ANIMAL HEALTH & WELFARE (/ADVOCATE/CATEGORY/ANIMAL-HEALTH-WELFARE)

A study of Zoea-2 Syndrome in hatcheries in India, part 2

Monday, 6 November 2017

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Hatchery management, pathogen detection and results analysis

Indian shrimp hatcheries have experienced larval mortality in the zoea-2 stage, with molt deterioration and resulting in heavy mortality.

Editor's note: This is part 2 of a series. ***Click here to read part 1*** *(https://www.aquaculturealliance.org/advocate/zoea-2-syndrome-india/).*

During the study, nine hatcheries were affected by the Zoea-2 Syndrome and six hatcheries – including two nauplii rearing centers in Andhra Pradesh – were not affected and had healthy larval production cycles. All hatcheries used breeding populations of imported, specific pathogen free (SPF) Pacific White shrimp (*Penaeus vannamei)* for seedstock production. Broodstock animals were fed fresh polychaetes, squid, oysters and an artificial pelleted diet.

In the spawning tanks, ethylenediaminetetraacetic acid (EDTA) was used as a chelating agent for heavy metals, and the fungicide treflan was also used. After each spawning, the eggs were washed with formaldehyde (100 ppm for 30 seconds), with iodine (50-100 ppm for 1 minute) and with seawater before stocking. (Table 1).

Kumar, Zoea-2, Table 1

Hatchery	Location	Zoea-2 Syndrome	Egg washing	Probiotics used	Antibiotics used	Algae used	Tempera
A	Marakanam, TN	Affected	Formaldehyde, iodine	B. subtilis, B. litcheniforms	Oxytetracycline	Chaetoceros	30
B	Marakanam, TN	Affected	Formaldehyde, iodine	Bacillus sp.	Oxytetracycline	Skeletonema, Thalassiosira	29
C	Marakanam, TN	Normal	Formaldehyde, iodine	Bacillus sp. Y Pseudomonas	Oxytetracycline, Erythromycin	Chaetoceros, Skeletonema	29
D	Marakanam, TN	Affected	Formaldehyde, iodine, treflan	Bacillus sp., Lactobacillus sp.	Oxytetracycline	Chaetoceros, Thalassiosira	30
E	Marakanam, TN	Normal	Formaldehyde, iodine	Bacillus sp.	None	Chaetoceros, Skeletonema	30
F	Marakanam, TN	Normal	Formaldehyde, iodine	B. subtilis y Rhodopseudomonas	Oxytetracycline	Chaetoceros, Thalassiosira	29
G	Nellore, AP	Affected	Formaldehyde, iodine	B. subtilis, B. Pumilis, B. Megaterium	Oxytetracycline, Erythromycin	Chaetoceros, Thalassiosira	28
H	Nellore, AP	Affected	Formaldehyde, iodine, treflan	Bacillus sp.	Oxytetracycline	Chaetoceros, Thalassiosira	29
I	Nellore, AP	Normal	Formaldehyde, iodine	Bacillus sp., y Streptococcus sp.	Oxytetracycline, Erythromycin	Chaetoceros	29
J	Ongole, AP	Affected	Formaldehyde, iodine	Bacillus sp., Lactobacillus sp.	Oxytetracycline	Chaetoceros, Thalassiosira	30
		Normal	Formaldehyde, iodine	Bacillus sp., Lactobacillus sp.	Oxytetracycline	–	29
K	Ongole, AP	Affected	Formaldehyde, iodine, treflan	Bacillus sp., Levadura	None	Chaetoceros, Thalassiosira	28
L	Ongole, AP (NCR)	Normal	Formaldehyde, iodine, treflan	Lactobacillus sp.	Oxytetracycline	Skeletonema, Chaetoceros, Thalassiosira	30
M	Kakinada, AP (NCR)	Affected	Formaldehyde, iodine	Bacillus sp.	None	Chaetoceros, Thalassiosira	29
N	Kakinada, AP	Affected	Formaldehyde, iodine, treflan	B. punctatuts, B. Subtilis	Oxytetracycline	Chaetoceros, Thalassiosira	31
O	Kakinada, AP	Affected	Formaldehyde, iodine	Bacillus sp.	Oxytetracycline	Chaetoceros, Thalassiosira	29

Table 1. Parameters of water quality management practices in shrimp hatcheries investigated for zoea-2 syndrome. NRC: Nauplii Rearing Center.

Next, the nauplii in the stage N-VI were stocked in larval rearing units and reared to PL. This production cycle continued with daily spawnings, followed by daily stocking of nauplii (3 to 10 days) in the next larval rearing tanks. A continuous stocking of nauplii was observed for more than four days in nine hatcheries affected by Zoea-2 Syndrome and in one normal, unaffected hatchery (C).

Water quality parameters (pH, salinity and alkalinity) in all hatcheries were within normal ranges (Table 1) and did not seem to influence the occurrence of Zoea-2 Syndrome. At all the hatcheries, the seawater treatment protocol involved sedimentation, chlorination, de-chlorination and filtration (sand filter, activated charcoal and cartridge filters) followed by UV filtration and ozonation. Oxytetracycline and probiotic formulations containing *Bacillus* sp., *Streptococcus* sp. and *Lactobacillus* sp. were used in the hatcheries. The microalgae *Skeletonema*, *Chaetoceros* and *Thalassiosira* were used as feed during the zoeal stages.

Kumar, Zoea-2, Table 2

Hatchery	Zoea-2 Syndrome	Stocking	Deficiency in unit separation	Deficiency of adequate disinfection	Swimming activity	Phototaxis	Empty guts	Sudde feedir stoppa
A	Affected	Yes	No	Yes	No	No	Yes	Yes
B	Affected	Yes	Yes	Yes	No	No	Yes	Yes
C	Normal	Yes	No	No	Yes	Yes	No	No
D	Affected	Yes	No	Yes	No	No	Yes	Yes
E	Normal	No	Yes	No	Yes	Yes	No	No
F	Normal	No	No	Yes	Yes	Yes	No	No
G	Affected	No	Yes	Yes	No	No	Yes	Yes
H	Affected	Yes	Yes	Yes	No	No	Yes	Yes
I	Normal	No	Yes	No	Yes	Yes	No	No
J	Affected	Yes	No	No	No	No	Yes	No
	Normal	No	No	No	Yes	Yes	No	No

K	Affected	Yes	Yes	Yes	No	No	Yes	Yes
L	Normal	No	No	No	Yes	Yes	No	No
M	Affected	No	No	Yes	Yes	Yes	No	No
N	Affected	Yes	Yes	Yes	No	No	Yes	Yes
O	Affected	Yes	No	Yes	No	No	Yes	Yes

Table 2. Observations on larvae and vibrios isolated from normal and affected hatcheries with the Zoea-2 syndrome.

After each larval production cycle, the tanks and equipment were disinfected with hypochlorite solution (20 to 30 ppm active ingredient); the water pipes were disinfected by filling with disinfectant solution (chlorine, 500 ppm; potassium permanganate ($KMnO_4$), 20 ppm; formaldehyde, 200 ppm; muriatic acid, 10 percent), and the air pipes were disinfected by fumigation with formaldehyde (200 ppm).

Two hatcheries (B, G) had no separate algae culture units, and in three hatcheries (E, H, N), the same workers were allowed to rotate to/from different units. In one hatchery (G), the same air blower was shared by two larval breeding units. Lack of disinfection was also observed during and between the larval production cycles in eight hatcheries affected by the Zoea-2 Syndrome and in two normal, unaffected hatcheries (Table 2).

A batch of nauplii produced in a hatchery (J) was stocked on the fourth day in larval rearing tanks in a larviculture unit that had previous batches of affected larvae. Some of the nauplii from the same batch were stocked in another unit in a different section in the same hatchery. The nauplii stored in the section with older, affected larvae developed the Zoea-2 Syndrome and did not metamorphose into mysis and PL. The same nauplii stocked in the other section in the same hatchery had no anomalies and metamorphosed into healthy mysis and PL.

In another case, the nauplii breeding center (M) produced PLs without any problems, while the same batch of nauplii stocked in the hatchery (K) of the producers (which already had Zoea-2 Syndrome) was affected by the syndrome. The incidences of the zoea syndrome were low in the hatcheries without maturation units (hatcheries L, M: these data are not presented).

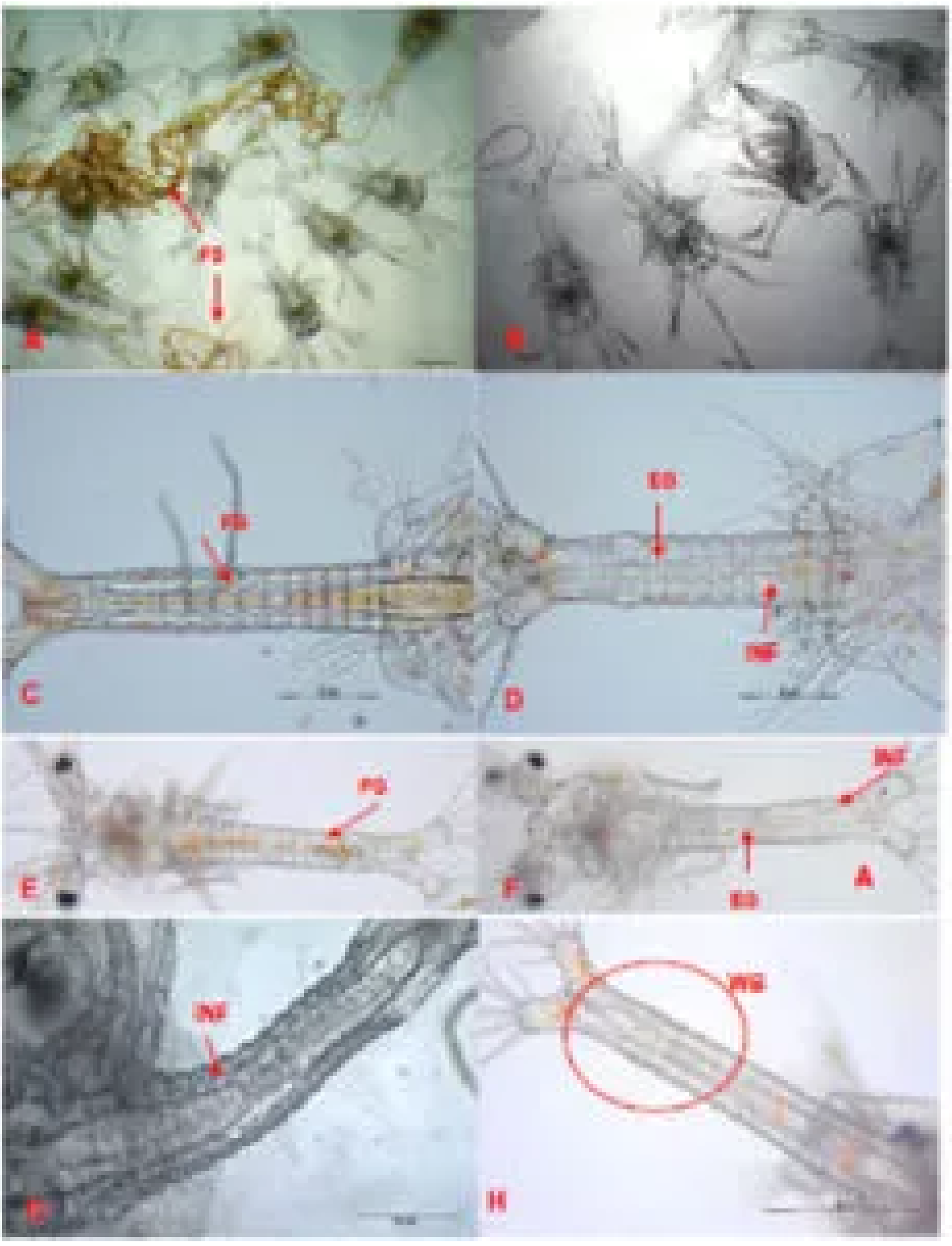
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Fig. 1: Light microscopy observations of normal larvae and larvae affected by Zoea-2 Syndrome. A: Normal zoea with full intestine and fecal strand. B: Affected zoea with empty intestine and absence of fecal strand. C, E: Normal zoea with full intestine without abnormalities. D, F, G: Infected zoea showing empty intestine, with inflammation and abnormalities in the intestinal epithelium; H: Infected zoea showing tearing of epithelial cells as white balls or spheres (circle). FS: fecal strands; FG: full intestine; EG: empty intestine; INF: inflammation, WB: white sphere or spheres.

Bacteriology

A total of 29 dominant vibrios were isolated from the 15 hatcheries. Of the nine affected hatcheries, *Vibrio alginolyticus* was found predominantly in eight, followed by *V. mimicus* in five and *V. vulnificus* in two hatcheries. In six hatcheries with no Zoea-2 Syndrome*, V. alginolyticus* and *V. mimicus* were predominant in three, followed by *V. cincinatensis* in two. In one hatchery (J), *V. alginolyticus* was predominant, followed by *V. mimicus* and *V. furnissi.*

Detection of viral pathogens

To determine if the presence of any known viral agent may have had a role in causing the Zoea-2 Syndrome, all the samples of larvae collected from the different hatcheries were screened for the OIE-registered viral pathogens, including WSSV, MBV, IHHNV, YHV, IMNV, TSV and CMNV. All larval samples collected from the hatcheries affected by the Zoea-2 Syndrome were negative for these DNA and RNA viruses.

Light microscopy exam

Recently collected live and healthy animals, as well as affected animals (after 36 to 48 hours of stage zoea I), were observed under a light microscope. Normal, unaffected zoea showed an active peristaltic movement of the intestine filled with food and long fecal strands projected from the anus (Fig. 1A, C, E). The affected zoea were less active and exhibited an almost empty intestine, with weak peristaltic movement and without fecal strands. The intestinal lumen showed inflammation (Fig. 1B, D, F, G and H). In histology, the hepatopancreas of normal zoea had intact tubules with developing B, F, and E cells (Fig. 2A, B), while the hepatopancreas of affected zoea showed severe necrosis and detachment of epithelial cells from the basal membrane of the hepatopancreatic tubule epithelium (Fig. 2C, D). The longitudinal histological sections of the intestine showed hypertrophy (Fig. 3E, F), vacuolization in columnar epithelial cells (Fig. 3C, D, E), peritrophic membrane disintegration (Fig. 3D, E) and tearing / desquamation of the epithelial cells of the basal membrane epithelium in the lumen of the intestine (Fig. 3D, E, F), compared to normal zoea that did not exhibit these systemic abnormalities (Fig. 3A, B).

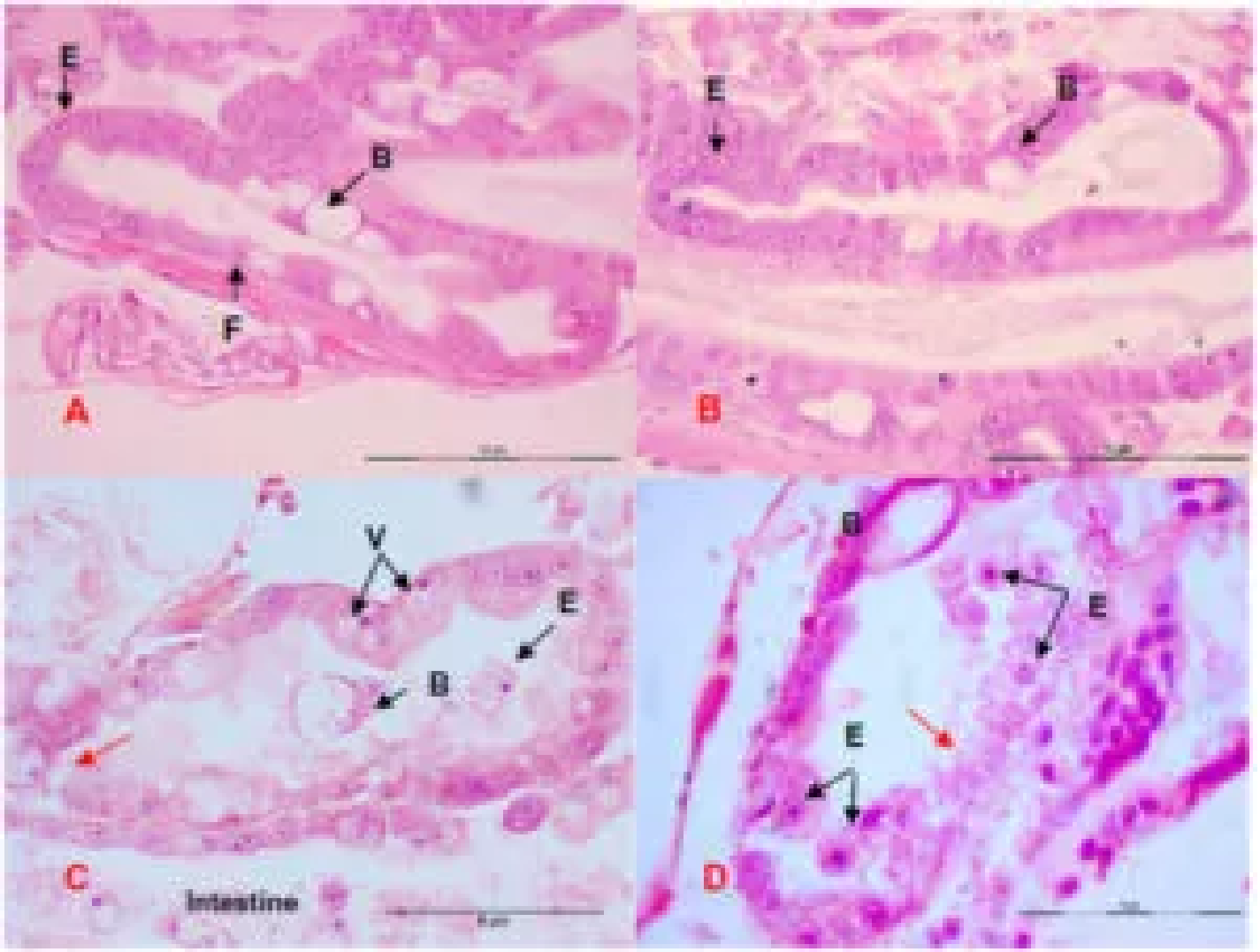
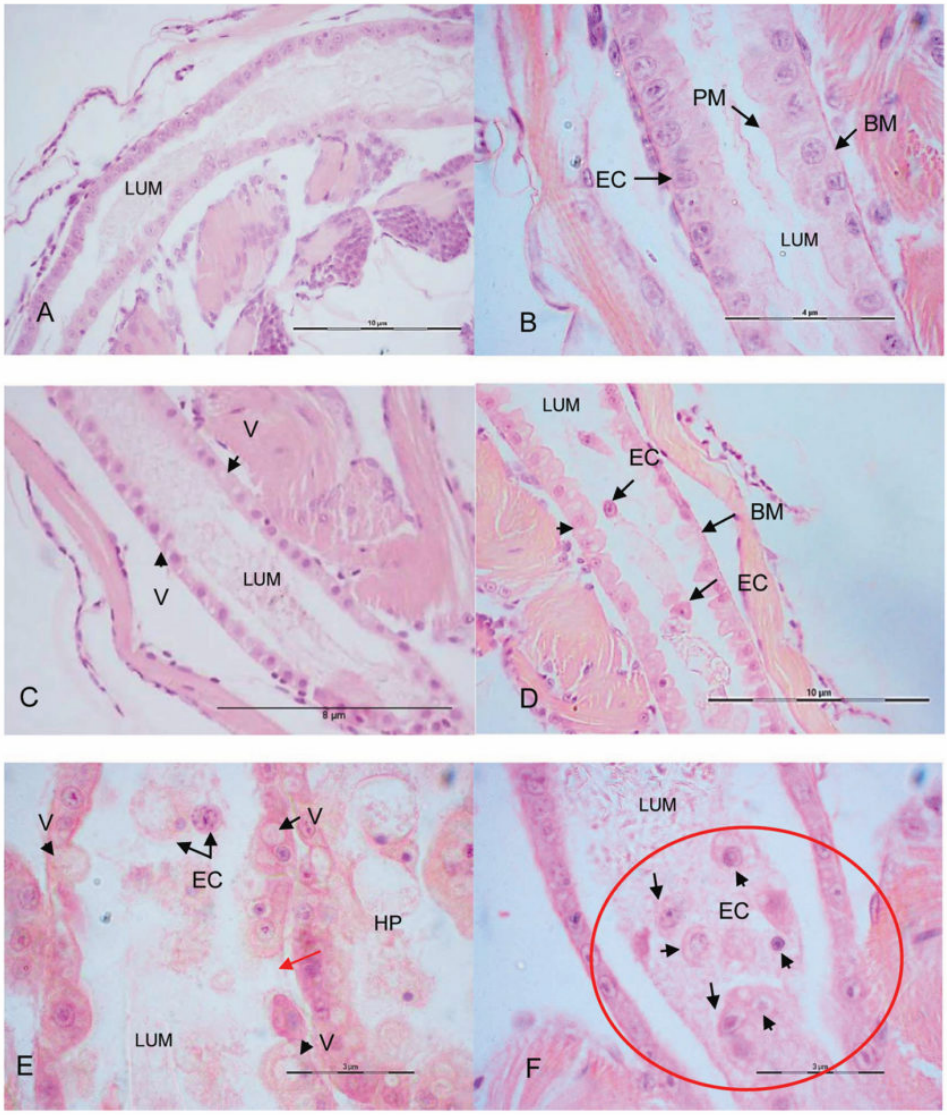


Fig. 2. Histology of the hepatopancreas (longitudinal sections). A, B: Normal hepatopancreas of zoea showing intact tubules with developing B, F and E cells. C: Hepatopancreas of affected zoea showing vacuolization, severe necrosis and detachment of B and E cells. D: Hepatopancreas of affected zoea showing severe necrosis, highly disintegrated tubular epithelium (red arrow) and detachment of epithelial cells from the basement membrane towards the lumen. E: embryonic cells; B: B cells; F: F cells; V: vacuolization (for further information, consult Aquaculture 479: 759-767 (2017).



Histology of the intestine (longitudinal sections). A, B: Intestinal epithelium of normal zoea with intact, normal epithelial cells. C, D, E: Hypertrophied epithelial cells, vacuolization in the intestinal epithelium, highly disintegrated peritrophic membrane (red arrow) and detached from the epithelial cell (black arrow) of larvae affected by the Zoea-2 Syndrome in the lumen. F: intestinal epithelium observed with detachment of epithelial cells (circle) accumulated in the lumen of the posterior intestine of affected zoea. LUM: lumen; EC: epithelial cell; PM: peritrophic membrane; BM: basal membrane; V: vacuolization; HP: hepatopancreas (for further information, consult Aquaculture 479: 759-767 (2017).

Electron transmission microscope

Ultrastructural studies revealed detachment of microvilli from the epithelial cells in hepatopancreatic tubules (Fig. 4B) compared to normal hepatopancreas with intact microvilli (Fig. 4A). Similarly, intestines in affected zoea showed disintegration and detachment of the peritrophic membrane; and also necrosis, desquamation and epithelial cell detachment from the basal membrane in the intestinal epithelium (Fig. 4D) compared to the normal intestine with an intact epithelium (Fig. 4C). No viral particles were observed in the ultramicroscopic sections.

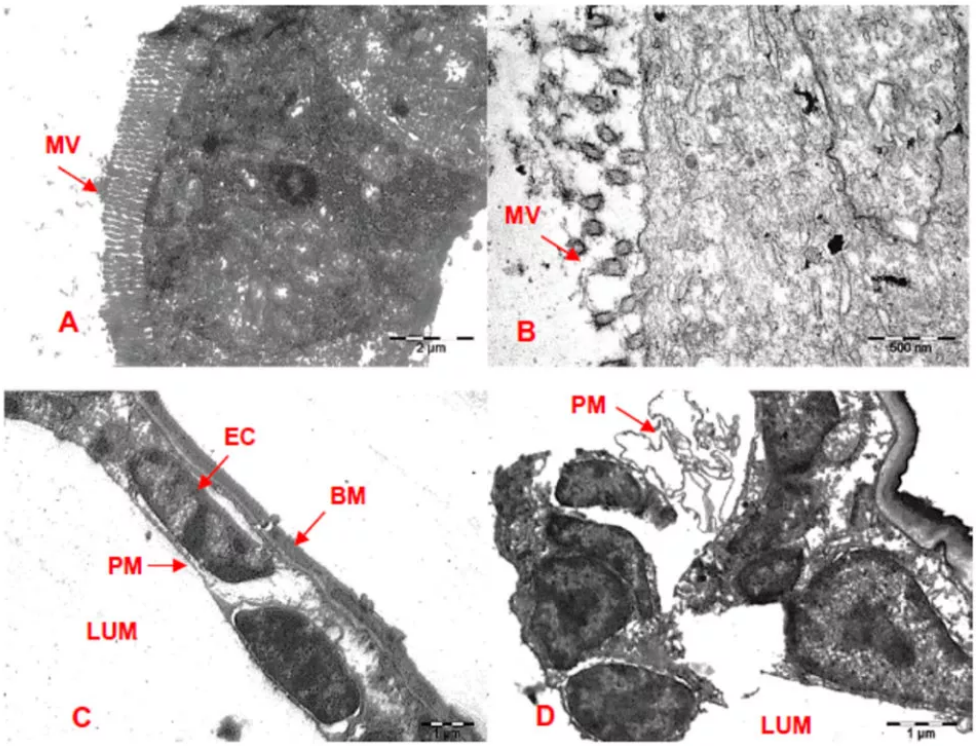


Fig. 4: Ultra-microscopic observations of larvae affected by zoea-2 syndrome. A: Normal hepatopancreatic epithelial tubule showing microvilli. B: Affected hepatopancreatic epithelial cells that show microvilli detachment in affected larvae. C: Intact intestinal epithelium with normal epithelial cells. D: Affected intestinal epithelium showing detachment of peritrophic membrane and desquamation / detachment of epithelial cells from the basal membrane. MV: microvilli; PM: peritrophic membrane; BM: basal membrane; EC: epithelial cells; LUM: lumen.

Statistical and epidemiological analysis

During the study it was observed that the incidences of zoea syndrome were more common in hatcheries with prolonged larval production cycles with continuous storage of nauplii for more than four days in the same larval breeding unit; with lack of adequate disinfection between cycles; and with broodstock animals that did not have a separate maturation unit, separate larval rearing and algae culture units, and different workers and equipment/implements for different units (Table 2).

To find an association between the incidence factors of the zoea-2 syndrome and the exposure of hatcheries mentioned above, the probability coefficient (p-value) was calculated. The p-value for the two hatchery factors – the storage of nauplii for more than four days in the same unit and the lack of adequate disinfection – was >1. Although the p-value with respect to the lack of separate units was >1, its confidence interval (CI 0.38-25.5) goes through 1. The storage of nauplii for more than four days in the same unit and the lack of adequate disinfection were both significantly associated with the increased incidence of zoea-2 syndrome, while the lack of separate units had no significant association (Table 3).

Kumar, Zoea-2, Table 3

Hatchery factors	Possibility index	Lower confidence interval (95%)	Upper confidence interval (95%)	P value
Nauplii storage, over 4 days in same unit	48	2.4697	932.9003	0.008
Deficiency in unit separation	3,125	0.382	25.5669	0.35
Deficiency in appropriate disinfection	20	1.4161	282.4627	0.03

Table 3. Values of possibility indices determined between incidences of zoea-2 syndrome and exposure to some hatchery factors.

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