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Zoea-2 syndrome of *Penaeus vannamei* in shrimp hatcheries

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ABSTRACT

Mortalities of Pacific white shrimp, *Penaeus vannamei* during zoea stages were investigated in fifteen Indian shrimp hatcheries located on the east coast of India. Popularly known as zoea-2 syndrome, is characterized by reduction in feeding rate of late zoea 1 and early zoea 2 stage larvae, impairment in metamorphosis followed by high mortalities. Microscopic studies revealed systemic abnormalities in affected larvae and pathological manifestation in hepatopancreas and intestine. Microbiological screening revealed the predominance of *Vibrio alginolyticus* in majority of the hatcheries (nine) affected by zoea-2 syndrome. Histological examination on the hepatopancreas and intestine revealed vacuolization, sloughing of epithelial cells and disintegration of peritrophic membrane of intestinal epithelium. OIE listed viral pathogens of shrimp were absent in affected larvae as confirmed by OIE polymerase chain reaction (PCR) protocols. Ultrastructural observation of the pathological manifestation in hepatopancreas and intestine could not reveal presence of any pathogens. Data on the water quality parameters were in the normal ranges and did not seem to have any bearing on the outbreak of zoea-2 syndrome in the hatcheries. Feeding schedules (*Skeletonema*, *Chaetoceros*, *Thalassia*) were uniform throughout the larval cycles and found to be not associated with the zoea-2 syndrome. Pathological manifestations of hepatopancreas and intestine indicated impairment of capacity of digestion and absorption resulting in delayed moulting and subsequent death of larvae in a gradual, progressive manner with cumulative mortality reaching of 30–100% at zoea II stage. Continuous stocking of nauplii over three to four days within the same hatchery larval rearing unit was found to exacerbate the incidence in the nine affected hatcheries (OR-48, CI 2.5–932.9). It could be construed from our studies that aetiology of zoea syndrome may not be due to known infectious agents.

1. Introduction

Shrimp aquaculture is a highly dynamic and rapidly growing enterprise dominated by exotic SPF *Penaeus vannamei* since its introduction in 2009 in India. The success of commercial shrimp industry mainly depends on the availability of healthy and quality seed. The intensive shrimp larviculture has substantially improved over the decade due to the rising demand from the growing farming sector. Presently in India, 276 shrimp hatcheries are involved in the production of SPF *P. vannamei* seeds, catering to the shrimp aquaculture sector (CAA, 2017). This increasing trend in intensification and commercialization has exacerbated the epizootics of diseases.

Diseases are significant challenges to shrimp larval rearing systems particularly of bacterial diseases such as luminescent bacterial disease which causes severe economic consequences to the hatchery operations (Austin and Zhang, 2006). Apart from the diseases of viral and bacterial origin, large scale losses of eggs and larvae have been reported due to larval mycosis caused by *Legenidium* spp. and *Sirolpidium* spp. and larval fouling caused by protozoans such as *Zoothamnium* and *Vorticella* (Karunasagar et al., 2001).

However, lately, in the post vannamei introduction, Indian shrimp hatcheries have been experiencing mortality of larvae at zoea II stage with impairment in molting resulting in heavy mortalities. Similar larval losses were reported earlier with "zoea-2 syndrome" in *P. vannamei* shrimp larvae in Ecuador, Mexico and the United States (Morales and Cuéllar-Anjel, 2008) in 1993. The occurrence of mortalities of *P. vannamei* zoeal stages reported frequently in Indian shrimp hatcheries prompted us to investigate the problem holistically, considering the possible involvement of biotic and abiotic factors.

2. Materials and methods

2.1. Sampling

Fifteen commercial shrimp hatcheries situated along the east coast of India (Fig. 1) were included in the investigation including six from

Abbreviations: OIE, office international des epizooties; PCR, polymerase chain reaction; CAA, coastal aquaculture authority; SPF, specific pathogen free; OR, odds ratio; CI, confidence interval

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Fig. 1. Map showing the location of shrimp hatcheries investigated for the occurrence of Zoea-2 syndrome during 2015–16.

Tamil Nadu (Kancheepuram and Vilupuram districts) and nine from Andhra Pradesh (Nellore, Prakasham and East Godavari districts). Larval samples from zoea syndrome affected and healthy larval cycles were collected in live condition. Live zoea were observed under light microscopy and a portion of larvae was preserved in Davidson AFA (OIE, 2016) fixative for histology. Zoeal samples were fixed in 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer for electron microscopic observations. Larvae were also preserved in 90% ethanol and RNAlater (Ambion) for screening pathogens by PCR analysis.

2.2. Microbiological examination

About a half-a-gram of larvae samples were washed in sterile phosphate buffered saline (PBS), homogenized and inoculated on thiosulphate citrate bile salt sucrose (TCBS) agar and the Zobell marine agar (ZMA). Total plate count (TPC) was performed by plating serial ten-fold dilutions by spread plate method in duplicate on ZMA. The plates were incubated at 30 ± 1 °C and observed after 24 h. Pure cultures of the dominant heterotrophic bacterial flora were identified based on phenotypic characteristics (Garrity et al., 2006; Noguerola and Blanch, 2008).

2.3. DNA extraction

The genomic DNA was extracted from larval samples as described by Rajendran et al. (2016). Briefly, larval samples were homogenized and digested for 10 min at 95 °C in 500 μL of lysis buffer (50 mM Tris, 1 m methylene diamine tetra-acetic acid (EDTA), 500 mM NaCl, 1% SDS) and 0.1 mg proteinase K. The mixture was centrifuged at 12,000 rpm (Eppendorf 5810 R, Germany) for 10 min at 4 °C. After centrifugation, supernatant was collected carefully and two volumes of ethanol was added and kept at − 20 °C for 1 h. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. The DNA pellet was washed with 70% cold ethanol, air-dried, re-suspended in nuclease-free water and stored at − 20 °C.

2.4. RNA extraction and cDNA synthesis

RNA was extracted from larval samples using TRIzol™ Reagent (Invitrogen, USA) following manufacturer's protocol. The quantity and quality of the extracted RNA was evaluated using a nano spectrophotometer (Implen, Germany) and stored at − 80 °C. Reverse transcription was carried out using iScript cDNA synthesis kit (BioRad, USA) in 10 μL reactions as per the manufacturer's instructions and the cDNA was stored at − 20 °C until further use.

2.5. Screening for viral pathogens

Nucleic acids extracted from larval samples were used for testing viral pathogens by PCR. For the detection of WSSV, nested PCR protocol Kimura et al. (1996) was used. Other DNA and RNA viruses viz., infectious hypodermal and haematopoietic necrosis (IHHNV), monodon baculovirus (MBV), hepatopancreatic parvo-like virus (HPV), yellow head virus (YHV), Taura syndrome virus (TSV), infectious myonecrosis

virus (IMNV) were detected by OIE recommended PCR assays (OIE, 2016). The covert mortality syndrome virus (CMNV) was tested by nested RT PCR protocol described by Zhang et al. (2014). The PCR was carried out in a thermal cycler (Eppendorf, USA). An aliquot of amplified PCR product was resolved on 2.0% agarose-Tris-acetate-EDTA (TAE) gels stained with 0.5 μg mL^{-1} ethidium bromide and the amplified DNA alongside a 100 bp DNA marker was visualized under UV illumination using a gel documentation system (Bio-Rad Laboratories, USA).

2.6. Light microscopic examination

Larval samples collected in live condition from normal and affected hatchery were observed under light microscopy. For histology, zoeal samples were fixed in Davidson's AFA fixative for 48 h and processed further using routine histological techniques (Bell and Lightner, 1988). Briefly, the zoeal samples were dehydrated through graded alcohols (70%, 90%, and 100%) each for 60 min. After dehydration, tissues were cleared twice with xylene for 60 min and infiltrated with paraffin wax for 2 h and then blocks were prepared using Leica EG 1160 tissue embedding system (Leica Microsystems, Germany). Tissue sections of 4–5 μm thickness were obtained on clean microscopic slides from paraffin blocks using Leica RM 2145 microtome (Leica microsystems, Germany) and the sections were further processed by staining with hematoxylin and eosin using standard procedure. The stained tissue sections were mounted in DPX and observed under a microscope (Zeiss, Germany).

2.7. Transmission electron microscopy

Larval samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 8 h at 8 °C and post-fixed in 0.1% osmium tetroxide prepared using the same buffer at 8 °C for 2 h and has been processed as per the protocols of Naveenkumar et al. (2013). Sections were examined by using JEM 1400 (JEOL Ltd., Tokyo, Japan) Transmission Electron Microscope at an accelerated voltage of 80 kV and photomicrographs were taken using the Olympus Keenview CCD Camera attached to the microscope at the Cancer Research Institute (WIA), Adyar, Chennai.

2.8. Statistical and epidemiological analysis

The odds ratio, which measures the strength of the association between disease and exposure to a risk factor, was estimated by using Epi Info™ 7.1.2 single table analysis at the 95% level of confidence by Taylor series approximation and two tailed *p* values by use of Fisher's exact test.

3. Results

3.1. Hatchery management

During the investigation nine hatcheries were affected by zoea-2 syndrome and six hatcheries including two nauplii rearing centers in Andhra Pradesh were unaffected and had healthy seed production cycles. All hatcheries used imported SPF *P. vannamei* brood stocks for seed production. Fresh polychaetes, squids, oyster and artificial semi-moisture pellet feed were used as brood stock diet. In spawning tanks EDTA as a heavy metal chelating agent and treflan for fungicide were used. After spawning, eggs were washed with formaldehyde (100 ppm for 30 s), iodine (50–100 ppm for 1 min) and sea water and stocked in hatching tanks (Table 1). Then nauplii at N-VI stage were stocked at larval rearing units and reared up to post larvae. This production cycle continued with daily spawning followed by daily stocking of nauplii (3–10 days) in subsequent larval rearing tanks. Continuous stocking of nauplii more than four days was observed in nine zoea-2 syndrome

Table 1
Water quality parameters and management practices in shrimp hatcheries investigated for zoea-2 syndrome.

Sl no.	Hatchery	Location	Z-IIS status	Egg washing	Probiotics used	Antibiotics used	Algae used	Temp	pH	DO	Salinity	Alkalinity
1	A	Marakanam, TN	Affected	Formaldehyde, iodine	*B. subtilis, B. litcheniforms*	Oxytetracycline	*Cheatoceros*	30	7.1	5	34	145
2	B	Marakanam, TN	Affected	Formaldehyde, iodine	*Bacillus* sp.	Oxytetracycline	*Skeletonema, Thalassia*	29	7.7	4	30	120
3	C	Marakanam, TN	Normal	Formaldehyde, iodine	*Bacillus* sp. and *Pseudomonas*	Oxytetracycline, Erythromycin	*Cheatoceros, Skeletonema*	29	8.1	5	31	115
4	D	Marakanam, TN	Affected	Formaldehyde, iodine, treflan	*Bacillus* sp., *Lactobacillus* sp.	Oxytetracycline	*Cheatoceros, Thalassia*	30	8	5	31	110
5	E	Marakanam, TN	Normal	Formaldehyde, iodine	*Bacillus* sp.	Nil	*Cheatoceros, Skeletonema*	30	7.5	4	30	135
6	F	Marakanam, TN	Normal	Formaldehyde, iodine	*B. subtilis* and *Rhodopseudomonas*	Oxytetracycline	*Cheatoceros, Thalassia*	29	7	6	31	120
7	G	Nellore, AP	Affected	Formaldehyde, iodine	*B. subtilis, B. pumilis, B. megaterium*	Oxytetracycline, Erythromycin	*Cheatoceros, Thalassia*	28	7.5	5	30	115
8	H	Nellore, AP	Affected	Formaldehyde, iodine, treflan	*Bacillus* sp.	Oxytetracycline	*Cheatoceros, Thalassia*	29	7.3	4	31	140
9	I	Nellore, AP	Normal	Formaldehyde, iodine	*Bacillus* sp., *Streptococcus* sp.	Oxytetracycline, Erythromycin	*Cheatoceros*	29	8	4	31	125
10	J	Ongole, AP	Affected	Formaldehyde, iodine	*Bacillus* sp., *Lactobacillus* sp.	Oxytetracycline	*Cheatoceros, Thalassia*	30	7.8	4	30	110
			Normal	Formaldehyde, iodine	*Bacillus* sp., *Lactobacillus* sp.	Oxytetracycline		29	8	5	30	130
11	K	Ongole, AP	Affected	Formaldehyde, iodine, treflan	*Bacillus* sp., *Yeast*	Nil	*Cheatoceros, Thalassia*	28	7.5	6	30	120
12	L	Ongole, AP (NRC)	Normal	Formaldehyde, iodine, treflan	*Lactobacillus* sp.	Oxytetracyclin	*Skeletonema, Cheatoceros, Thalassia*	30	8	5	31	120
13	M	Kakinada,AP (NRC)	Normal	Formaldehyde, iodine	*Bacillus* sp.	Nil	*Cheatoceros, Thalassia*	29	7.5	6	29	115
14	N	Kakinada, AP	Affected	Formaldehyde, iodine, treflan	*B. punctatuts, B. subtilis*	Oxytetracyclin	*Cheatoceros, Thalassia*	31	8	6	30	135
15	O	Kakinada, AP	Affected	Formaldehyde, iodine	*Bacillus* sp.	Oxytetracyclin	*Cheatoceros, Thalassia*	29	7.3	4	29	130

NRC — nauplii rearing centre, Z-IIS — Zoea-2 syndrome.

Table 2
Observations on larvae and vibrios isolated from normal and zoea-2 syndrome affected hatcheries.

S. no	Hatchery	Zoea-2 syndrome	Stocking of nauplii > 4 days	Lack of separate units	Lack of proper disinfection	Swimming activity	Photo taxis	Empty gut	Suddenly stops feeding	Arrested peristaltic movement of gut	Inflammation of gut	Faecal strings	White balls or white sphere like structure	Bacteria isolated
1	A	Affected	Y	N	Y	N	N	Y	Y	Y	Y	N	N	*V. alginolyticus*, *V. mimicus*
2	B	Affected	Y	Y	Y	N	N	Y	Y	Y	Y	N	Y	*V. alginolyticus*
3	C	Normal	Y	N	N	Y	Y	N	N	N	N	Y	N	*V. alginolyticus*, *V. campbelli*
4	D	Affected	Y	N	Y	N	N	Y	Y	Y	Y	N	Y	*V. alginolyticus*, *V. mimicus*
5	E	Normal	N	Y	N	Y	Y	N	N	N	N	Y	N	*V. alginolyticus*
6	F	Normal	N	N	Y	Y	Y	N	N	N	N	Y	N	*Vibrio metschnikovii*, *V. mimicus*
7	G	Affected	N	Y	Y	N	N	Y	Y	Y	Y	N	N	*V. alginolyticus*
8	H	Affected	Y	Y	Y	N	N	Y	Y	Y	Y	N	Y	*V. alginolyticus*, *V. vulnificus*
9	I	Normal	N	Y	N	Y	Y	N	N	N	N	Y	N	*V. alginolyticus*, *V. proteolyticus*
10	J	Affected	Y	N	N	N	N	Y	N	Y	Y	N	Y	*V. alginolyticus*, *V. mimicus*, *V. furnissi*
		Normal	N	N	N	Y	Y	N	N	N	N	Y	N	*V. mytili*, *V. mimicus*
11	K	Affected	Y	Y	Y	N	N	Y	Y	Y	Y	N	Y	*V. alginolyticus*, *V. mimicus*
12	L	Normal	N	N	N	Y	Y	N	N	N	N	Y	N	*V. cincinatensis*
13	M	Normal	N	N	Y	Y	Y	N	N	N	N	Y	N	*V. cincinatensis*, *V. mimicus*
14	N	Affected	Y	Y	Y	N	N	Y	Y	Y	Y	N	Y	*V. cincinatensis*, *V. vulnificus*
15	O	Affected	Y	N	Y	N	N	Y	Y	Y	Y	N	N	*V. alginolyticus*, *V mimicus*

L & M — nauplii rearing centre, Y — yes, N — no.
Separate unit — includes physically separate algal, maturation, and larval rearing unit and separate workers and separate implements for individual units.

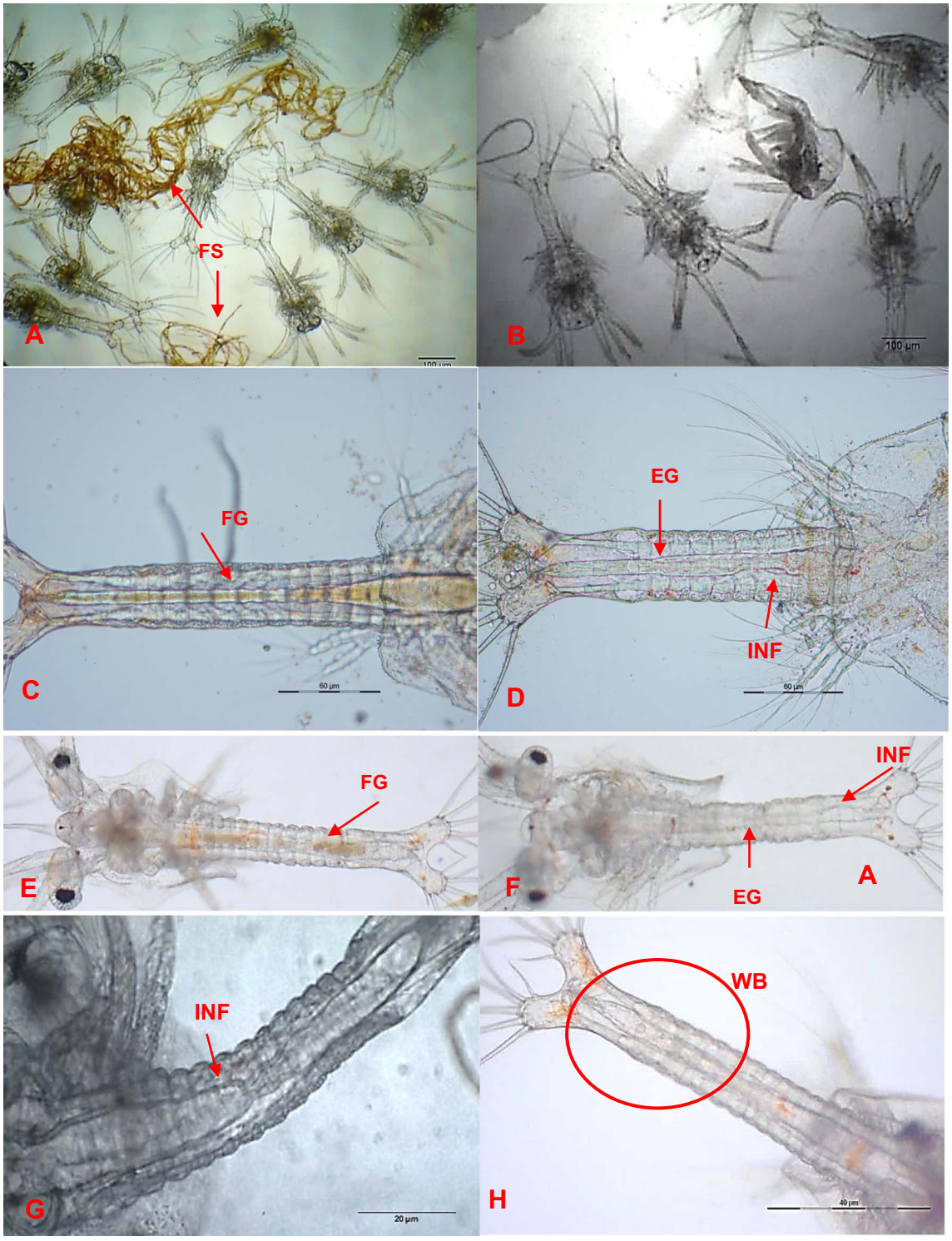

Fig. 2. Light microscopic observations of normal and Zoea-2 syndrome affected larvae.
A — Normal zoea with full gut and fecal strands; B — affected zoea with empty gut and absence of fecal strand; C & E — normal zoea with full gut with no abnormalities, D, F, G — infected zoea showing empty gut with inflammation like disruptions in intestinal epithelium; H — infected zoea showing sloughed of epithelial cells as white balls or white sphere like structures (circle). FS — fecal stands; FG — full gut; EG — empty gut; INF — Inflammation, WB — White ball or sphere like structures.

affected hatcheries and in one normal hatchery C (Table 2). Water quality parameters pH, salinity and alkalinity in all the hatcheries were in the normal range (Table 1) and did not seem to influence occurrence of zoea-2 syndrome. *Skeletonema*, *Cheatoceros*, *Thalassia* were used as algal feed during the zoeal stages. In all the hatcheries seawater treatment protocol involved sedimentation, chlorination, dechlorination, and filtration with sand filter, activated carbon filter, cartridge filter followed by UV filtration and ozonation. Oxy-tetracycline and probiotic formulations containing *Bacillus* sp., *Streptococcus* sp., *Lactobacillus* sp., were used in hatcheries (Table 1). Following every larval production cycle, tanks and implements were disinfected with hypochlorite solution (20–30 ppm active ingredient), water pipelines were disinfected by filling with disinfection solution (chlorine (500 ppm), potassium permanganate ($KMnO_4$ — 20 ppm), formaldehyde (200 ppm), muriatic

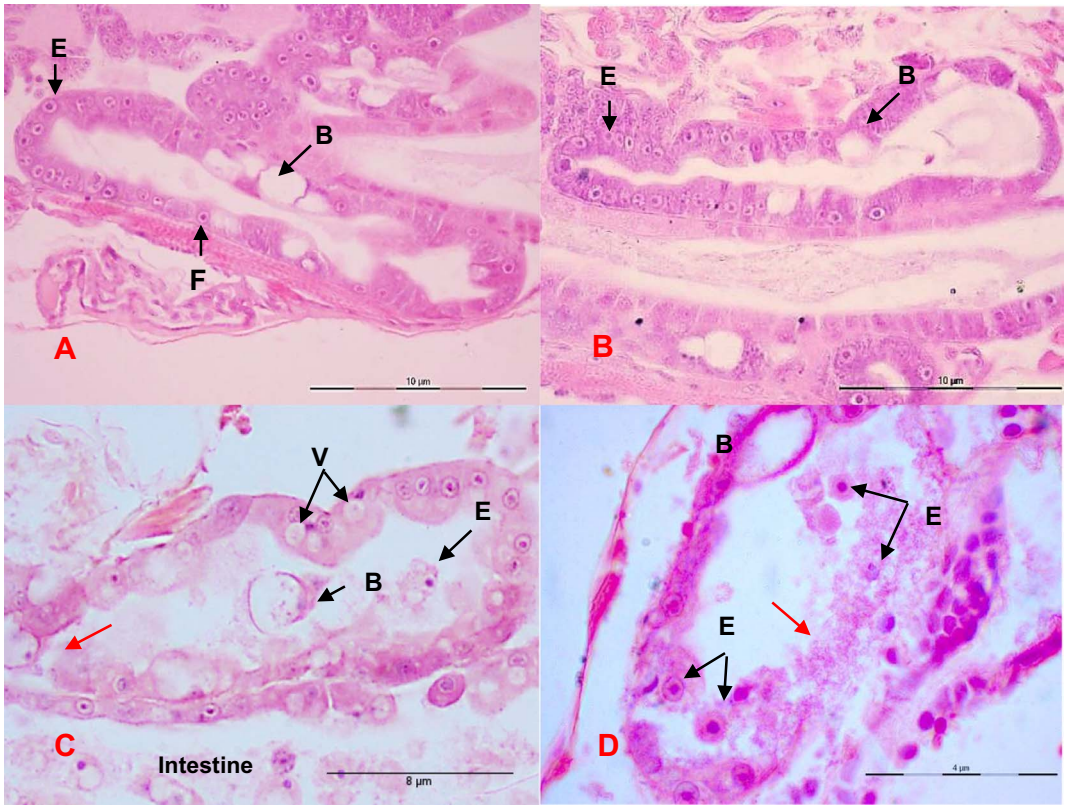

Fig. 3. Histology of hepatopancreas (longitudinal sections).
A & B — Normal hepatopancreas of zoea showing intact tubules with developing B cell and F cell, and E cell; C — hepatopancreas of affected zoea showing vacuolization, severe necrosis and sloughing of B cell and E cell; D — hepatopancreas of affected zoea showing severe necrosis, highly disintegrated tubule epithelium (red arrow) and rounded up, sloughing and detachment of epithelial cell from basement membrane in to the lumen. E — E (embryonic cells) cell; B — B cell, F — F cell, V — vacuolization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acid (10%)) and air pipelines were disinfected by fumigation with formaldehyde (200 ppm). Two hatcheries B and G did not have separate algal culture units and in three hatcheries (E, H, N), same workers were allowed to work in different units and in two hatcheries (K, I) same implements were used across different larval rearing tanks and across different units and in one (G) hatchery same blower was shared by two larval rearing units. Lack of proper disinfection was also observed during the larval production cycle and between the cycles in eight zoea-2 syndrome affected hatcheries and two normal hatcheries (Table 2). A batch of nauplii produced in hatchery 'J' were stocked in larval rearing tanks on fourth day in one larval rearing unit having earlier stocks of affected larvae and a portion of nauplii from the same batch stocked in another fresh larval rearing unit in a different section in the same hatchery. The nauplii stocked in the section with older affected larvae developed zoea syndrome and failed to metamorphose into mysis and PL. The same nauplii stocked in the other section in the same hatchery had no abnormalities and metamorphosed into healthy mysis and PL. In another instance, the nauplii rearing centre 'M' produced PL without any issues, whereas the same batch of nauplii stocked within the producers' hatchery 'K' (already having zoea-2 syndrome) were affected with zoea-2 syndrome. Incidences of zoea syndrome were low in hatcheries which did not have maturation units (hatcheries L, M) (data not shown).

3.2. Bacteriology

A total of 29 dominant vibrios were isolated from all the fifteen hatcheries. From nine affected hatcheries *Vibrio alginolyticus* was found predominant in eight, followed by *V. mimicus* in five and *V. vulnificus* in two hatcheries. Among six hatcheries, that did not have zoea-2 syndrome, *V. alginolyticus* and *V. mimicus* were predominant in three followed by *V. cincinatensis* in two hatcheries (Table 2).

In hatchery 'J', *V. alginolyticus* was found to be predominant followed by *V. mimicus* and *V. furnissi* from zoea stocked on fourth day of first larval rearing unit which got affected with zoea-2 syndrome. From the same batch of nauplii, *V. mytili* and *V. mimicus* were found predominant bacteria, which were stocked in fresh larval rearing unit of the same hatchery and did not have zoea-2 syndrome. Then from hatchery 'K', from same batch of nauplii, *V. alginolyticus* found predominantly isolated followed by *V. mimicus* in the affected zoea whereas *V. cincinatensis* was found to be predominant followed by *V. mimicus* in the nauplii rearing centre 'M' which did not have zoea-2 syndrome.

3.3. Detection of viral pathogens

To find the role of any known viral agent in zoea-2 syndrome, all the larval samples collected from different hatcheries were subjected to screening of OIE listed and known viral pathogens such as WSSV, MBV, IHHNV, YHV, IMNV, TSV, and CMNV. All zoeal larval samples collected from zoea-2 syndrome affected hatcheries were negative for these DNA and RNA viruses.

3.4. Light microscopic examination

Freshly collected live healthy and affected zoea of *P. vannamei* observed under microscope (after 36–48 h of zoea I stage). Normal zoea were showing active peristaltic movement of gut filled with feed and long fecal strings projected from the anus (Fig. 2A, C, E). Affected zoea were less active and displayed almost empty gut with very week peristaltic movement with no fecal strings. The intestinal lumen showed inflammation (Fig. 2B, D, F, G and H). In histology, hepatopancreas of

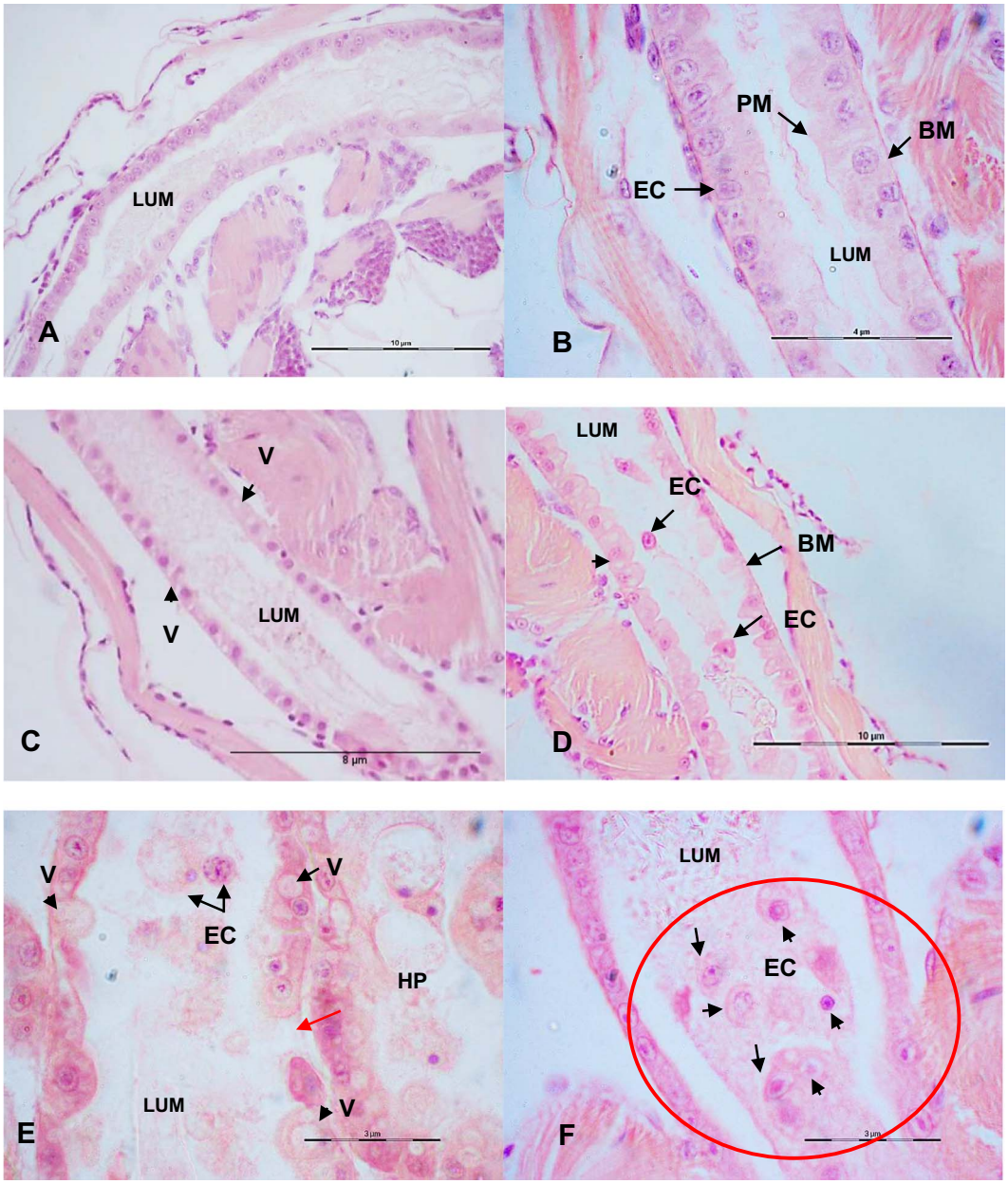

Fig. 4. Histology of intestine (longitudinal sections).
A & B — Intestinal epithelium of normal zoea with intact normal epithelial cells; C, D & E — hypertrophied epithelial cells, Vacuolization in intestinal epithelium, highly disintegrated peritrophic membrane (red arrow) and detached/sloughed of epithelium cell (black arrow) of zoea-2 syndrome affected larvae into the lumen; F — intestinal epithelium noticed with sloughed of epithelial cells (circle) accumulated in the lumen of posterior intestine of affected zoea. LUM — lumen, EC — epithelial cell, PM — peritrophic membrane, BM — basement membrane, V — vacuolization, HP — hepatopancreas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the normal zoea had intact tubules with developing B, F, and E cell (Fig. 3A, B), whereas, the hepatopancreas of affected zoea displayed severe necrosis and rounding up, sloughing and detachment of epithelial cells from the basement membrane of hepatopancreatic tubule epithelium (Fig. 3C, D). The longitudinal histological sections of intestine showed hypertrophy (Fig. 4E, F), vacuolization in epithelial columnar cells (Fig. 4C, D, E), disintegration of peritrophic membrane (Fig. 4D, E) and sloughing/desquamation of epithelial cells from the basement membrane of epithelium accumulated in the lumen of intestine (Fig. 4D, E, F) compared to the normal zoea with no systemic abnormalities (Fig. 4A, B).

3.5. Transmission electron microscopy

Ultrastructural studies revealed sloughing of microvilli from the epithelial cells in the hepatopancreatic tubule (Fig. 5B) compared to normal hepatopancreas with intact microvilli (Fig. 5A). Similarly, affected zoeal intestine showed disintegration and sloughing of peritrophic membrane, necrosis, desquamation and detachment of epithelial cell from basement membrane in intestinal epithelium (Fig. 5D) compared to the normal intestine with an intact epithelium (Fig. 5C). No viral like particles could be observed in the ultramicroscopic sections.

3.6. Statistical and epidemiological analysis

In this study it was observed that incidences of zoea syndrome were more in hatcheries a) having prolonged larval production cycles with continuous stocking of nauplii more than four days in the same larval rearing unit b) lack of proper disinfection between the cycles and c)

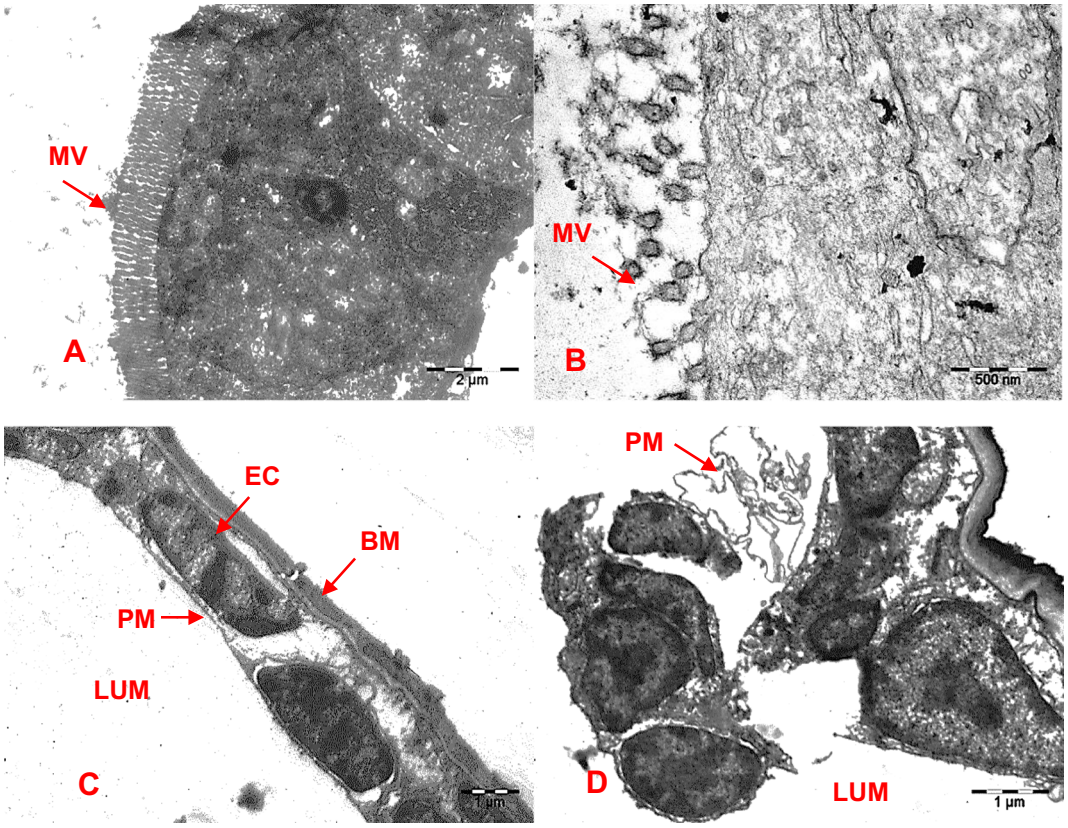

Fig. 5. Ultra-microscopic observations of zoea-2 syndrome affected larvae.
A — Normal hepatopancreatic tubule epithelial showing normal microvilli; B — affected hepatopancreatic epithelial cell showing sloughing of microvilli in affected larvae; C — intact intestinal epithelium with normal epithelial cell; D — affected intestinal epithelium showing sloughing of peritrophic membrane and desquamation/detachment of epithelial cells from basement membrane. MV — microvilli; PM — peritrophic membrane; BM — basement membrane; EC — epithelial cells; LUM — lumen.

Table 3
Odds ratio (OR) values determined between the incidences of zoea-2 syndrome and exposure to hatchery factors.

Hatchery factors	Odds ratio	Confidence interval (95%)		*p* value*
		Lower	Upper	
Stocking of nauplii more than four days in the same unit	48	2.4697	932.9003	0.008
Lack of separate units	3.125	0.382	25.5669	0.35
Lack of proper disinfection	20	1.4161	282.4627	0.03

Exact confidence limits calculated using Epi Info, version 7.1.2. If the 95% confidence interval excludes 1.0, the association is statistically significant at $p < 0.05$. If the 95% confidence interval includes 1.0, the association is not statistically significant at $p < 0.05$.

* Two tailed *p* values estimated by Fisher's Exact test.

hatcheries which did not have separate maturation unit, larval rearing unit, and algal culture unit, separate workers and separate implements for different units (Table 2). To find association between zoea-2 syndrome incidences and to the exposure of above mentioned hatchery factors, odds ratio was calculated. The odds ratio value for the two hatchery factors namely, stocking of nauplii more than four days in the same unit and lack of proper disinfection was > 1. Although the odds ratio value with regard to lack of separate units was > 1, but its confidence interval (CI 0.38–25.5) passes through 1. Both stocking of nauplii more than four days in the same unit and lack of proper disinfection was significantly associated with increase in the zoea-2 syndrome incidence, whereas, lack of separate units was not having significant association (Table 3).

4. Discussion

At present, Indian shrimp hatcheries, solely depend on imported SPF *P vannamei* brood stocks for the seed production. In recent years, zoea-2 syndrome emerged as a significant challenge to *P. vannamei* larviculture causing severe economic consequences to shrimp hatchery operators. During the study, out of fifteen commercial shrimp hatcheries in Tamil Nadu and Andhra Pradesh, nine hatcheries were affected with zoea-2 syndrome. It was observed that gradual progressive mortalities with cumulative occurrence reaching 30–100% in the zoeal stages have been recurrent in the *P. vannamei* hatcheries in India. Generally, the most critical stage in larval rearing is nauplii VI — zoea I. At Z I stage, the zoea start extensive feeding mainly on algae. Healthy zoea feed actively and have full gut with no abnormalities. After 36–48 h, of zoea I, larvae suddenly stop feeding and develop systemic abnormalities and suffer mortalities. Gross signs of non-feeding and lethargy in the affected larvae could be linked to lack of proper nutrition during the larval metamorphosis which may further hinder the successful larval development in the larval cycle and this might lead to poor survival (D'Souza and Loneragan, 1999; Jamali et al., 2015). Microscopic and ultrastructural studies revealed underlying pathological changes in the digestive system and associated organs, particularly hepatopancreas and intestine resulting the impairment in the nutrient absorption and starvation in the affected animals. This impairment in the nutrient absorption could be leading to the diminishing activity, delayed molting and mortality in zoea-2 syndrome affected larvae, as reported from *Exopalaemon carinicauda* larvae (Zhang et al., 2015), where starvation significantly affected the growth, survival and development. Sloughing of microvilli and epithelial cells in hepatopancreas, the inflammation and desquamation of epithelial cells in intestinal epithelium point to an underlying pathological process during zoea-2

syndrome. The ultrastructural studies and PCR did not reveal the presence of any pathogens including the known shrimp DNA and RNA viruses in the affected zoea.

During the microbial investigation, considerably higher association with *V. alginolyticus* was observed in the zoea-2 syndrome affected larvae. However its role as pathogen could not be resolved since it is also associated as natural flora of healthy larvae. Its association with zoea-2 syndrome was highlighted in many previous studies (Vandenberghe et al., 1999) but still their role in larval mortality due to zoea-2 syndrome is not clear. Several authors have described the association of *Vibrio alginolyticus* in penaeids and non-penaeids (Lavilla Pitogo et al., 2000; Lee et al., 1996; Mohney et al., 1994) and *Macrobrachium rosenbergii* larval mortality due to antibiotic resistant *V. alginolyticus* (Jayaprakash et al., 2006). With the occurrences of vibriosis in both healthy and diseased animals, the hypothesis of opportunistic nature of vibriosis in penaeid shrimp is widely accepted (Egidius, 1987).

The present study has indicate that zoea-2 syndrome is possibly due to accrued conditions in the larval rearing tanks during the larval cycles even with constant and uniform water quality parameters and management practices. Further, zoea-2 syndrome appears to be related to prolonged production cycle in the hatcheries and continuous stocking more than three to four days in the same larval rearing units may probably cause zoea-2 syndrome incidence. Since zoea-2 syndrome was not seen in the fresh larval units of the hatchery, its incidence might have been caused by the cumulative effect of unknown factors accumulated during larval cycles in the affected hatcheries. It is also observed in this study that lack of proper disinfection during and between the larval production cycles and lack of separate units could be other predisposing factors in zoea-2 syndrome occurrence.

In almost all commercial shrimp hatcheries, despite following extensive water treatment with sand filters, cartridge filters, UV filters and chlorination, the bacterial load got increased during the larval production cycle. With some undefined negligent management practices, the vibrios being opportunistic pathogens could cause infections resulting in delayed molting for three to four days and mortality of zoea at *Z*-II stages. The loss due to zoea-2 syndrome in a hatchery (100 million nauplii stocking capacity) is figured to approximately 12 to 40 lakhs Indian rupees ₹ (18–61 thousand USD $), where the larval production is entirely dependent on imported specific pathogen free brood stock thereby escalating the cost of seed production. Ensuring strict good management practices and following proper disinfection and shutdown periods between the larval production cycles and reducing the number of days of stocking nauplii in < 3–4 days in the same unit and improved algal quality by serial dilution/batch culture and ensuring separate re-circulatory systems, physically separate units for maturation, spawning, larval rearing, algal culture (Indoor and outdoor), and separate water and air supply units and separate workers and separate implements for individual unit would help reduce the incidences of zoea-2 syndrome.

In conclusion, the present study has revealed that zoea-2 syndrome, in *P. vannamei* hatcheries is not caused by known infectious agents. There are indicators of impairment of digestive system of zoea and unknown factors affecting zoeal metamorphosis. An integrative multidimensional investigation, involving physiological factors within zoea may perhaps useful in understanding the causes of impairment of digestive system and role of opportunistic pathogens.

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