercially important species such as common carp (*Cyprinus carpio*), tilapia (*Oreochromis mossambicus*), and milk fish (*Chanos chanos*) are considered resistant (Lilley et al., 1998).

A distinctive inflammatory response is produced by the innate immune defenses of fish against the invading *A. invadans*. Macrophages encapsulate the invading mycelium to form granulomas (Chinabut et al., 1995; Wada et al., 1996; Thompson et al., 1999). Superoxide anion production in response to EUS infection has been demonstrated in silver barb, *Barbodes gonionotus*, and gourami, *Osphronemus gouramy* (Miles et al., 2001). Hematological studies help to assess the health status of fish exposed to disease (Evenberg et al., 1986; Cruz-Lacierda and Shariff, 1995; Pathiratne and Rajapakshe, 1998).

The present study was conducted in Tripura, one of the northeastern states of India where EUS is common in various fish species. The low winter temperature (20-25°C) in this region is ideal for EUS outbreaks. Bata (*Labeo bata*) is a commercially important minor carp species that is often polycultured with Indian major carps such as catla (*Catla catla*), rohu (*Labeo rohita*), and mrigal (*Cirrhinus mrigala*). During sample collection for the present study it was observed that large catla (approximately 800 g) were unaffected and coexisted with EUS-affected bata. This observation prompted us to investigate whether the oomycete from EUS-affected bata could induce EUS in small catla.

Experimental infection studies are important for understanding the infectivity and role of *A. invadans* in the etiology of this necrotizing ulcerative condition. EUS has been reproduced in fish (Catap and Munday, 2002; Kiryu et al., 2002; Sosa et al., 2007; Oidtmann et al., 2009). The present communication describes inter-species transmission of the EUS pathogen and associated physiological responses. The objectives of this work were (a) to describe the development of the characteristic ulcerative lesions in catla by intramuscular injection of *A. invadans* zoospores isolated from bata, (b) to elucidate the infectious nature of the disease during cohabitation, and (c) to evaluate the involvement of selected immunological, hematological, and biochemical responses in EUS.

Materials and Methods

Fish and husbandry. Apparently healthy catla (mean weight 70 g) were collected from a local fish farm. After collection, they were immediately brought to the laboratory and acclimatized in 1000-l indoor circular tanks supplied with dechlorinated and aerated tap water at ambient temperature (23-26°C). The fish were fed twice daily (rice bran and mustard oil cake in a ratio of 1:1). Water was maintained at optimum levels for the fish species throughout the experiment: dissolved oxygen 6.88±0.56 mg/l, pH 7.14±0.77, ammonia 0.029±0.011 mg/l, nitrite 0.016±0.01 mg/l. Water was periodically exchanged to remove waste feed and fecal material.

Oomycete isolation, identification, culture, and sporulation. EUS-affected bata (mean weight 75 g) were collected from a local fish pond in January-February, 2010. *Aphanomyces invadans* from the affected bata were isolated following the method of Lilley et al. (1998). Pieces of affected muscles (2-4 mm^3) were excised aseptically and placed in a petri dish containing the isolation medium, glucose-peptone-PenStrep broth (GP-PenStrep; Willoughby and Roberts, 1994; Lilley et al., 1998). Inoculated media were incubated at 25°C and examined in an inverted microscope within 12 h. Emerging hyphal tips were repeatedly transferred to fresh plates of GP-PenStrep agar until the cultures were free of bacterial contamination. The organism was subcultured by aseptically cutting a block of agar (3-4 mm) from the periphery of a colony and placing it upside-down in a petri dish containing fresh agar.

Muscle and liver tissues from naturally-ulcerated bata were used for histopathology. Standard procedures were followed for tissue fixation, processing, sectioning, and staining. The isolates were identified based on histopathology and morphology of the cultured organism.

To induce sporulation, an agar plug (3-4 mm) of actively growing mycelium was placed on a petri dish containing glucose-peptone-yeast broth (Lilley et al., 1998) and incubated for four days at approximately 20°C. Autoclaved pond water was prepared by mixing and autoclaving one part water from an EUS-affected pond with two parts distilled water. The agar plug was washed out of the resulting mat by sequential transfer through five petri dishes containing autoclaved water and overnight incubation at 20°C in the water. After 12 h, achlyoid clusters of primary cysts formed and the release of motile secondary zoospores was apparent under microscope. After sporulation, zoospores were collected and their densities estimated with a hemocytometer.

Infection by intramuscular injection. Sixty apparently healthy catla were kept in three 1000-l indoor circular tanks at 20 fish per tank for acclimatization, maintained as described above. The optimal dose of *A. invadans* spores for injection was 1×10^5/ml autoclaved pond water. The fish were injected with 0.2 ml of the spore concentration. Spores were injected intramuscularly into the flank of the fish just below the dorsal fin using a 27-gauge 12.7-mm needle and a 1.0 ml syringe. Fish injected with 0.2 ml autoclaved pond water were used as controls. Fish were examined for gross pathological changes. Muscle and liver were taken from affected fish after decapitation and fixed in 10% phosphate buffered formalin for histopathology. Catla were sampled 12 days after injection and immunological, hematological, and biochemical parameters were measured.

Infection by cohabitation. Forty-five apparently healthy catla were kept with nine EUS-affected bata in three 1000-l indoor circular tanks at 15 healthy catla plus three affected bata per tank for acclimatization, maintained as described above. The fish were checked daily for development of EUS-like lesions. Muscle and liver were taken from affected fish after decapitation and fixed in 10% phosphate buffered formalin for measurement of immunological, hematological, and biochemical parameters. Affected catla were sampled after 12 days of cohabitation with EUS-affected bata.

Collection of blood and separation of serum and plasma. Blood from experimentally-infected and apparently healthy catla was drawn from the caudal vein using a sterile 1-ml hypodermal syringe and a 24-gauge needle. The blood was allowed to clot at room temperature and the serum was separated and stored in sterilized vials at -20°C, until further use. To prepare plasma, blood was collected from the fish using EDTA as an anticoagulant and centrifuged at 1000 rpm for 10 min. The supernatant (plasma) was collected and stored in sterile tubes at -20°C, until further use.

Isolation of head kidney leukocytes. Head kidney leukocytes were isolated following the method described by Kamilya et al. (2008) with some modification. Following blood collection and euthanasia, the head kidney was removed aseptically from experimentally-infected and apparently healthy catla. A cell suspension was obtained by teasing the head kidney tissues with forceps in complete RPMI-1640 medium containing penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% fetal calf serum (Hi-media, India). The cell suspension was washed by centrifugation for 10 min at 1000 rpm. The pellet was resuspended in RPMI-1640 medium, carefully layered on top of the leukocyte isolation medium, HiSep (Hi-media, India), and centrifuged at 1500 rpm for 30 min. Cells at the medium-HiSep interface were transferred to clean tubes and washed twice by centrifugation at 1000 rpm for 10 min. Purified leukocytes were counted using a hemocytometer and their number adjusted to 1×10^6 cells/ml in RPMI-1640 medium. Cell viability was determined by the trypan blue exclusion test.

Nitric oxide assay. Production of nitric oxide was assessed by measuring absorbance of nitrite from the supernatant using the Griess reaction (Green et al., 1982). Head kidney leukocytes (1×10^6/ml) were cultured in complete RPMI-1640 medium in 96-well microtiter plates for 24-72 h at 25°C in a humidified 5% CO_2 incubator. After incubation, the plate was centrifuged and 100 µl supernatant was removed from each well. One hundred µl of 1% sulfanilamide in 2.5% phosphoric acid was added to each sample,

followed by 100 µl 0.1% N-naphthyl-ethylenediamine in 2.5% phosphoric acid, and absorbance was measured at 540 nm.

Superoxide anion production. Production of superoxide anion (O_2^-) by head kidney leukocytes was evaluated following the method of Kamilya et al. (2008). Leukocytes (1×10^6 cells/ml) were seeded into the wells of a 96-well microtiter plate. Nitroblue tetrazolium (NBT) was dissolved in RPMI-1640 medium to a final concentration of 2 mg/ml. Medium containing NBT was filter-sterilized and 50 µl NBT was added to the leukocyte culture. After incubating the plate for 25 min at room temperature, the supernatant was removed from each well and the cells were fixed by adding 200 µl 70% methanol for 1 min. Unreduced NBT was removed by washing cells several times with 70% methanol. Reduced NBT was dissolved by adding 120 µl 2 M KOH to each well, followed by 140 µl dimethyl sulphoxide (DMSO). Optical density was measured at 595 nm.

Leukocyte proliferation assay. The proliferative response of the head kidney leukocytes was determined by the MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay method (Mosmann, 1983) with modifications. Head kidney leukocytes (1×10^6 cells/ml) were distributed into the wells of a 96-well microtiter plate. The mitogen, concanavalin A, at concentrations of 50 µg/ml was added to each well. The plate was incubated for 72 h at 25°C. After incubation, 20 µl MTT solution (dissolved in phosphate buffered saline at a concentration of 5 mg MTT/ml and sterilized by filtration) was added to the wells and the plate was incubated at 25°C for 4 h. After centrifugation of the plate, the media was removed and 200 µl DMSO was added to each well and mixed for 2 min. The microplate was read on a microreader at 595 nm.

Lysozyme activity. The serum lysozyme activity was determined following a turbidimetric method (Ellis, 1990), modified to a microtiter plate assay. Twenty-five µl of each serum sample was added to a 175 µl suspension of the lysozyme sensitive gram-positive bacterium *Micrococcus lysodeikticus* (0.2 mg/ml in 0.05 M sodium phosphate buffer, pH 6.2). The reaction was carried out with constant shaking and absorbance was recorded at 450 nm after 1 and 5 min. One unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001/min.

Hematological parameters. Hematological parameters were measured using standard procedures and included total red blood cell count, total white blood cell count, packed cell volume, and hemoglobin content for experimentally-infected and apparently healthy catla.

Biochemical parameters. Biochemical parameters were measured using a biochemical test kit (Crest Biosystems, India) as per the manufacturer's instructions and included serum glutamate pyruvate transaminase (SGPT) activity, serum glutamate oxaloacetate transaminase (SGOT) activity, serum alkaline phosphatase (ALP) activity and total plasma protein for all experimentally-infected and apparently healthy catla.

Statistical analyses. Data were statistical analyzed using SPSS-15.0 for Windows software (SPSS Inc., Chicago, IL, USA). Results are presented as means±standard deviation. Mean values were compared by one-way ANOVA. Significance was inferred at $p<0.05$.

Results

Clinical signs, gross pathology, and histopathology of naturally-ulcerated bata. Lesions on the body surface of the naturally-ulcerated bata were moderately advanced. Diseased fish had varied clinical signs, including hemorrhage, ulcers, grey erosions. and penetrating wounds on different parts of the body including the caudal peduncle and the dorsal and ventral surfaces. Some affected fish had more advanced lesions in the form of circular or open ulcers with complete loss of dermis and scales. Histopathological examination of muscle and liver tissues from the bata revealed the presence of hyphae and granuloma (Fig. 1). Hyphae penetrated the muscle section and some hyphae and associated granuloma were elongated, without surrounding inflammatory cells. The clinical signs and gross pathology of the ulcerated fish, plus the presence of mycotic granuloma in affected tissues, indicated a positive diagnosis for *A. invadans*.l

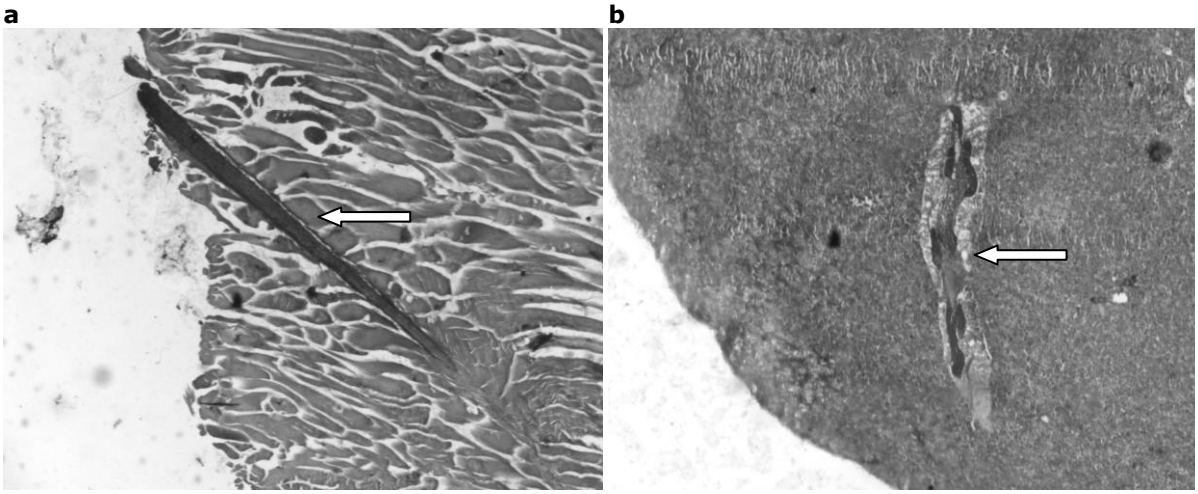

Fig. 1. Naturally-infected *Labeo bata*: (a) *Aphanomyces invadans* hyphae (arrow) penetrate the muscle, (b) elongated fungal hyphae and associated granuloma (arrow) in the liver.

Isolation and culture of oomycetes. Cultures from muscle tissue revealed non-septate, broad, sparsely branching hyphae with diameters ranging 11-15 µm. Following sporulation, filamentous zoosporangia and achlyoid clusters of primary zoospores formed (Fig. 2). Sporangia had one or two lateral evacuation tubes. No other fungus-like organisms morphologically consistent with those present in the bata ulcers were recovered in culture. The cultural features further confirm that *A. invadans* was the causative agent for the ulceration in the bata.

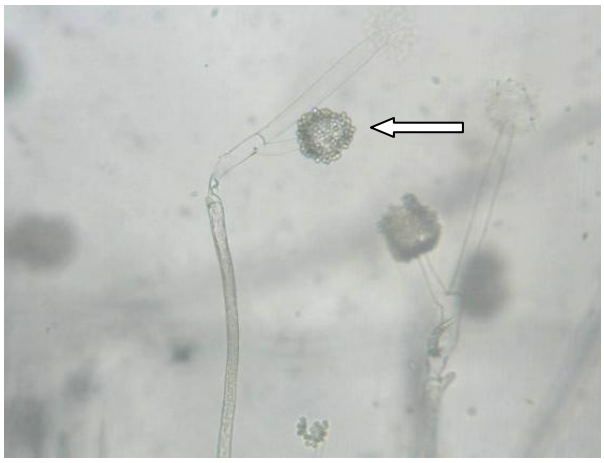

Fig. 2. Achlyoid cluster (arrow) of primary zoospores of *Aphanomyces invadans*.

Intramuscular injection. Catla challenged by intramuscular injection exhibited erythema at the injection site. Lesions appeared as petechiae at the site of the injection six days post infection (dpi). By 10 dpi, the lesions were swollen and ulcerative, resulting in sloughing of the scales around the lesions. Control fish did not display behavioral or clinical signs of disease throughout the experiment. Histopathological examination revealed hyphae and granulomatous reactions in muscle and liver tissue sections, similar to those described above for the bata.

Cohabitation. Catla exposed to cohabitation with EUS-affected bata exhibited lesions 8 dpi. Lesions initially appeared as petechiae on the dorsal surface of the body. By 12 dpi, the lesions were ecchymotic and appeared as areas of ulceration. Histopathological examination revealed hyphae and granulomatous reactions in muscle and liver tissue sections, similar to those described above for the bata.

Immunological, hematological, and biochemical parameters. Immunological parameters were generally significantly higher in experimentally-infected catla than in apparently healthy catla (Table 1). The red and white blood cell counts of experimentally-infected catla were significantly higher than those of apparently healthy catla while packed cell volume and hemoglobin content did not statistically differ. While all biochemical parameters in injected catla were significantly higher than in the control, only total protein was statistically differed in cohabitation catla.

Table 1. Immunological, hematological, and biochemical parameters (means±standard deviation) in the Indian major carp, *Catla catla*, experimentally infected by *Aphanomyces invadans.*

		Infected by...	
	Apparently healthy	*Cohabitation*	*Intramuscular injection*
Immunological parameters (OD = optical density)			
Superoxide anion production (OD = 595 nm)	0.047±0.011	0.095±0.010*	0.097±0.012*
Nitric oxide production (OD = 540 nm)	0.46±0.073	0.55±0.1*	0.56±0.06*
Lymphocyte proliferation (OD = 595 nm)	0.39±0.046	0.44±0.026*	0.44±0.026*
Lysozyme activity (U/ml)	93.33±26.13	112.0±16.78	136.0±39.52*
Hematological parameters			
Red blood cells ($\times 10^5$/mm)	16.70±0.094	16.24±0.092*	16.42±0.016*
White blood cells ($\times 10^4$/mm)	4.05±0.05	5.02±0.21*	4.86±0.36*
Packed cell volume (%)	12.33±1.53	10.67±1.15	11.0± 1.73
Hemoglobin content (g/dl)	5.43±0.15	4.80±0.17	4.87±0.31
Biochemical parameters			
Total plasma protein (g/dl)	5.86±0.18	6.77±0.28*****	6.82±0.25*****
Serum glutamate pyruvate transaminase (U/ml)	21.33±1.15	24.33±1.52	28.0±2.0*****
Serum glutamate oxaloacetate transaminase (U/ml)	27.67±1.53	30.0±2.0	35.33±1.52*****
Alkaline phosphatase (KA units)	1.35±0.10	1.54 ±0.08	1.72±0.15*****

* significantly differs from apparently healthy fish ($p<0.05$)
Five fish from each treatment were assayed in triplicate.

Discussion

The ponds from which the EUS-infected bata were collected contained larger catla than those used in the study and they were apparently uninfected, suggesting that large fish are less susceptible to *A. invadans* than small fish. Indeed, catla greater than 1 year old seem to be resistant to infection by *A. invadans* (Pradhan et al., 2007).

Cohabitation of healthy catla with EUS-positive bata resulted in EUS-like lesions in the catla, indicating that EUS infection is waterborne. The infectious nature of the disease is suggested by the transmission of EUS under natural conditions (Balasuriya et al., 1990; Cruz-Lacierda and Shariff, 1995). In addition, an earlier cohabitation study showed the development of EUS-like lesions under simulated natural conditions (Lio-Po et al., 2003).

Histopathological examination revealed inflammatory responses against the invading organism, often the formation of granulomas, as previously described (Chinabut et al., 1995; Wada et al., 1996; Thompson et al., 1999; Saylor et al., 2010). This inflammatory response is governed by the innate immune defenses of the fish where granulocytes are thought to play an important role. However, the mechanism by which granulocytes participate in the inflammatory process against invading oomycetes is not well understood. Immune functions of granulocytes such as reactive oxygen and nitrogen species and lysozyme production play an important role in eliminating pathogens, especially bacteria (Secombes, 1990; Yin et al., 1997). The increase in these parameters in EUS-infected catla indicates the possible involvement of these mechanisms in inhibiting the replication of *A. invadans*. However, the increased responses, which generally occur at an early stage of any infection, might not be effective enough to prevent the spread of infection in later stages. The apparent ability of *A. invadans* to withstand such responses may explain the susceptibility of catla to EUS. Both experimentally-infected catla groups showed increased leukocyte proliferation in response to the mitogen, concanavalin A, indicating that leukocytes are polyclonally activated in EUS-infected fish. An enhanced proliferative response was also observed in Atlantic menhaden with ulcer disease syndrome (Faisal and Hargis, 1992). The possible impact of leukocyte activation and proliferation on the development of granulomatous reactions associated with the disease remains to be elucidated.

EUS-infected catla were anemic as evidenced by a decreased total red blood cell count, packed cell volume, and hemoglobin content. Anemia might be due to

hemodilution caused by a loss of body fluid from hemorrhagic/necrotic lesions in infected catla as observed in EUS-affected Asian cichlid fish, *Etroplus suratensis* (Pathiratne and Rajapakshe, 1998). Decreased hemoglobin and hematocrit levels in EUS-affected *Ophicephalus striatus* have also been attributed to hemodilution (Cruz-Lacierda and Shariff, 1995). The increased total leukocyte counts in EUS-infected catla might be responsible for the enhanced immunological responses; the leukocyte count was higher and the hemoglobin content lower in blood from EUS-affected Indian fish than in healthy fish (Kar et al., 2000).

Levels of total plasma protein, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and serum alkaline phosphatase (ALP) were higher in both experimentally-infected groups than in the apparently healthy fish. One of the reasons for increased total protein concentration in serum might be the formation of anti-*A. invadans* antibodies in the serum. Elevated levels of antibodies to *A. invadans* have been reported from striped snakehead (*Channa striata*; Thompson et al., 1997; Miles et al., 2001) and rainbow trout (*Oncorhynchus mykiss*; Thompson et al., 1999) and high values of SGOT, SGPT, and ALP were reported from EUS-affected fish (Kar et al., 2000). Generally, the enzymes SGOT and SGPT are found in different tissues, with a higher concentration in the liver (Nyblom et al., 2006). Increased SGOT, SGPT, and ALP may be due to hepatocyte damage by the *A. invadans* hyphae. The changes in these enzymes were smaller in fish infected by cohabitation than in intramuscularly-injected fish, perhaps because motile zoospores were directly injected into the muscle where zoospore germination and proliferation were rapid, leading to heightened tissue destruction and spread of the organism.

In conclusion, we successfully demonstrated the experimental induction of EUS in small catla suggesting the infectious nature of the disease. The fact that catla are susceptible to EUS has significant implications for fish farmers in the north-eastern region of India. Keeping in view the strong transmission potential, polyculture between Indian major carps and *L. bata* should be reassessed. The altered immunological, hematological, and biochemical parameters indicate a strong physiological response against infection, but this response was not effective at controlling the replication or spread of the organism. The physiological responses, especially immune responses, of the fish to *A. invadans* need to be further examined.

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