

Embryonic Development, Larval Rearing, and Digestive Tract and Enzyme Ontogeny of Hilsa Shad, *Tenualosa ilisha*

Debasis De^{S†*}, Prem Kumar^{S††}, Panantharayil S. Shyne Anand[†], Gouranga Biswas^{††}, Suchita Mukherjee^{††}, Tapas Kumar Ghoshal^{††}, Vetta R. Suresh[‡], and Koyadan Kizhakedath Vijayan[‡]

[†]ICAR- Central Institute of Brackishwater Aquaculture, Chennai, Tamil Nadu India

^{††}Kakdwip Research Centre of ICAR- Central Institute of Brackishwater Aquaculture Kakdwip, West Bengal, India

[‡]ICAR-Central Inland Fisheries Research Institute, Barrackpore West Bengal, India



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ABSTRACT

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Production of fish larvae in captivity and its rearing are the key factors behind the successful mass propagation of any commercial important aquaculture species. Towards this goal, efforts were carried out to standardize fry production and larval rearing of hilsa shad, *Tenualosa ilisha*. Artificial fertilization of hilsa was done through dry stripping method. Experiments were carried out to understand the embryonic development, larval rearing, ontogenic development of gut and digestive enzymes. Significantly ($P < 0.01$) higher ($86.33 \pm 0.88\%$) fertilization and hatching rate ($57 \pm 0.58\%$) was recorded when hydration and incubation were done using river water (salinity 0.19 ppt, hardness 950–980 ppm). After an incubation period of 22–24 h at $23 \pm 1.0^\circ\text{C}$ temperature, larvae hatched out. Newly hatched larvae measured 2.20 ± 0.23 mm in total length with large yolk sac and 4 to 6 small oil globules. On the 5th day after hatching, the alimentary canal was noticed and the mouth opened between 3rd and 8th days. The aperture of mouth was found to be 82 μm . Larval rearing at different salinity for 15 days revealed the highest survival ($53.67 \pm 3.75\%$) when reared at 1.5 ppt. Presence of digestive enzyme (amylase, acidic protease activity, alkaline protease, and lipase) was first detected on 5th- day post-hatching. Fourteen days old larvae were characterized by the absence of oil globule and a well-developed intestine with food particle in the gut. Considering the mouth opening and presence of digestive enzymes on 3rd day and 5th respectively exogenous feeding of suitable particle size may be introduced from 3rd day of hatching onwards.

ADDITIONAL INDEX WORDS: *Hilsa*, larval development, gut, yolk sac, oil globule, digestive enzyme.

INTRODUCTION

The hilsa shad (*Tenualosa ilisha*), belonging to the family of Clupeidae, is an important commercial fish of the Indo-Pacific region, especially Bangladesh, India and Myanmar (Bhaumik, 2015), where its fishery generates employment and income for millions of people (Boblme, 2012; Sahoo *et al.*, 2018). It is a euryhaline, anadromous fish which inhabits freshwater rivers, estuaries and marine environments. Presently, the catch of this species has declined in these countries due to obstruction in natural migration for breeding, overfishing, water pollution and sedimentation in rivers (Boblme, 2012; Sahoo *et al.*, 2018). Therefore, there is an urgent need to propagate this fish in captivity for farming and to restore the population of this species in their natural habitats through mass ranching. For conservation and aquaculture of this species, numerous attempts on breeding through stripping and artificial fecundation using wild matured broodstock have been attempted since 1908 to till 1962 (Sahoo *et al.*, 2018) however, larval rearing was not successful.

Reproductive biology of the species reveals that hilsa breed throughout the year with two main spawning seasons, *i.e.*, September–October and January–March (Miah, 2015; Rahman *et al.*, 2017). In the past, though many researchers have tried the breeding of hilsa, very low hatching rate and survival of larvae were recorded (Rahman *et al.*, 2017; Raj, 1917; Wilson, 1909).

In general, teleost larvae show a common sequence and pattern of the development of organ systems. However, the timing of organogenesis in terms of both structure and functionality is highly species-specific (Trevino *et al.*, 2011). Therefore, a species-specific study on larval development is essential to understand the feed and feeding management. Knowledge of larval development and digestive tract ontogeny is essential for understanding the nutritional physiology of fish larvae. This information may also help to improve weaning, rearing, and feeding strategies of larvae (Hamlin *et al.*, 2000; Onal *et al.*, 2008; Sharma *et al.*, 2016). Recently, larval rearing of hilsa in the tank system was reported (Chattopadhyay *et al.*, 2019). However, there is no published literature on embryonic development and gut ontogeny or onset of digestive enzymes in hilsa larvae. Therefore, the present study aims to elucidate the embryonic, larval development and gut ontogeny and onset of digestive enzymes of hilsa.

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^S Authors equally contributed

*Corresponding author: debasisde@ciba.res.in

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METHODS

Stripping and Artificial Fecundation

Females brooders (450±12 g to 660.5±25.5 g / 376.5±3.5 to 380±75 mm) and males brooders (225±52.33 g to 280±12 g / 262.8±12.96 to 265±45 mm) were collected during its spawning migration using gill net from the Hooghly river at Godakhali, West Bengal, India (Latitude 23°44'17"N and Longitude 86°20'55"E). Fertilization was done through dry stripping method (Hoitsy, 2012; Malhotra *et al.*, 1969, 1970; Mathur *et al.*, 1974; Sen *et al.*, 1990). Fertilized eggs were oxygen packed and transported to the laboratory at Kakdwip Research Centre of ICAR-CIBA, West Bengal, and incubated at 23±1.0°C in natural photoperiod. Embryonic developmental stages were observed at different hours, starting from 5 hours post-fertilization (hpf). During incubation, 70% of water was exchanged with fresh filtered water.

Effect of Water From Different Sources on Fertilization and Hatching

To understand the effect of water sources on fertilization, water from three sources, *i.e.*, river, filtered riverine water from the treatment plant and subsurface water, having a hardness of 980, 950 and 550 ppm respectively, were used for hydration of egg-milt mixture on boat in the Hooghly river at Godakhali, West Bengal. Fertilized eggs were identified as per the standard method (Raja, 1985).

To study the effect of water quality on hatching rate, 1000 nos. fertilized eggs collected from each source water (river water, filtered riverine water from the treatment plant and subsurface water) were incubated in 5 L water from two sources, *i.e.*, filtered riverine water from the treatment plant and subsurface water in triplicate. Gentle aeration was provided throughout the study. The temperature was maintained at 23 to 25°C, as reported earlier (Jones and Menon, 1951):

$$\text{Fertilization rate (\%)} = \frac{\text{Fertilized egg count}}{\text{Total egg count}} \times 100 \quad (1)$$

$$\text{Hatching rate (\%)} = \frac{\text{Count of larvae hatched out}}{\text{Total count of fertilized egg}} \times 100 \quad (2)$$

Effect of Salinity on Larval Rearing

The effect of salinity on larvae survival was studied for 15 days in an indoor experiment with five levels of water salinity (0, 0.5 ppt, 1 ppt, 1.5 ppt, and 2 ppt) in aquarium tanks. Newly hatched larvae (200 no) were randomly stocked in 5 L water. The water temperature of the larval rearing tank was maintained at 25±2°C. From the third day to 6th day post-hatching, 100 ml of phytoplankton containing *Isochrysis galbana* (7.42×10⁴ nos./ml) and *Chlorella salina* (7.58×10⁴ nos./ml) at 1:1 ratio was supplied (Table 1). On the seventh day, onwards 50 ml rotifers, *Brachionus plicatilis* (45 nos./ml) and 100 ml mixed phytoplankton were added in rearing tanks. The dose of rotifer and phytoplankton was increased @ 10 ml/tank/day basis. Daily, 50% of the water was replaced with water of respective salinity. All the live feeds were added in the rearing tanks at 8:00 h and 16:00 h daily.

Embryonic Development and Ontogeny of the Digestive Tract

For a study on larval development and digestive tract ontogeny, larvae were raised in freshwater following larval rearing protocols of Kumar *et al.* (2018) with slight modifications. Fertilized eggs were observed under a trinocular microscope at different hours of incubation starting from 5 hpf till hatching. The hatched larvae, collected from larval rearing tank at different days post hatching (dph), were also observed under a microscope until oil globule utilization. During microscopic observation of the live sample, measurement of total length (TL), mouth gap, yolk sac volume, oil globule diameter was carried out with the aid of a trinocular microscope with the image-analyzing software (ProgResCapture Pro 2.7). The yolk sac volume (V) was calculated, according to Korzelecka-Orkisz *et al.* (2010):

$$V = \frac{\pi}{6} * Lh^2 \quad (3)$$

where 'h' is yolk sac height, and 'L' is yolk sac length.

After hatching larvae were distributed at the density of 50 larvae L⁻¹ in 12 different glass aquarium (50 L) filled with clean filtered freshwater. Larval rearing tanks were inoculated with algae (*Chlorella* spp.) at a density of 1-5×10³ cells mL⁻¹. Throughout the experiment, gentle aeration was provided, and 25% of water was exchanged daily.

Histology

For histological analysis, samples of six larvae were collected from each larval rearing tank at 1, 3, 9, and 14 dph and fixed in 10% neutral buffer formalin (NBF). NBF fixed larvae were dehydrated through a series of increasing ethyl alcohol concentrations (70-100%), cleared in xylene, and impregnated with paraffin wax. Wax blocks were sectioned to 5 µm thicknesses using a microtome (HM 325, Thermo Scientific) and stained with hematoxylin and eosin, as described by Roberts (1989). The tissue slides were cleaned in xylene, mounted in DPX, and then observed under the light microscope.

Estimation of Digestive Enzymes

To know the first appearance of digestive enzymes in the gut of hilsa larvae, hundreds of live larvae were collected on 3rd, 5th, 7th, 8th, 9th, 10th, 11th, 12th, and 15th dph. Larvae were collected before feeding (Ma *et al.*, 2005) and were homogenized in cold 0.25 M sucrose solution. Homogenized sample was centrifuged at 6000 rpm for 20 min at 4°C. Protein content was measured using the Lowry method (Lowry *et al.*, 1951). Gut digestive enzymes such as amylase, protease, cellulase and lipase activity were measured following Bernfeld (1955), Walter (1984), Miller (1959), and Ogunbiyi and Okon (1976), respectively.

Statistical Analysis

The means and standard errors of the performance indicators were subjected to one-way ANOVA followed by multiple comparison post hoc test. Statistical analysis was done using SPSS for Windows v.16.0 program (SPSS Inc., Chicago, IL, USA). All values are presented as means ± standard error (SE) of the mean unless otherwise stated.

Table 1. Feeding schedule of plankton for larval rearing of hilsa during initial 15 days.

Age of larvae (dph)	Plankton species supplied	Plankton offered (Nos./d/tank)	Feeding Frequency	Feeding Time
3-6	<i>Isochrysis galbana</i> , <i>Chlorella salina</i> (1:1)	7.50×10^6	2	08:00 h and 16:00 h
7-15	<i>Isochrysis galbana</i> , <i>Chlorella salina</i> (1:1)	7.50×10^6 - 13.00×10^6	2	08:00 h and 16:00 h
7-15	<i>Brachionus plicatilis</i>	2.25×10^3 - 5.85×10^4	2	08:00 h and 16:00 h

Table 2. Water quality and fertilization percentage of hilsa in different types of water.

Water types	Salinity (ppt)	Hardness (ppm)	Fertilization (%)**
River water	0.19 ± 0.00	980 ± 5.20	86.33 ± 0.88^a
Filtered river water	0.17 ± 0.01	950 ± 2.31	74.00 ± 0.58^b
Subsurface water	0.47 ± 0.01	550 ± 8.08	62.67 ± 0.88^c

** $P < 0.01$, ^{a,b,c} values bearing different superscript in a column differ significantly.

RESULTS

Fertilization and Hatching Efficiency

Fertilization rate was significantly ($p < 0.01$) higher in river water ($86.33 \pm 0.88\%$) as compared to filtered riverine water from the treatment plant ($74.00 \pm 0.58\%$) and subsurface water (Table 2). Hatching percentage was maximum ($57.00 \pm 0.58\%$) when the fertilization, as well as hatching, was allowed to happen in the same water, i.e., filtered riverine water from the treatment plant (Figure 1).

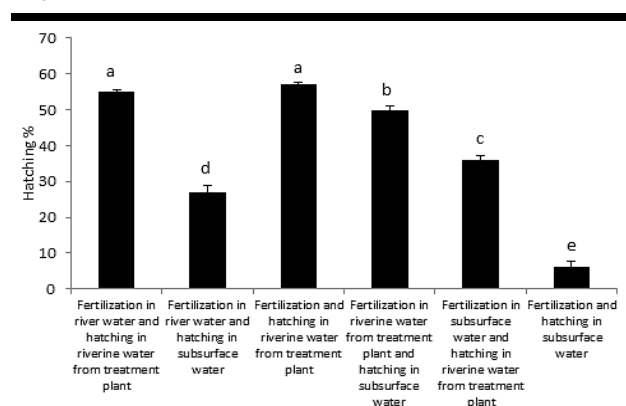


Figure 1. Hatching percentage of hilsa eggs hydrated and incubated in water with different salinity and hardness.

Embryonic Development

The germinal disc was observed at 5 hpf. After that, the eggs entered into the morula stage. Gastrulation, which is marked by epiboly, started 7 hpf. At 9 hpf, germ rings could be observed in the middle. By 9-11 hpf, advanced stages of the blastoderm cap were noticed. Closing of the blastopores was observed at 12 hpf. Notochord formations started in 17-19 hpf. Rudimentary eyes were also noticed after 18 hpf. Eggs were ready for hatching at about 21-23 hours, and finally, the hatch-out took place 22-24 hpf (Figure 2).

Effect of Salinity on Larval Survival

From the experiment on salinity optimization on larval rearing, it was observed that at 7 dph and 15 dph larval survival was significantly ($p < 0.01$) higher at 1.5 ppt salinity (Table 3).

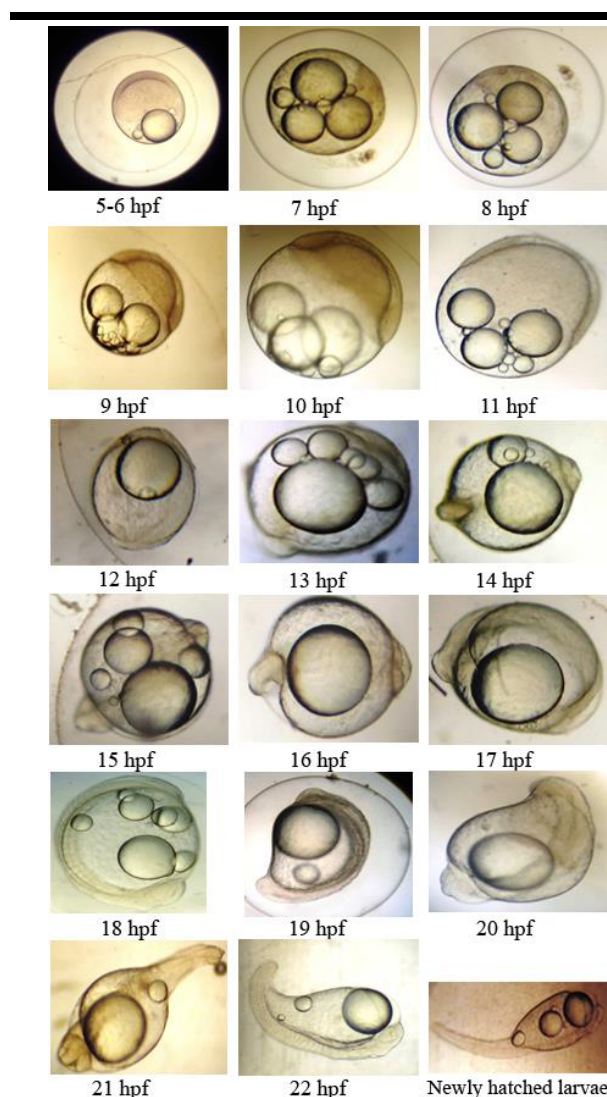


Figure 2. Photomicrograph showing embryonic development of hilsa, *Tenualosa ilisha* at different hours post fertilisation (hpf), (10X).

Table 3. Survival of hilsa larvae reared at different salinity.

Survival	Salinity (ppt)				
	0	0.5	1.0	1.5	2.0
7 dph**	17.68±2.03 ^c	44.67±4.33 ^b	51.00±2.31 ^b	73.33±2.60 ^a	45.00±5.20 ^b
15 dph**	12.00±0.58 ^c	12.00±1.73 ^c	12.67±1.53 ^c	53.67±3.75 ^a	20.67±0.88 ^b

**P < 0.01, ^{a,b,c} values bearing different superscript in a row differ significantly; dph: days post hatching.

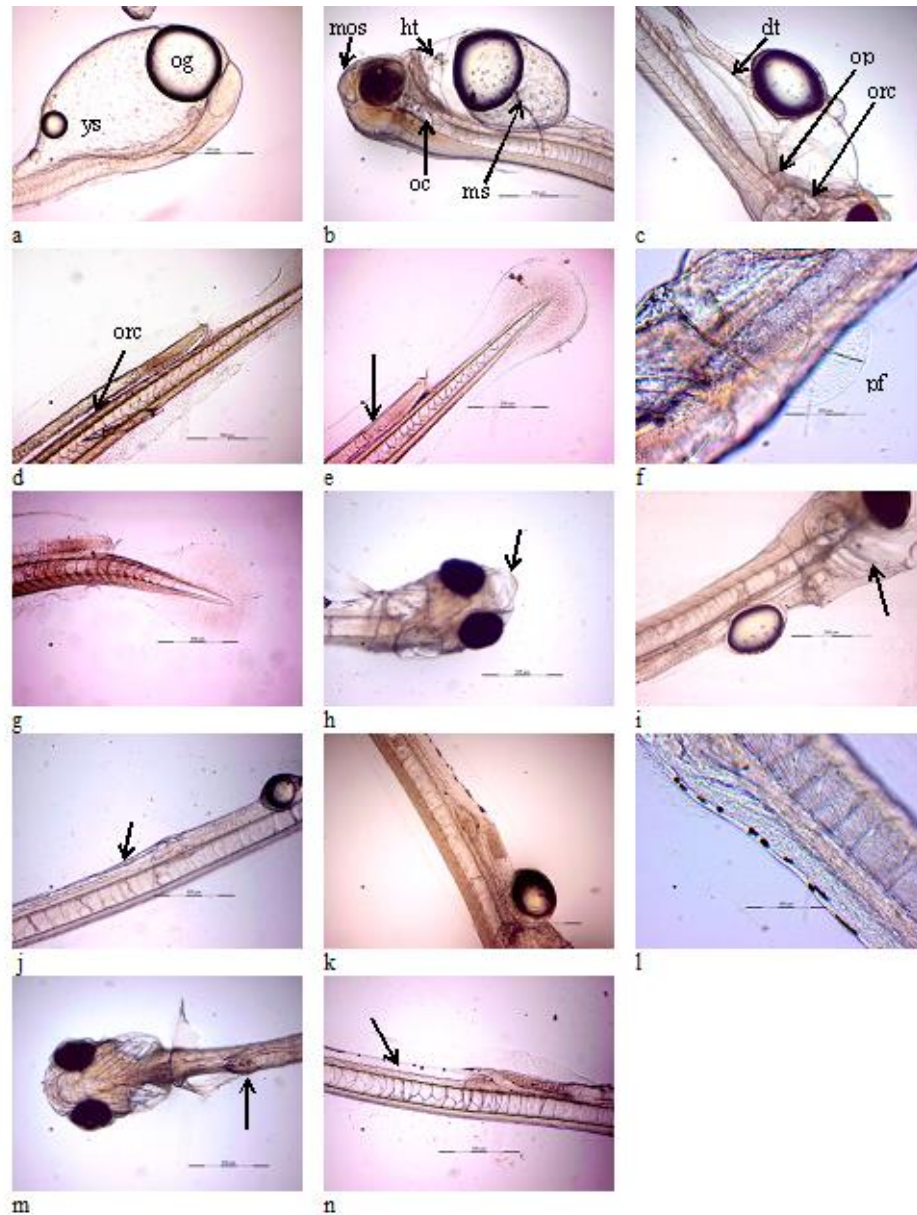


Figure 3. Photomicrograph showing larval development of hilsa, *Tenulosa ilisha* (4X, 10X) (a) Newly hatched larvae (0 dph) with a large yolk sac (ys) and 4 to 6 oil globules (og), (b) 2 dph larvae with two chamber heart (ht), mouth slit (mos), partial pigmented eye and melanophores spot (ms) on yolk, (c) 4 dph larvae with oil globule, oropharyngeal cavity (orc), esophagus (op) and digestive tube (dt) in formation, (d) 5 dph larvae, arrow indicate intestinal wall with crumple, (e) 6 dph larvae, arrow indicate developed rectum, (f) 7 dph larvae with developed pectoral fin (pf), (g) 8 dph larvae, (h) 9 dph larvae, arrow indicate developed jaws, (i) 10 dph larvae, arrow shows with gill rakers, (j) 11 dph larvae, arrow indicate algal cells in gut (k) 12 dph larvae, (l) 13 dph larvae, (m) 14 dph larvae, arrow indicates absence or very little oil globule (n) 15 dph larvae arrow indicate food particles in gut with no oil globule.

Developmental Stages of Larvae

Significant morphological developments in the hilsa larvae observed on different days after hatching are given below.

Newly Hatched Larvae

The newly hatched larvae were comma-shaped, unpigmented, transparent, with a large oval shaped yolk sac (volume: $0.419 \pm 0.147 \text{ mm}^3$) and 4-6 small oil globules, and one large oil globule (Figure 3a). The length of newly hatched larvae was $2.20 \pm 0.23 \text{ mm}$.

One dph Larvae

The average size, yolk sac volume, and oil globule diameter of one dph larvae were $3.64 \pm 0.20 \text{ mm}$, $194 \pm 0.04 \text{ mm}^3$ and $0.410 \pm 0.047 \text{ mm}$, respectively. At this stage, otic capsules moved towards hindbrain, and eye lens was seen. Histological observation showed the presence of acidophilic yolk sac, undifferentiated digestive tract, liver, and pancreas (Figure 4a).

Two dph Larvae

The two days old larvae measured $4.35 \pm 0.22 \text{ mm}$ in total length with approximately 90-100 number of myotomes. The volume of yolk sac measured $0.097 \pm 0.043 \text{ mm}^3$, and the diameter of oil globule was $0.416 \pm 0.024 \text{ mm}$. The larvae were characterized by the existence of a well-developed two-chambered heart, mouth slit, pectoral fin bud, partial pigmented eye, and melanophore spots on yolk (Figure 3b).

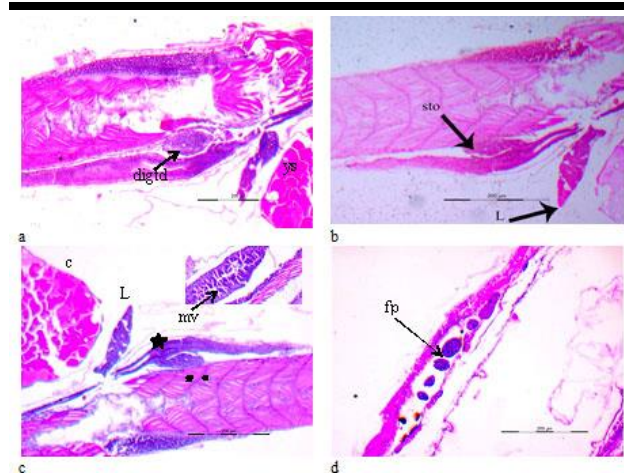


Figure 4. Histological observation of hilsa, *Tenulosa ilisha* larvae (4 X, 10 X). (a) 1 dph larvae showed acidophilic yolk sac (ys) and undifferentiated digestive track (digtd), (b) 3 dph larvae with v-shaped stomach (sto) and liver (L), (c) 9 dph larvae with well-developed intestine showing microvilli (mv), well-differentiated cardiac (single star) and pyloric stomach (double star) and (d) 14 dph larvae with food particle (fp) in gut.

Three dph Larvae

The average total length, yolk sac volume, and oil globule diameter were $4.82 \pm 0.57 \text{ mm}$, $0.07 \pm 0.01 \text{ mm}^3$ and $0.417 \pm 0.16 \text{ mm}$, respectively. The otic vesicle has shifted closer to the lens placode; both eyes were fully pigmented, both upper and lower

jaws formed, mouth opened. Histological observation showed the presence of v-shaped stomach, the anterior cardiac stomach, and the posterior pyloric stomach. The liver and pancreas were seen on the dorsal side of the yolk sac (Figure 4b).

Four dph Larvae

The four days old larvae measured $4.77 \pm 0.66 \text{ mm}$ in total length with $0.353 \pm 0.03 \text{ mm}$ diameter of oil globule (Figure 3c). Four days old larvae had no yolk sac. It had a well-developed mouth, pectoral fin and gut consisted of the oropharyngeal cavity, esophagus and digestive tube formation.

Five dph Larvae

Average length and oil globule diameter of five days old larvae were $5.56 \pm 0.75 \text{ mm}$ and $0.341 \pm 0.03 \text{ mm}$, respectively. At this stage, coiling of intestine was discernible (Figure 3d).

Six dph Larvae

Average length, oil globule diameter and mouth slit of six days old larvae were $5.77 \pm 0.45 \text{ mm}$, $0.328 \pm 0.002 \text{ mm}$, and $183.02 \pm 1.05 \text{ }\mu\text{m}$, respectively. Formation of the rectum with slight congestion near hindgut was also noticed (Figure 3e).

Seven dph Larvae

Average total length, oil globule diameter and mouth gap of seven days old larvae were 5.87 ± 0.22 , $0.324 \pm 0.03 \text{ mm}$, and $184.71 \pm 2.55 \text{ }\mu\text{m}$, respectively. Well-developed pectoral fin rays were observed (Figure 3f). Histological observation showed the presence of microvilli in the intestinal mucosa (Figure 4c).

Eight to Fifteen dph Larvae

The average total length of eight, nine, ten, eleven, twelve, thirteen, fourteen and fifteen dph larvae were 5.92 ± 0.15 , 5.96 ± 0.12 , 5.98 ± 0.18 , 5.98 ± 0.23 , 6.11 ± 0.40 , 6.12 ± 0.03 , 6.14 ± 0.22 and $6.16 \pm 0.24 \text{ mm}$, respectively (Figure 3). Eight days old larvae showed the opening of the anal pore (Figure 3i). Nine days larvae had well developed lower and upper lips (Figure 3h). Ten days old larvae had well-developed gill rakers (Figure 3i). Eleven, twelve, and thirteen days old larvae showed the presence of algal cells in the gut (Figures 3j, 3k, and 3l). Scarce or very little oil globule was visible on the fourteenth day (Figure 3m) and histology showed the presence of food particle in the gut (Figure 3d). Oil globule exhausted completely on 15th days, and anal pore was open (Figure 3n).

Digestive Enzymes

Digestive enzymes in the larvae were estimated at different days after hatching, and it was found that no digestive enzyme activity was detected before the 4th day after hatching. On 5th - day post-hatching, amylase, cellulase, acidic protease, alkaline protease, and lipase activities were detected. Alkaline protease and lipase activities increased ($p < 0.01$) with the age of larvae (Table 4).

DISCUSSION

Tenulosa ilisha lives in the marine, estuary, and riverine waters at different stages of their life cycle (Bhaumik, 2015). They start their life in fresh water as breeding takes place in freshwater.

Table 4. Digestive enzyme activity in hilsa larvae at different days post hatching (dph).

Parameter	3 dph	5 dph	7 dph	8 dph	9 dph	10 dph	11 dph	12 dph	15 dph
Amylase** (Unit/ml/min)	ND	3.59±0.03 ^{ab}	3.22±0.12 ^a	3.46±0.02 ^{ab}	6.21±0.32 ^c	3.71±0.05 ^b	7.35±0.06 ^d	4.01±0.01 ^d	9.93±0.08 ^e
Cellulase* (Unit/ml/min)	ND	0.22±0.07 ^a	0.25±0.04 ^a	0.89±0.01 ^d	0.53±0.06 ^b	0.73±0.03 ^c	0.16±0.04 ^a	0.33±0.07 ^a	0.72±0.08 ^c
Acidic protease** (Unit/ml/min)	ND	4.64±0.50 ^{bc}	0.65±0.10 ^a	0.80±0.05 ^a	2.35±0.70 ^{ab}	4.72±0.20 ^{bc}	6.55±0.20 ^c	1.56±0.60 ^{ab}	10.81±2.6 ^d
Alkaline protease** (Unit/ml/min)	ND	2.40±0.10 ^a	4.33±0.6 ^a	4.50±0.14 ^a	5.86±1.10 ^a	5.43±0.00 ^a	14.53 ±0.50 ^b	17.14±2.90 ^b	30.08±0.20 ^c
Lipase** (Unit/ml/min)	ND	2.50±0.00 ^a	3.83±0.67 ^b	4.33±0.17 ^b	4.50±0.29 ^b	5.67±0.17 ^c	7.50±0.00 ^d	7.67±0.17 ^d	8.50±0.00 ^e

* $p < 0.05$; ** $p < 0.01$; ND-Not Detected; dph-days post hatching.

They remain in fresh water till they reach to 15-16 cm in length (Raja, 1985) and then migrate to sea. The adult phase they spent in the sea, and they come back to freshwaters for breeding purpose. They reach maturity at the age of one year completion. Male and female hilsa attains maturity at a length of 260-290 and 310-330 mm respectively. Around 0.1-2.0 million eggs are found in a matured female hilsa (Islam *et al.*, 1989). As hilsa is a very sensitive fish, artificial breeding attempt in laboratory failed as in most of the occasion mortality of brood took place during transportation. With this experience, breeding was tried on a boat in Hooghly river at Godakhali, a freshwater region and potential breeding ground for hilsa (Bhaumik and Sharma, 2012) in the Hooghly river which comes just after the brackishwater region. In this region, the Hooghly river flows downstream into the Hooghly-Bhagirathi system. For successful fertilization, the quality of eggs is very important (Kinsey, Sharma, and Kinsey (2007). Eggs should not be "over-ripening" (Bromage, 1995) or under-matured. The selection of the brooders is, therefore, very important. In the current study, female broods with stage VI eggs were selected and stripped.

The first reported attempt at hilsa breeding was done by Wilson in 1909 (Sahoo *et al.*, 2018). After that, many unsuccessful attempts were made. These efforts were failed because of the lack of knowledge of feeding behaviour, the inappropriate quality of water used for rearing, and the inability of the fish to adapt (Sahoo *et al.*, 2018). In the present study, the effort was made to understand the ideal conditions necessary for fertilization, hatching, and rearing. Fertilization was done using the dry stripping method as it was reported to be most suitable for successful breeding of hilsa (Sahoo *et al.*, 2018). In the present study, the fertilization rate was ranging from 62.67-86.33% in different treatment groups. The hatching took around 22-24 h at 23±1.0°C, which is in agreement with the earlier report (Rahman *et al.*, 2017). Jones and Menon (1951) reported that hatching takes place in about 23 to 26 h at an average temperature of 23°C. Kulkarni (1950) reported that the time required for hatching was 18 to 26 h at 28.0°C to 28.5°C. In the present experiment, three different types of water were used. The first type was the river water. The second was filtered riverine water from the treatment plant, *i.e.*, plankton free as well as silt free. The third type, groundwater, differs from the above two types in salinity as well as in hardness. In the current

study, river water with a hardness of 980 ppm resulted in the highest fertilization rate, which indicates water hardness plays a crucial role for fertilization and hatching as reported in ornamental fish, *Danio rerio* (Chanu *et al.*, 2010). When the fertilized eggs were incubated in the same type of water as used for fertilization, hatching rate was found to be higher. The hatching rate was lower when water used for fertilization and incubation of egg was different. In the present study, the hatching percentage was found to decrease with decreasing hardness of the water. The current study suggests that for fertilization and hatching to be most effective, the hardness of the water used for fertilization and incubation of eggs needs to be similar (≥ 950 ppm) to source water where from brooders were collected.

In the past, many researchers have studied the development of the hilsa egg (De and Sen, 1988; Jones and Menon 1951; Rahman *et al.*, 2017). The eggs start to swell after the fertilization process like other clupeids as reported by Jones and Menon, 1951. Observations of the present study were also in agreement with the earlier studies.

In larval development of fish, first appearance, differentiation and physiological function of organ depend on their life history, environmental condition and feed availability (Lazo *et al.*, 2011; Sharma *et al.*, 2016; Zambonino-Infante *et al.*, 2008). The study on the developmental biology of fish larval stages will help to understand the nutritional physiology during early stages (Rust, 2002). Newly hatched larvae of hilsa actively swim at bottom, they have closed mouth and anus, which are similar to most of the teleosts studied (Kumar *et al.*, 2018; Lazo *et al.*, 2011; Onal *et al.*, 2008; Sharma *et al.*, 2016). Mouth of hilsa opened at 3-8 dph, which is similar to the most studied teleost such as cyprinids (Wallace *et al.*, 2005), percoids (He *et al.*, 2012; Teles *et al.*, 2015), silurids (Kumar *et al.*, 2018; Pradhan *et al.*, 2012), salmonids (Rust, 2002) and cichlids (Trevino *et al.*, 2011), where mouth opening is reported from 1 to 2 dph. The hilsa larvae had two kinds of energy reserves, such as yolk and oil globule which are very common in fish (Bjelland and Skiftesvik, 2006), and differ from catfish (Kumar *et al.*, 2018) and mahseer (Sharma *et al.*, 2016). In this study, it is found that the yolk is closely associated with the liver in newly hatched larvae, where yolk substances go to the liver through the venous circulation. A similar observation was made in wedge

sole (Herrera *et al.*, 2010; Morrison, 1993). In the present study, eye pigmentation occurred at three dph in hilsa. Therefore, the pigmentation of the eye indicates the preparedness for exogenous feeding in hilsa after yolk sac utilization. This is a typical feature of indirectly developing species. In hilsa, endogenous feeding on yolk sac lasts till 4dph, and after that, it depends on oil globule till 14 dph. Exogenous feeding in hilsa starts at 11 dph (before complete exhaustion of oil globule). Therefore, 11 to 14 dph can be considered as endo-exo feeding phase as oil globule content is not completely depleted until 14 dph. Heavy mortality of larvae was observed immediately after oil globule utilization, which might be due to fast swimming or filter feeding nature of larvae. Development and differentiation of the digestive system from the primitive stage to the functional stage proceed gradually. In newly hatched hilsa larvae, the digestive tract is like a tube with undeveloped digestive glands. Until four dph larvae depend on yolk reserves. In the present study, the mouth opened at 3 dph, digestive system components such as buccopharynx, oesophagus, intestine, liver and pancreas get differentiated at 3-4 dph, *i.e.*, prior to first exogenous feeding, following pattern similar to precocial species (Rust 2002; Mai *et al.*, 2005; Trevino *et al.*, 2011; Kumar *et al.*, 2018). In hilsa, major development of digestive system took place during 3 to 11 dph, as reported in altricial (He *et al.*, 2012; Pradhan *et al.*, 2012; Teles *et al.*, 2015; Wallace *et al.*, 2005) or precocial fishes (Rust 2002; Sharma *et al.*, 2016; Trevino *et al.*, 2011). In the digestive system, the liver is an important organ for lipid digestion through the production of bile (Lazo *et al.*, 2011; Rust, 2002; Zambonino-Infante *et al.*, 2008). In 1 dph hilsa larva, the liver could be observed between yolk sac and intestine, whereas in other species such as gill head seabream (Sarasquete *et al.*, 1995) and golden mahseer (Sharma *et al.*, 2016) liver appeared at two dph.

In this study, the detailed ontogeny of intestine, liver, and pancreas through histology was not carried out. The heart of newly hatched hilsa larvae received blood from pronephros and was still not compartmentalized. At 2dph, the heart gets compartmentalized, which indicates the commencement of the circulatory system. In teleost larvae, the gas exchange occurs through the skin until the gill lamellae are developed, the yolk sac being proposed as a possible site for a gas exchange due to its larger surface area (Pelster, 2008). For this reason, the main function of gill in larvae is reported to be osmoregulation (Falk-Petersen, 2005; Rombough and Moroz, 1997). Gill rakers/lamellae began to develop at ten dph in hilsa so that it would help in filter feeding and respiration. This was also supported by the beginning of exogenous feeding at 11 dph.

In the experiment on larvae rearing, it was observed that water with low salinity (1.5 ppt) ensued better survival of hilsa larvae. When salinity was increased beyond 1.5 ppt survival declined as at that stage hilsa larvae might not be able to maintain ionic homeostasis. There is no parallel report to compare the optimum salinity requirement for larval rearing of hilsa.

Chattopadhyay *et al.* (2018) reported larval survival of 13.30-61.31% in freshwater circular fiber reinforced plastic tank fed with chlorella followed by co-feeding with *Brachionus*. In the current study, the mixed phytoplankton used for larval rearing consisted of *Isochrysis galbana* and *Chlorella* sp.

Though earlier reports confirmed mouth opening of hilsa on 5th-day post-hatching (Jones and Menon, 1951), in the present study mouth opening of larvae was observed between 3rd and 8th day. The time required for mouth opening may vary, hence in the current study microalgae was supplied on 3rd day onwards. As the mouth aperture was bigger, hilsa larvae could easily take the microalgae immediately after the opening of the mouth. On seventh day onwards rotifer was provided along with microalgae to the larvae. Though small sized copepods were provided by an earlier worker for rearing hilsa larvae (Jones and Menon, 1951), in the current study, copepods were not provided during initial 15 days due to their known predatory effects on the fish larvae (Jackson and Lenz, 2016).

Digestive enzymes were estimated to understand the type of feed suitable to the larvae and also to understand the ability of larvae to digest phytoplankton at the initial stage. As per the authors' knowledge, no report is available on the first appearance of digestive enzyme in hilsa larvae. Till the third day, no enzyme activity was observed in hilsa larvae, which implies that feed will hardly be digested even if the feed is consumed. From a microscopic study, on the third day, a fine threadlike alimentary canal was observed. After the fifth day, amylase, cellulase, acidic protease, alkaline protease, and lipase activity were detected. At this stage, larvae were ready to consume and digest the feed in the presence of a digestive enzyme (Ribeiro *et al.*, 1999). Amylase and cellulase concentrations kept fluctuating but did not show any systematic trend in the early larval stage. They started steadily increasing after 9 to 11 days, indicating their preparedness to digest exogenous feed. There was a steady increase in alkaline protease and lipase levels. Presence of cellulase in the larvae may be from microbial source come from water or feed (Kolkovski *et al.*, 1993).

CONCLUSION

The study revealed that water hardness play a major role in fertilization and hatching of hilsa egg. It was observed that the digestive tract and associated glands of hilsa larvae developed at three dph. At four dph yolk sac was completely utilized, at 11 dph first exogenous feeding starts and at 15 dph, oil globule gets exhausted. It was also observed that the digestive system appeared to be developed prior to the onset of first exogenous feeding, and larvae first feed on phytoplankton. The first-hand information on embryonic and larval development and the onset of digestive enzymes will help to understand the larval rearing procedure for aquaculture and conservation of this species. Further, the baseline knowledge generated in this work will assist in researching the field of larval nutrition and digestive physiology.

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LITERATURE CITED

- Bernfeld, P., 1955. Amylases, α , and β , *Methods in Enzymology*, 1, 149-158.
- Bhaumik, U., 2015. Migration of hilsa shad in the Indo-Pacific region-A review. *International Journal of Current Research and Academic Review*, 3 (11), 139-155.
- Bhaumik, U. and Sharma, A.P., 2012. Present Status of Hilsa in Hooghly-Bhagirathi River, *In: CIFRI Bulletin* 179, pp. 42.
- Bjelland, R.M. and Skiftesvik, A.B., 2006. Larval development in European hake (*Merluccius merluccius* L.) reared in a semi-intensive culture system. *Aquaculture Research*, 37, 1117-1129.
- Boblme., 2012. Report of the Hilsa Fisheries Assessment Working Group II, 24-25 April 2012, Mumbai, India. *BOBLME-Ecology*, 10, pp. 1-34.
- Bromage, N.R., 1995. Broodstock management and seed quality - general considerations. *In: Bromage N.R. and Roberts, R.J. (eds.), Brood-stock management and egg and larval quality*. Boston: Wiley-Blackwell, pp. 1-24.
- Chanu, T.I.; Rawat, K.D.; Sharma, A.; Das, A.; and Devi, B.N., 2010. Effects of water hardness on egg hatchability and larval survival of aquarium fish, *Danio rerio*. *Journal of Aquaculture*, 18, 1-7.
- Chattopadhyay, D.N., Chakraborty, A., Ray, P.K. Mandal, R.N., Banik, S.K., Suresh, V.R. and Ghosh, K., 2018. Larval rearing of hilsa shad, *Tenulosa ilisha* (Hamilton 1822). *Aquaculture Research*, DOI: 10.1111/are.13934
- De, D.K., and Sen, P.R., 1988. Observations on the embryonic and early larval development of Indian shad, *Tenulosa ilisha* (Ham.). *Journal of Inland Fisheries Society of India*, 18, 1-12.
- Falk-Petersen, I.B., 2005. Comparative organ differentiation during early life stages of marine fish. *Fish and Shellfish Immunology*, 19, 397-412.
- Hamlin, H.J.; von Herbing, I.H., and Kling, L.J., 2000. Histological and morphological evaluations of the digestive tract and associated organs of haddock throughout post-hatching ontogeny. *Journal of Fish Biology*, 57, 716-732.
- He T., Xiao Z.; Liu Q Ma D.; Xu S., Xiao., and Y, Li J., 2012. Ontogeny of the digestive tract and enzymes in rock bream *Oplegnathus fasciatus* (Temminck et Schlegel 1844) larvae. *Fish Physiology and Biochemistry*, 38, 297-308.
- Herrera, M.; Hachero-Cruzado, I.; Naranjo, A., and Mancera, J.M., 2010. Organogenesis and histological development of the wedge sole *Dicologlossacuneata* M. larva with special reference to the digestive system. *Reviews in Fish Biology and Fisheries*, 20 (4), 489-497.
- Hoitsy, G.; Woynarovich, A. and Moth-Poulsen, T., 2012. Guide to the small scale artificial propagation of trout. Budapest: FAO, pp. 22.
- Islam, M.S.; Huq, O.M.; Hossain, M., Asad, S.A. and Das, N.N., 1989. Maturity and spawning of Hilsa shad, *Hilsa ilisha* of Bangladesh. Hilsa investigations in Bangladesh, Marine Fishery Resources Management, Report BOBP/ REP36, RAS/81/051, 95 p.
- Jackson, J.M. and Lenz, P.H., 2016. Predator-prey interactions in the plankton: larval fish feeding on evasive copepods. *Scientific reports*, 6, 33585.
- Jones, S. and Menon, P. M.G., 1951. Observations on the life-history of the Indian shad, *Hilsa ilisha* (Hamilton). *Proceedings of the Indian Academy of Sciences-Section. B*, 33 (3), 101-124.
- Kinsey, W.H.; Sharma, D. and Kinsey, S.C., 2007. *Fertilization and egg activation in fishes*. *In: Patrick J. B.; Joan C.; and Esther L. (eds.), The Fish Oocyte* Dordrecht: Springer. pp. 397-409.
- Kolkovski, S.; Tandler, A.; Kissil, G.W. and Gertler, A., 1993. The effect of dietary exogenous digestive enzymes on ingestion, assimilation, growth and survival of gilthead seabream (*Sparus aurata*, Sparidae, Linnaeus) larvae. *Fish Physiology and Biochemistry*, 12 (3), 203-209.
- Korzelecka-Orkisz, A., Smaruj, I., Pawlos, D., Robakowski, P., Tański, A., Szulc, J., and Formicki, K., 2010. Embryogenesis of the stinging catfish, *Heteropneustes fossilis* (Actinopterygii: Siluriformes: Heteropneustidae). *Acta Ichthyologica Piscatoria*, 40 (2), 187-197.
- Kulkarni, C.V., 1950. Breeding habits, eggs and early life history of Indian shad, *Hilsa ilisha* (Hamilton) in the Narbada river. *Proceeding of National Academy of Science India*, B, 15 (2), 169-176.
- Kumar, P.; Biswas, G.; Ghoshal, T.K.; Kailasam, M., and Vijayan, K.K., 2018. Embryonic and larval developments of brackish water catfish, *Mystus gulio* (Hamilton and Buchanan, 1822) induced with human chorionic gonadotropin and consequent larval rearing. *Aquaculture Research*, 49 (7), 2466-2476.
- Lazo J.P.; Darias, M.J.;Gisbert, E., 2011.Ontogeny of the digestive tract. *In: Holt, G.J. (ed.), Larval Fish Nutrition*. Wiley, Chichester, pp. 5-46.
- Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 193 (1), 265-275.
- Ma, H.; Cahu, C.; Zambonino, J.; Yu, H.; Duan, Q.; Le Gall, M.M. and Mai, K., 2005. Activities of selected digestive enzymes during larval development of large yellow croaker (*Pseudosciaena crocea*). *Aquaculture*, 245 (1-4), 239-248.
- Mai, K.; Yu, H.; Ma, H.; Duan, Q.; Gisbert, E.; ZamboninoInfante, J.L., and Cahu, C.L., 2005. A histological study on the development of the digestive system of *Pseudosciaena crocea* larvae and juveniles. *Journal of Fish Biology*, 67, 1094-1106.
- Malhotra, J.C.; Mathur, P.K.; Kamal, M.Y.; Chandra, R., and Desai, V.R., 1969. Successful artificial propagation of *Hilsa ilisha* (Ham.) near Allahabad. *Current Science*, 38, 429-430.
- Malhotra, J.C.; Mathur, P.K.; Kamal, M.Y., and Mehrotra, S.N., 1970. Observations on the hatching of fertilized eggs of *Hilsa ilisha* (Ham.) in confined freshwater. *Current Science*, 39, 538-539.
- Mathur, P.K.; Malhotra, J.C.; Mehrotra, S.N., and Shah., K.L., 1974. Experiments on the nursery rearing of spawn of *Hilsa ilisha* (Hamilton) in freshwater ponds. *Journal of Inland Fisheries Society of India*, 6, 205-210.
- Miah, M.S., 2015. Climatic and anthropogenic factors changing spawning pattern and production zone of Hilsa fishery in

- the Bay of Bengal. *Weather and Climate Extremes*, 7, 109-115.
- Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31 (3), 426-428.
- Morrison, C.M., 1993. Histology of the Atlantic cod, *Gadus morhua*: An atlas. Part four. *Canadian Special Publication of Fisheries and Aquatic Sciences*, 119C, 496.
- Ogunbiyi, O.A. and Okon, E.E., 1976. Studies on the digestive enzymes of the African fruit bat *Eidolon helvum* (Kerr). *Comparative Biochemistry and Physiology*, 55 (4), 359-361.
- Onal, U.; Langdon, C., and Celik, I., 2008. Ontogeny of the digestive tract of larval percula clownfish, *Amphiprion percula* (Lacepède 1802) a histological perspective. *Aquaculture Research*, 39, 1077-1086.
- Pelster, B., 2008. Gas exchange. In: Finn, R.N., and Kapoor, B.G. (eds.), *Fish Larval Physiology*. American Fisheries Society: Bethesda, pp. 91-118.
- Pradhan, P.K.; Jena, J.K.; Mitra, G.; Sood, N., and Gisbert, E., 2012. Ontogeny of the digestive tract in butter catfish *Ompok bimaculatus* (Bloch) larvae. *Fish Physiology and Biochemistry*, 38, 1601-1617.
- Rahman, M.A.; Ahmed, T.; Pramanik, M.M.H. and Flura, H.M., 2017. On-board breeding trial of hilsa (*Tenualosa ilisha*, Ham. 1822) and testing of larval rearing in Bangladesh. *Journal of Aquaculture Research and Development*, 8 (2), 1000471.
- Raj, B.S., 1917. On the habits of the hilsa and their artificial propagation in the Coleroon. *Journal and proceedings of the Asiatic Society of Bengal*, 13, 184.
- Raja, B.T.A., 1985. A review of the biology and fisheries of *Hilsa ilisha* in the Upper Bay of Bengal. Bay of Bengal Program. Marine Fisheries Management. Report.
- Ribeiro, L., Zambonino-Infante, J.L., Cahu, C., and Dinis, M.T., 1999. Development of digestive enzymes in larvae of *Solea senegalensis*, Kaup 1858. *Aquaculture*, 179 (1-4), 465-473.
- Rombough, P.J. and Moroz, B.M., 1997. The scaling and potential importance of cutaneous and branchial surfaces in respiratory gas exchange in larval and juvenile walleye *Stizostedion vitreum*. *Journal of Experimental Biology*, 200, 2459-2468.
- Rust, M.B., 2002. Nutritional physiology. In: Halver, J.E. and Hardy, R.W., (eds.), *Fish Nutrition*, 3rd edn. Academic Press: Amsterdam, pp. 367-452.
- Roberts, R.J., 1989. Nutritional pathology of teleosts. In: Roberts, R.J. (ed.), *Fish Pathology*, Bailliere Tindall, London, pp. 337-362.
- Sahoo, A.K.; Wahab, M.A.; Phillips, M.; Rahman, A.; Padiyar, A.; Puvanendran, V.; Bangera, R.; Belton, B.; De, D.K.; Meena, D.K. and Behera, B.K., 2018. Breeding and culture status of hilsa (*Tenualosa ilisha*, Ham. 1822) in South Asia: a review. *Reviews in Aquaculture*, 10 (1), 96-110.
- Sarasquete, C.; Polo, A., and Yu, fera M., 1995. Histology and histochemistry of the development of the digestive system of larval gilthead seabream, *Sparus aurata* L. *Aquaculture*, 130, 79-92.
- Sen, P.R.; De, D.K., and Nath, D., 1990. Experiments on artificial propagation of hilsa, *Tenualosa ilisha* (Hamilton). *Indian Journal of Fisheries*, 37, 159-162.
- Sharma, P.; Akhtar, M.S.; Singh, A.K.; Das, P., and Sarma, D., 2016. Histomorphological changes in digestive tract of golden mahseer (*Tor putitora*) during different developmental stages. *Fish Physiology and Biochemistry*, 42 (6), 1681-1698.
- Teles, A.; Costa, W.M. Ammar D.; Müller, Y.M.; Nazari, E.M., and Cerqueira, V.R., 2015. Ontogeny of the digestive tract of *Centropomus parallelus* larvae. *Fish Physiology and Biochemistry*, 41, 549-559.
- Trevino, L.; Alvarez-González, C.A.; Perales-García, N.; Arevalo-Galan, L.; Uscanga-Martínez, A.; Marquez-Couturier, G.; Fernandez, I., and Gisbert, E., 2011. A histological study of the organogenesis of the digestive system in bay snook *Petenia splendida* Gunther, 1862 from hatching to the juvenile stage. *Journal of Applied Ichthyology*, 27, 73-82.
- Wallace, K.N.; Akhter, S.; Smith, E.M.; Lorent, K.; and Pack, M., 2005. Intestinal growth and differentiation in zebrafish. *Mechanism of Development*, 122, 157-173.
- Walter, H.E., 1984. *Proteinases: methods with hemoglobin, casein and azocoll as substrates*. In: Bergmeyer, H.U. (eds.), *Methods of enzymatic analysis*. Weinheim: Verlag-Chemie, pp. 270-277.
- Wilson, H.C., 1909. Artificial propagation of Hilsa in the Coleroon. Government of Madras, India, Revenue Department, G.O., 1219.
- Zambonino-Infante, J.; Gisbert, E.; Sarasquete, C.; Navarro, I.; Gutierrez, J. and Cahu, C.L., 2008. Ontogeny and physiology of the digestive system of marine fish larvae. In: Cyrino, J.E.O.; Bureau, D., and Kapoor, B.G. (eds.), *Feeding and Digestive Functions of Fish*. Science Publishers, Enfield, pp. 277-344.