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Investigation on the mortality of juveniles in captive stock of the Indian halibut *Psettodes erumei*

Joe K. Kizhakudan[1], P. Ezhil Praveena[2], B.A. Venmathi Maran[3], S.K. Otta[2], T. Bhuvaneswari[2], J.J.S. Rajan[2], S. Krishnamoorthi[1] & *K.P. Jithendran[2]
[1]Madras Research Centre, Central Marine Fisheries Research Institute,
75 Santhome High Road, Chennai-600 028, India
[2]Aquatic Animal Health and Environment Division, Central Institute of Brackishwater Aquaculture,
75 Santhome High Road, Chennai-600 028, India
[3]Marine Ecosystem Research Division, Korea Institute of Ocean Science & Technology,
P. O. Box 29, Ansan, Seoul 425-600, Republic of Korea
*[E-mail: kpjithendran@yahoo.com]

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A study was undertaken to investigate the cause of mortality in the captive stock of Indian halibut, *Psettodes erumei* (Bloch & Schneider, 1801) (Psettodidae). Halibuts were severely infected with two species of parasitic copepods, *Protochondracanthus alatus* (Heller, 1868) and *P. trilobatus* (Pillai, 1964) (Poecilostomatoida, Chondracanthidae) and also with protozoan *Amyloodinium* sp. (Blastodinida, Oodiniaceae) on gills. Since the parasitic copepods have high host specificity on *P. erumei*, a large number of copepods were collected. Gill lamellae appeared pale and excess mucous secretion was observed on the body surface. Histopathological changes were mainly in the gill tissues with severe lamellar hypertrophy and hyperplasia, lamellar fusions with the presence of trophonts of *Amyloodinium* sp. Wild caught tilapia, and juveniles of mullet and milkfish used as live feed were suspected to be the cause of transmission to the rearing system. Infected fishes showed loss of appetite, irregular swimming, restlessness, exhaustion and gaping at the surface. Samples tested were negative for betanodavirus and systemic bacterial infection. Management intervention and multiple treatment of rearing facility using formalin @ 200 ppm controlled the infection, and half of the stock could escape from mortality.

[Keywords: Indian halibut, *Psettodes erumei,* Copepods, *Protochondracanthus alatus,trilobatus*, *Amyloodinium* sp., Pathogenic infection, Mortality, Treatment]

Introduction

Among the many marine fishes cultured under controlled environment, flatfish cultivation under captive condition is gaining prominence[1]. Flatfish farming is increasing steadily in many parts of the world, and the members of this group are represented in the list of 'new species of aquaculture' suitable for both sea-based and land-based farming systems[2]. A lot of development has taken place in temperate and Mediterranean countries like Norway, Spain, France, United States and Israel in domestic rearing and large-scale aquaculture of temperate halibuts and sub-temperate turbots[1,3,4]. However, captive rearing of the tropical Indian halibut *Psettodes erumei* (Bloch & Schneider, 1801) (Psettodidae) is yet to be achieved. Domestication of *P. erumei* in India is being attempted at the Kovalam Field laboratory of CMFRI to establish a viable rearing technology and substantiate the falling production of this commodity in the wild. Live juveniles and sub-adults from Chennai coast are being reared in captivity to understand their growth, feeding, breeding biology and amenability to captive maturation.

Health management measures are a vital part of intensified culture practices, lack of which results in severe loss due to mortality. Sound aquatic animal health is based upon prevention of disease[3]. Diseases due to parasites pose severe problem in the culture and captive maintenance of a variety of marine fishes[5-8]. In the aquatic environment dinoflagellates are also common and are found to be symbiotically associated with

several vertebrates[9-10]. The present study describes a case of mortality associated with mixed parasitic infections in captive stock of *P. erumei* juveniles reared at Kovalam Field Laboratory of CMFRI, and the management interventions for successful treatment.

Materials and Methods

Juveniles of *P. erumei* were collected from the shallow waters off southeast coast of India (opp. Chinnacuddalore N 12^{O} 26' 938" E 80^{O} 08' 704" and opp. Chemmenchery, N 12^{O} 44' 672" E 80^{O} 16' 267") and transported in live condition using fibreglass reinforced plastic (FRP) tanks to the Field Laboratory of Madras Research Centre of CMFRI located at Kovalam, south of Chennai, India, as a part of captive broodstock development programme. Fishes were acclimatized and maintained in three circular FRP tanks of three ton capacity with 3 m^2 floor-space, filled with filtered and aerated sea water (salinity 30-35 ppt; temperature 28-30^{o}C; pH 7.8-8.3) after a preliminary quarantine protocol during which the fishes were kept in marine quarantine tanks for five days, given 200 ppm formalin bath (30 min) for two days and then released into the holding tanks. The fishes (n=300) maintained in the facilities were of 170-220 mm in total length (TL) at the time of initial stocking, @100 numbers per tank. However, during the time of this investigation, the mean length and weight recorded were 228 mm (range=180-275 mm) and 155 gm (range=125-300 gm), respectively. The input water source to the rearing tank was through sediment base, filtered through series of slow sand filters/pressure sand filters. Due to the bottom dwelling habit of the flatfish, the holding system required specific modifications. The bottom of the tank was provided with marine sediments (1000-1800 µm) pre-treated with dry lime and chlorine (chlorate crystals); sun-dried and washed thoroughly. The fish caught from the wild remained shy for nearly 5-10 days of initial handling and later started accepting live feed (tilapia, milkfish and mullet (TL=70-150 mm), collected from the backwaters nearby the research station on a daily basis, screened through quarantine tanks for 24 hours and acclimatised to the required salinity.

Severe mortality was noticed in the halibuts maintained in the rearing tanks with signs of gasping and distress, a month after stocking, during August-September 2012, coinciding with monsoon season. The fishes of various size groups in different tanks exhibited low-grade mortality initially, 2-3 fish/day followed by a phase of rapid mortality for one week leading to 50% loss of stock within a month of initial stocking. The first signs were change in appearance from golden brown or sand coloured banded fish to deep chocolate brown. Fish started swimming in circles, moving upside down with exhaustive respiratory movements, gaping of mouth causing intake of water. Fishes came to the surface very frequently and later the gills became very pale. Within 12 hours after onset of these clinical symptoms, the fish started dying progressively reaching cumulative mortality of about 50%. The affected fishes secreted excess mucous during the final stages.

All dead fishes were examined for any gross clinical lesions. However, for a comprehensive necropsy, two live fishes (280-300 g, 180-275 mm length) in advanced stage of clinical signs were subjected to detailed investigations at Aquatic Animal Health Laboratory of Central Institute of Brackishwater Aquaculture (CIBA), Chennai. The fishes were sacrificed and detailed postmortem examination was done.

For bacteriological examination, blood sample was collected by puncturing the ventral vein with the help of a sterile syringe. The collected blood was inoculated into Zobell Marine Agar (ZMA) broth and incubated at 30°C for 48 hours. Besides this, tissue samples of brain and eye were collected in absolute alcohol to examine the fishes for viral diseases like viral nervous necrosis by polymerase chain reaction (PCR) using primers forward (5'-CGA GTC AAC ACG GGT GAA GA-3') and reverse (5'-CGT GTC AGT CAT GTG TCG CT-3'). PCR was performed as described earlier[11].

The gills were excised and gill filaments were removed, placed in a petri-dish containing normal saline and examined under stereozoom microscope. Similarly, the visceral organs were also dissected out and examined for any internal parasites. A representative number of wet mounts of scrapings from the skin, fins, gills and other internal organs were prepared and examined under binocular microscope for the presence of ecto and endoparasites. Parasites attached on the gills were

examined live and were carefully removed using fine forceps microphotographed and preserved in 70% ethanol for identification. The copepods were processed by following wooden slide procedure[12] and identified[13].

Tissues samples such as gills, liver, spleen, heart, brain, stomach and kidney were fixed in 10% neutral buffered formalin and processed by standard histological method for histopathological examination. Tissue sections (4-5 µm) were cut using Leica RM 2245 microtome and stained with Haematoxylin and Eosin (H&E) and mounted with DPX mountant. The histological changes were recorded using an Olympus CX41 microscope with digital camera C7070 attachment.

Results

The initial signs of morbid fishes were darkening of the skin colour from golden brown or sand coloured banded fish to deep chocolate brown (Fig. 1A & B). Infected fishes exhibited abnormal swimming behaviour like swimming in circles, anorexia, depression, moving upside down with severe respiratory distress, gaping of mouth and sudden collapse. In few fishes intake of water could also be seen. Fishes swim near the surface frequently with dyspnoea and later the gills become very pale. In the terminal stages the affected fishes secreted excess mucous. Mortality was observed within 12 hours after onset of these clinical symptoms.

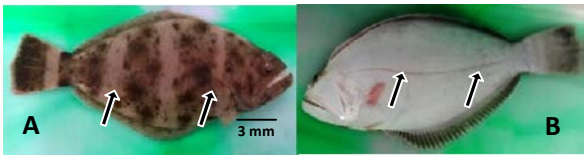

Fig. 1-Clinically affected Indian halibut *Psettodes erumei* showing the discoloration of the body; (A) dorsal side; (B) ventral side. Arrows indicate the pale discoloration, scale bar: 3 cm

On necropsy, the affected fishes did not exhibit any visible gross changes. All the external and internal organs appeared normal. During the course of investigation, the gill lamellae showed presence of grossly visible parasitic copepods with long coiled egg sacs filled with eggs and wet mount examination of the gills showed the presence of large number of microscopic oval shaped structures resembling dinoflagellates which were found attached to the gills in large numbers (Figs. 2-4). Many of them were also seen freely outside the gills, either in single cell stage or in dividing stages. However, the body surface was found to be free from parasitic infection.

Even after 48 hours of incubation, there was no turbidity in ZMA broth and therefore, the fishes were negative for any systemic bacterial infection. Similarly, all the samples subjected for PCR analysis were also found to be negative for betanodavirus infection.

A total of 4-5 copepods were collected from the gills of each infected fish, however the protozoan infections were plenty. The copepods were identified as poecilostomatoids of two different species such as *Protochondracanthus alatus* (Heller, 1868) and *P. trilobatus* (Pillai, 1964) belonging to the family Chondracanthidae. Both chondracanthids *P. alatus* and *P. trilobatus* were found attached together on the halibuts. The former is longer, and the latter is smaller. It can be mistaken as a juvenile of the former in size, but can be well differentiated by some characteristic features. The diagnostic features for these two species are given below:

Diagnostic features of two copepods

Protochondracanthus alatus **(Heller, 1868)** (Fig. 2A-C).

The body is long (4.32-4.68 mm) and well elongated (Fig. 2A). Head is found with two protrusions of small knob-like processes laterally. A pair of vermiform processes near oral region is present (Fig. 2B). It has a long cylindrical trunk with a pair of long lateral processes and short posterior processes at end. Genital somite is wider than long, found with dwarf male (Fig. 2C). Egg sacs are found to be longer than the body size.

Protochondracanthus trilobatus **(Pillai, 1964)** (Fig. 3A-C).

Body is not well elongated as found in *P. alatus* and short (2.78-2.84 mm) in length (Fig. 3A). Head is round-shaped, without two knob-like protrusions (Fig. 3B). It also lacks the oral processes. The two lateral trunk processes that hang out from the neck portion are also short. Genital somite is slightly wider than long (Fig. 3C). Egg sacs are found either longer than or equal to the body size.

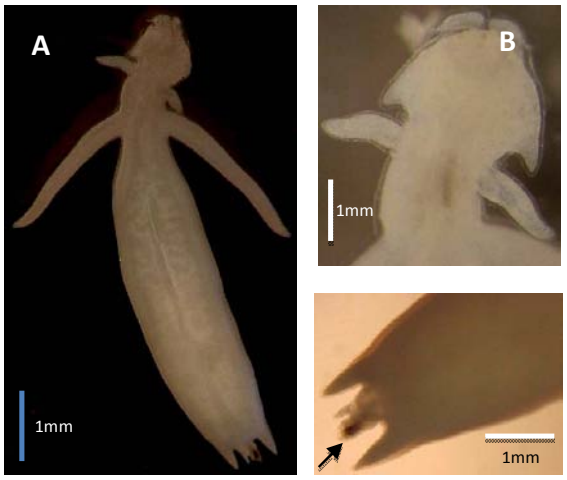

Fig. 2- *Protochondracanthus alatus* (Heller, 1868); (A) female habitus showing body with cylindrical trunk and caudal ramus; (B) enlarged head with two vermiform processes; (C) enlarged genital somite showing caudal ramus, arrow indicates the attachment of dwarf male

Fig. 3- *Protochondracanthus trilobatus* (Pillai, 1964); (A) female habitus with ovigerous eggs, showing body with cylindrical trunk and caudal ramus; (B) enlarged head; (C) enlarged genital somite showing caudal ramus

Diagnosis of Dinoflagellates

The pear shaped to ovoid cysts observed in the gill tissues were diagnosed as dinoflagellate *Amyloodinium* sp. embedded in the vascular tissues of the gill lamellae (Fig. 4A). Tomonts in various stages of development were also detected while examining the gills (Fig. 4B).

Histopathology

Histopathological changes were observed only in gills while all the other organs such as liver, spleen, heart, brain, stomach and kidney appeared to be normal and no lesions were evident. The gill tissues showed severe lamellar hypertrophy and hyperplasia in both the primary and secondary gill lamellae (Fig. 5A). The parasitic stage trophont was seen encysted in the gill tissue (Fig. 5B). There was a massive proliferation of branchial epithelium surrounding the invading protozoan. Haemaorrhagic infiltrations with lamellar fusion and disorganisation were seen in almost all the gill tissues (Fig. 5A).

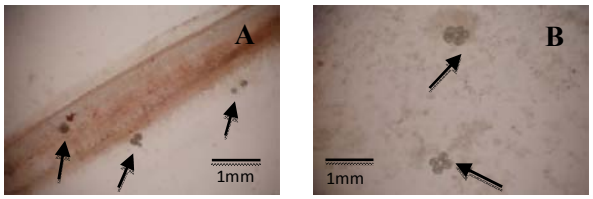

Fig. 4- (A) *Amyloodinium* trophonts (arrows) on gill tissue of Indian halibut, *Psettodes erumei*, 40x, (B) Tomont (reproductive stage) of the parasite, *Amyloodinium* sp., 100x (arrows)

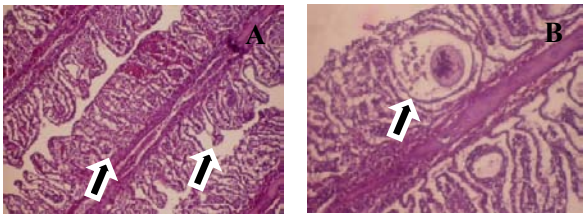

Fig. 5- Histopathological section of the gill filaments of Indian halibut, *Psettodes erumei*; (A) fusion of lamella (arrows) H&E 40X; (B) dinoflagellate, *Amyloodinium* sp. in gill sections (arrow) H&E 100X

Management interventions

Several doses of diluted formaldehyde were tried to separate the attached forms of parasites. On confirmation of parasites among the fish, two successive treatments of 100 ppm formalin for one hour duration at one day interval gave complete termination of the parasites from the fishes held in captivity. The adult and juvenile stages of many copepod parasites could be collected at the bottom of the tanks after each successive treatment. For treatment of fishes, live animals were collected without much handling stress, using knotless

(cloth texture) scoop nets and shifted to a treatment tank with formalin. The treatment duration was one hour and the dose was prepared and kept conditioned at 28-29°C temperature, 35 ppt, salinity and pH of 8.2) with 100 ppm formalin in 300 L FRP tanks kept close to the rearing tank. The rearing tank water level was lowered to half the capacity and the similar treatment dosage was given to the substrate (to sanitise the facility from eggs and developing stages of the parasites) for the same duration and flushed out. After resettling, the fishes were inspected for random surface check and released back in the original rearing tanks. The survival at the end of the treatment was about 50% in the medium size groups.

Discussion

Intensive aquaculture of fish is not without its inherent problems, and these include disease outbreaks and consequences of introducing parasites to new hosts and/or new localities with the transportation of live fish[14]. They further highlighted epizootics caused by parasites which cause morbidity and mortality in both cultured and natural habitats with control measures. Recently, mass mortality of adult olive flounder *Paralichthys olivaceus* showing ascites occurred at many aquaculture farms in South Korea[7]. In India also the diseases due to parasites pose severe problem in the culture and captive maintenance of a variety of marine fishes[6, 8].

In the present study, the clinical course observed includes erratic swimming behaviour, isolation, change of body colouration to chocolate brown, loss of appetite and emaciation before succumbing to death. In August 2012, the coastal water was very turbid due to sudden turbulence in the seawater and there were reports of fish deaths on the beach at Kovalam and neighbouring area (personal observations, Joe K. Kizhakudan). In this case the affected fish gathered at the surface in areas where the dissolved oxygen concentrations are higher since oxygen mixed waters are supplied into the holding tanks at the surface *via* air lift pumps. Similar phenomenon was observed and reported earlier in USA[15]. Based on necropsy and detailed laboratory investigations, we could suggest that the cause of death was mainly due to the multiple parasitic infections. *Amyloodinium*, a dinoflagellate is known to penetrate deep into host epithelium using a rhizoid-like structure and cause substantial damage to tissues at the attachment site. Morbidity and mortality from amyloodiniosis can be severe, sometimes with rapid onset over a period of a few days. Affected fish may die suddenly, showing few clinical signs, but in most cases behavioral and physical changes were observed before death. If the site of infection is gill, which seems to be the most common, the primary clinical signs reported were respiratory disorder. These may include increased respiratory rate (rapid movement of the opercula), "piping," and gathering at the surface or in areas with higher dissolved oxygen concentrations, as well as reduced appetite.

As the affected fishes exhibited gasping symptoms and histopathological examination revealed heavy load of trophont in the gill tissue, it can be suggested that asphyxia might have caused the mortality in fishes. Paperna[5] has also observed that a single trophont feeds on multiple epithelial cells simultaneously and can cause extensive damage to the gill tissue. The main histological changes observed in these animals were hyperplasia of the gill branchial tissue. It might be due to the adaptive mechanism developed by the affected fishes in order to withstand the irritant due to the heavy load of protozoans[16]. Further, gills are involved in many important functions in fish, such as respiration, osmoregulation, and excretion. Although hyperplasia is due to the protective mechanism exhibited by the fishes in response to the heavy parasitic load, it impairs respiration, excretion of nitrogenous waste materials and disturbs osmotic balance[17]. It was found to lower the circulation at the gills, widen the blood spaces and contract the pillar cells[18]. The diffuse gill tissue impairment due to the presence of the copepod and the trophont might have caused loss of fish respiratory surface and there by asphyxiation and finally death of the fishes. Further the fishes were negative for all the bacteriological and virological tests carried out. Apparently, the microscopic observations indicate that the parasites are potentially pathogenic[14], and that heavy parasitism could compromise gill functions resulting in severe mortality.

The histological examination revealed the trophonts of protozoa encysted in the gill tissue

with massive proliferation of branchial epithelium surrounding the encysted parasitic trophonts. Both the primary and secondary gill lamellae were heavily infiltrated by erythrocytes. The lamellar fusion with disorganization was seen in almost all the gill tissues. Hypertrophy of the lamellar epithelium was seen in few areas. In the affected fish diffuse necrosis of the branchial tissue was observed.

Chondracanthidae is one of the major families of Copepoda, comprised with more than 40 genera including 150 species[13]. The flatfishes are considered as the most preferred host for chondracanthids. In this study, the infected copepods are found as host specific to Indian halibut[13,19]. It was so far reported from wild fishes caught off Sri Lanka[20], India[13, 19] and China[21]. Purivirojkul and Areechon[22] also reported parasitic copepods from marine fishes from the Gulf of Thailand and opined *Protochontracanthus* spp. to be specific to their hosts, *i.e. Psettodes erumei.*

We predict three possible routes of introduction of dinoflagellate in a closed system leading to *Amyloodinium* outbreak: 1) being a small sized life cycle stage, tomonts or infective dinospores can be introduced directly with incoming seawater, becoming a source of infection for fish in the system; 2) fresh introduction of fish infected with trophonts into a culture system to begin the reproductive process; and 3) introduction through food items especially the live wild fish. Once introduced, *Amyloodinium* infection can be controlled by chemical treatment, flushing and filtration[23,24]. However, the copepod, chondracanthids are known to be specific to flat fishes, it is unlikely to enter in culture system through other forage fishes used as live feed. Hence the fresh introduction of infected fish could be the possible route. These also emphasize that the need for strengthening the quarantine protocol and maintenance of water quality and pre-stock management to check the entry of infective stages and their multiplication in closed culture system.

Formalin has been used as an effective therapeutic compound in aquaculture in both freshwater and marine hatcheries[25]. In the current study, formalin was indeed effective against both the species of parasitic infections. Constraints in the use of formalin often arise due to side effects on hosts, especially when mixed infections with other parasites and secondary bacterial infections are common. Strict regulations also exist in some countries like Japan, where formalin is forbidden in off-shore cultures due to harmful effect on the environment and, others like USA[26] restricted its use to a maximum dose of 170 ppm at temperatures higher than 10^{o}C. Due to the inherent drawbacks of bathing of infected fish, especially in floating cages, and the growing environmental concern, the possibility of administering effective parasiticides in the feed would be more convenient and should be explored. Temporary exclusion of fish from the tanks also is likely alternative to try in a land-based system as in this study. The lesions associated with copepod and dinoflagellate indicates that these can turn to be serious pathogens in host fish in captivity. However, more attention should be paid to its transmission dynamics in land based culture system.

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