

# Ontogenetic Development of Digestive Tract and Enzymes Activity in Hatchery-reared Pink Ear Emperor, *Lethrinus lentjan* Larvae

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## Abstract

Ontogenetic development of the digestive tract and associated organs of *Lethrinus lentjan* larvae was learned by observing changes in external morphology, histology, and enzymatic changes from hatching to 40-day post-hatching (dph). Ontogenetic development of the *L. lentjan* digestive system can be divided into three major stages; the first stage from hatching of larvae to complete absorption of yolk-sac, which ends on 2 dph, the second stage from 3 to 15 dph i.e., from exogenous feeding to the formation of the gastric gland and third stage till completion of metamorphosis (35 dph). Pyloric caeca were developed after 20 dph, which was formed entirely by 27 dph. Enzymatic activities of amylase, lipase, trypsin and chymotrypsin were detected before the onset of exogenous feeding and pepsin was detected from 19 dph onwards in *L. lentjan*. So considering both histology of digestive tract and enzyme development, weaning of *L. lentjan* larvae to artificial diet can be practiced after 19-20 dph coinciding with the formation of pyloric caeca and production of pepsin enzyme for better digestibility and consequently better growth and survival. The present study can be expended as a reference guide to understand the digestive tract development and successful hatchery rearing of other economically important marine fishes.

## Introduction

The recent developments in marine finfish aquaculture, especially cage farming have led to an increase in demand for seed from marine finfish hatcheries and the need for species diversification. The supply of hatchery-produced juveniles to the mariculture sector is less because of high larval mortality rates (Tucker, 1998; Qin, 2013). A better understanding of the early larval rearing phase is necessary to improve

large-scale marine fish seed production (Watanabe and Kiron, 1994; Xavier et al., 2021). Limited knowledge of larval development and rearing protocol is the major bottleneck in hatchery production. Most of the marine fish larvae begin their exogenous feeding when their digestive system are still in a rudimentary stage of development. Thus, the structural and functional knowledge of the digestive system of fish species is an essential prerequisite for the hatchery phase of larval rearing. This would benefit for better growth and

survival since environmental and nutritional requirements in the early life stage are species-specific (Palinska-Zarska et al., 2014; Furgala-Selezniow et al., 2016; Laczynska et al., 2016; Stejskal et al., 2021). The digestion and absorption of nutrients occur in the gastrointestinal tract, and digestion is performed by the action of different enzymes of the stomach, exocrine pancreas and intestine (Zambonino-Infante and Cahu, 2001). Biochemical and physiological studies of the digestive system with structural development are in need to understand the digestive capacity of developing larvae. Fish with an efficient digestive system is necessary for an efficient food intake and complete nutrient utilization (Kjorsvik et al., 2004). Indeed a better understanding of fish digestive capacity and nutritional requirement is essential to improve larval growth and survival (Alvarez-Gonzalez et al., 2006; Ma et al., 2012). Hence, the knowledge of the digestive tract development with the changes in digestive enzyme production is necessary for understanding the nutritional physiology of larval fish.

*L. lentjan* (Pink ear emperor) belongs to the family Lethrinidae is a high-value marine finfish widely distributed in Indo-Pacific Ocean waters (Carpenter and Niem, 2001). Captive breeding through volitional spawning in recirculating aquaculture system (RAS) and larval rearing in the green water system of *L. lentjan* have been reported (Anil et al., 2019). Gomathi et al. (2021) studied the embryonic development of *L. lentjan* under culture conditions. Even though captive breeding and larval rearing have been standardized, there is a lack of knowledge in digestive system development. Information on gut ontogeny and digestive enzyme development is crucial to developing an appropriate protocol to wean fish larvae to compound diets (Baglolle et al., 1997; Cahu and Zambonino-Infante, 2001).

The present study sheds light on the ontogenetic development in relation to the main digestive enzymes (amylase, trypsin, chymotrypsin, protease, lipase and pepsin) and the morphological and functional change of the digestive system of *L. lentjan*, which can help to improve the larval rearing protocol, mainly feeding which in turn helps to increase growth and larval survival rates.

## Materials and Methods

### Fish Selected for The Study

Eggs and larvae of *L. lentjan* for the present study were obtained from volitional spawning of brooders in 10-ton RAS system in Vizhinjam Regional Centre ICAR-CMFRI, Vizhinjam.

### Eggs and Larval Rearing of *L. lentjan*

Larval rearing protocol for the study is by following Anil et al. (2019). Fertilized, transparent and floating eggs of *L. lentjan* were incubated in pre-set 2-ton

fertilized fiberglass-reinforced tanks (FRP) in triplicates. A green water system with copepod (*Parvocalanus crassirostris* and *Acartia southwelli*) as initial live feed was used for larval rearing. The stocking density was 20 larvae L<sup>-1</sup>. Bottom siphoning of tanks was done after hatching to remove the egg shells and dead eggs.

A green water system with the combination of algal species such as *Nannochloropsis salina*, *Isochrysis galbana* and *Chaetoceros calcitrans* were also used for larval rearing. Copepod adults and nauplii of *P. crassirostris* and *A. southwelli* (0.2-0.4 nos mL<sup>-1</sup>) were added during egg incubation to ensure the sufficient number of copepod nauplii during the first feeding of the larvae. From 3 days post hatching (dph) onwards, nauplii of *P. crassirostris* (40-45 µm width) and *A. southwelli* (45-55 µm) were added at the rate of 2-3 nos mL<sup>-1</sup> to ensure that the smallest copepod stage (1<sup>st</sup> naupliar stage) was available when the larvae starts feeding. From 6 dph onwards, enriched rotifers (with Origreen, Skeretting, France) of *Brachionus plicatilis* and *B. rotundiformis* (8-10 nos mL<sup>-1</sup>) were fed to larvae along with copepods. In later stages of 14-18 dph, larvae were fed with *Artemia* nauplii and from 18 dph onwards enriched *Artemia* (Origreen, Skeretting, France) were given. Artificial feed (Gemma Diamond, 300-500 µm, containing 62% prtotein, 11% lipid and 8.5% ash was fed from 16 dph onwards, which continued until 20 dph. From 20 dph onwards, pellet size of 200-400 µm was supplied along with 150 µm feed which was continued till 30 dph. Between 25 and 40 dph, the larvae were fed 300- 500 µm feed. Larvae were fed 2 to 3 times per day. An *in-situ* biological filter with an airlift was kept in tank to retain better water quality while feeding with artificial pellets.

Water quality parameters like temperature, turbidity, salinity, pH, alkalinity, dissolved oxygen, total ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were estimated daily and maintained in the optimal range. Water quality parameters maintained were as follows; temperature: 29±1°C, salinity: 33–35 gL<sup>-1</sup>, pH: 7.6–8, total ammonia: 0.204–0.302 mgL<sup>-1</sup>, D.O.: 4.7–5.6 mgL<sup>-1</sup>, alkalinity: 100–130 mgL<sup>-1</sup> CaCO<sub>3</sub>, CO<sub>2</sub>: 4.15–4.80 mgL<sup>-1</sup>; turbidity: 0.16–0.39 NTU, nitrate (NO<sub>3</sub>): 0.3–0.7 mgL<sup>-1</sup>, nitrite (NO<sub>2</sub>): 0.007–0.029 mgL<sup>-1</sup>. Light and dark hours maintained was 12 h L: 12 h D.

### Live Feed Culture and Enrichment for Larval Rearing

Both species of copepods, *P. crassirostris* and *A. southwelli*, were mass cultured in FRP tanks (1 ton) separately using algal species of *N. oculata* and *I. galbana*. Rotifers of Large and Small strains (*B. plicatilis* and *B. rotundiformis*, respectively) were also cultured in 2 ton FRP tanks using *N. oculata* (at a density of 3.1 ×10<sup>6</sup> cells mL<sup>-1</sup>). Harvested rotifers were enriched with algal-based enrichment medium (Origreen, Skeretting, France) for 2-4 h in separate tank. Freshly hatched *Artemia* nauplii of 400 µm (Five star, OSI brand cyst, Ocean Star International, USA) was used without

enrichment and nauplii after 8 h post hatch, were enriched for 5-6 hrs. Both enriched *Artemia* and rotifers were fed to larvae only after washing in seawater.

### Growth and General Morphological Observations

To study the general morphology and growth, fish larvae were randomly sampled from larval rearing tanks at 1, 3, 5, 7, 10, 13, 16, 19, 22, 25, 30, 35 and 40 dph. On each sampling day, around 10-30 fish larvae were randomly collected from each tank and anaesthetized using sodium bicarbonate buffered MS-222 (5-20 mgL<sup>-1</sup>). Since larvae were transparent from 0 to 10 dph, they were directly observed under the microscope to record the general morphology and gut development. Photographs and measurements were taken with Zeiss AXIO Lab A1 compound microscope with Axio camera ERc5s and ZEN 2 lite software and Leica S8APO stereo zoom microscope attached with Leica DFC 290 camera and Leica application suite version 4.1.0 software. Larval development was described in terms of total length: the measurements taken along the midline of the body from the tip of the snout to the end of caudal fin. Growth was determined in terms of specific growth rate (SGR) (%/day) and absolute growth rate (AGR) (mm/day), using the following equations given by Hopkins (1992).

$$SGR = \frac{(\ln \text{ final length} - \ln \text{ initial length})}{\text{Number of days}} \times 100$$

$$AGR = \frac{(\ln \text{ final length} - \ln \text{ initial length})}{\text{Number of days}}$$

### Histological Analysis

For the histological description of the gut's morphogenesis, 5-10 larvae were sampled at 1, 2, 3, 5, 7, 10, 13, 16, 19, 22, 25 and 30 dph. The samples were rinsed in distilled water and fixed in neutralized-buffered formaldehyde (5 to 10 % formalin). Afterwards, they were dehydrated with series of different alcohol concentrations, cleared in xylene, and embedded in paraffin blocks. The whole larval sagittal section of 4 to 5  $\mu\text{m}$  was taken using a rotary microtome. For general histo-morphological observations, hematoxylin and eosin staining procedure was followed (Pearse, 1985). Using DePex, the sections were mounted permanently on slides. Five randomly selected sections of fish larvae were examined under a Zeiss AXIO Lab A1 compound microscope and photographs were taken which was analyzed with Axio Cam ERc5s and ZEN 2 lite software, respectively.

### Gut Content Analysis

Gut content analysis was performed from 2 dph to 5 dph to investigate the first feeding of larvae on a daily basis. After that, gut content analysis was done at 5-day intervals to understand the larval feeding pattern,

especially at each weaning period. On each sampling day, 5-10 larvae were sacrificed for gut content analysis.

Gut dissection of larvae was done under the microscope using a small needle or by squeezing out the gut contents by giving a gentle pressure on cover slip and glass slide sandwiched with random sample of larvae. Photographs were taken using Zeiss AXIO Lab A1 compound microscope attached with Axio Cam ERc5s with ZEN 2 lite software.

### Enzyme Analysis

About 10 - 1000 larvae ranging total wet weights of 0.2 to 0.3 g were collected before the morning feeding at 1, 3, 5, 7, 10, 13, 16, 19, 22, 25, 30, 35 and 40 dph for digestive enzyme analysis. Then, they were maintained in glass beakers at least 1 h for complete assimilation or excretion of food remnants from the gut. It was followed by frozen storage at -80°C until enzyme assays. Enzyme extractions were performed within one month of sample collection. For this, whole larvae were taken in a glass tube, containing 0.25 M chilled sucrose solution, and homogenized with a mechanical homogenizer (Remi Equipment, India) in ice-cold conditions to avoid heating. Afterwards, the homogenates were centrifuged at 5000 rpm for 10 min at 4°C (Remi centrifuge, India). Then, the supernatant was collected carefully and stored in -20°C for enzyme assay.

Lowry's method was used to determine the protein content in the samples (Lowry et al., 1951). Tissue homogenate (0.1 mL) was first precipitated using 1 mL of 10% TCA. The protein residue was collected after centrifugation at 5000 rpm for 20 min. The residue was dissolved in 0.5 mL of 0.1 N NaOH, and then 0.1 mL of the dissolved protein residue was used for further analysis. Alkaline copper sulphate (5 mL) was added to dissolve protein residue and left for 10 min. To this, 0.5 mL of 1 N Folin's reagent was added and incubated for 30 minutes in the dark. OD (optical density) was recorded by measuring absorbance at 660 nm against the blank. Bovine Serum Albumin (BSA) was used as a standard.

Amylase activity was determined by the estimation of decline in sugar production due to the action of gluco-amylase and  $\alpha$ -amylase on carbohydrates using di-nitro-salicylic-acid (DNS) method (Rick and Stegbauer, 1974). Concisely, tissue homogenate and reaction mixtures (1% w/v) of starch solution and phosphate buffer (pH 6.9) were incubated at a temperature of 37°C for 30 min. To this, DNS is added and kept in a boiling water bath for 5 min. After cooling and diluting with distilled water, the absorbance was measured at 540 nm. Maltose was used as standard. The amylase activity was expressed as a mole of maltose released from starch per min at 37°C.

Estimation of total protease activity was done by the casein digestion method of Drapeau (1971). 1 % casein in 0.05 M Tris-phosphate buffer (pH 7.8) was used as the enzyme reaction mixture, which was kept in incubation for 5 min at 37°C. Then, the tissue

homogenate was added and kept aside 10 min for the reaction, which was further stopped by adding 10 % TCA followed by filtration of the entire content. One unit of enzyme activity was defined as the amount of enzyme needed to release soluble acid fragments equivalent to 0.001 at 280 nm per minute at 37°C and pH 7.8.

Determination of lipase activity was done by titrimetric method of Cherry and Crandell (1932). It was estimated by measuring the fatty acids released by the enzymatic of triglycerides in the stabilized emulsion of olive oil. The fatty acids released were estimated by titrating with sodium hydroxide solution. The assay system consists of 1.5 mL of stabilized lipase substrate and 1.5 mL of 0.1 M Tris-HCl buffer (pH of 8.0), to this 1.0 mL of the crude enzyme extract was added. This mixture was incubated for 24 h at 27°C and the reaction was stopped by adding 3 mL of 95% ethyl alcohol. It was then titrated against 0.01 N NaOH 0.9% (w/v) using phenolphthalein as indicator.

Trypsin and chymotrypsin activities were estimated by the casein digestion method (Kunitz, 1947). The reaction mixture for trypsin consist of phosphate buffer (pH 7.5), 1% casein as substrate and tissue homogenate was incubated at 37°C for 20 min. OD of the sample mixture was recorded at 280 nm after incubation. The reagent blank was prepared by adding tissue homogenate without incubation. For the estimation of chymotrypsin reaction, mixture consists of borate buffer with CaCl<sub>2</sub> at pH of 8, 1% casein as substrate, and tissue homogenate. The absorbance was measured at 280 nm. The trypsin and chymotrypsin activities were estimated from tyrosine standard curve, which expressed as µM of tyrosin at 37°C.

Pepsin activity estimated according to Anson, (1938). The substrate used was hemoglobin (1%) in glycine-sodium chloride-HCl buffer at pH of 3.0. A 25 µL of enzyme extract was incubated in 125 µL substrate and 100 µL buffer for 30 min. The reaction was stopped using 5% TCA. The mixture was centrifuged for 10 min for 2300 g at 4°C after an incubation period of 5 min.

Absorbance of the mixture was recorded at 280 nm. Blank reading was taken by adding TCA to the substrate prior to the addition of enzyme extract. One unit pepsin activity expressed as 1 µM equivalent of tyrosine liberated per min. Specific activity of the enzyme was expressed as U mg<sup>-1</sup> protein.

### Statistical Analysis

All measurements were carried out in triplicates. Statistical analysis of each digestive enzyme activity between the sampling dates was compared with one-way Analysis of Variance (ANOVA). Duncan's Multiple Range Test (DMRT) was performed to determine the differences among the means at 5% significance levels using SPSS version 16.0. All data were represented as mean ± standard error (SE).

### Results

#### Growth and Development of *L. lentjan* Larvae

The feeding protocol followed, and the growth of *L. lentjan* larvae in terms of total length (mm) during the larval development are depicted in Figure 1. The average total length of larvae was 1.345±0.015 mm (n=30) at hatching and 21.394±0.58 mm (n=30) at 40 dph. An AGR of 0.50 mm day<sup>-1</sup> and a SGR of 6.91 % day<sup>-1</sup> were observed during the larval rearing period of 40 days. The growth of *L. lentjan* larvae is followed by an exponential curve, which was fitted by  $Y = 1.8636e^{0.0652x}$  ( $R^2 = 0.9596$ ).

At hatching, the digestive tract was a straight tube lying dorsally to yolk sac and from 12 hour post hatch (hph) it became visible as a single tube shaped gut developing posterior to yolk sac (Figure 2a and 2b). Yolk sac length of 777.32±58.25 µm and oil globule diameter of 138.12±7.5 µm at the time of hatching reduced to 227.81±97.29 µm and 90.11±9.89 µm, respectively at 1 dph. Yolk and oil globule completely absorbed by 2-3 dph (Figure 2a, 2b, 2c and 2d). By 2 dph, the urinary

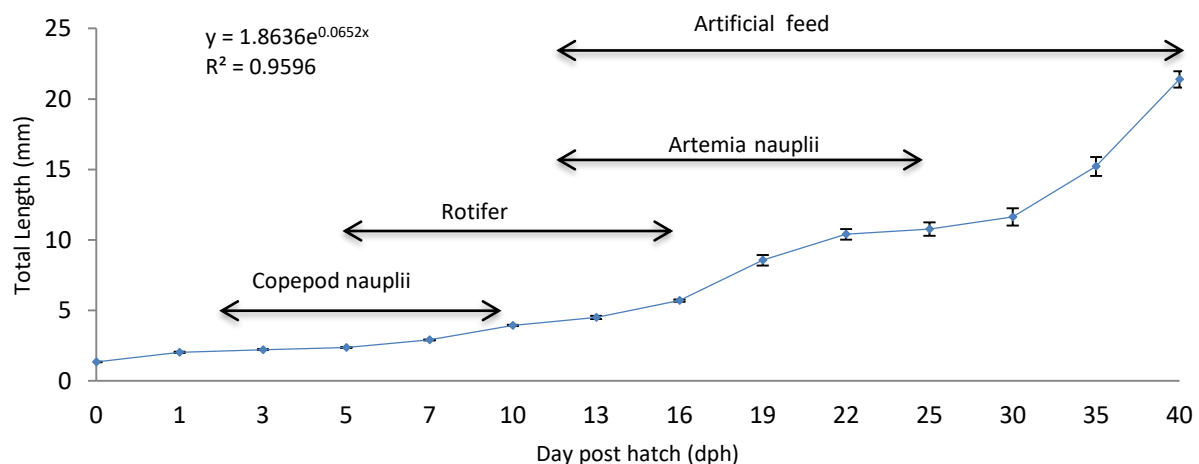


Figure 1. Total length of *L. lentjan* from 1 to 40 days post-hatch (dph). Feeding protocol is presented above the growth curve.

bladder anlage also opens to the digestive tract and this connection was further confirmed by the observation of liquid discharge from the urine bladder into the hindgut cavity (Figure 2a, 2b and 2c). Even though the anus was open, the mouth was closed on 0 and 1dph (Figure 2a and 2b). Although mouth opened on 2 dph, only algae were found on the gut of larvae until 3 dph (Figure 4a). From 4 dph onwards, copepod nauplii were identified in the mid gut of larvae (Figure 4b). Formation of stomach, liver, pancreas and intestinal valve were observed with enlargement in the size of digestive tracts as the larval development progressed. Pyloric caeca formation marking the last significant development in the digestive tract was observed on 27 dph (Figure 3j).

**Histology of Digestive Tract Development**

**Mouth and Buccopharynx**

Mouth and buccopharyngeal cavity were not formed in newly hatched larvae (Figure 2a and 3a). On 1 dph, mouth, buccal cavity and pharynx were undifferentiated and were still closed. Mouth slit was visible on 1 dph (Figure 2c and 3b). Mouth opened at 2 dph, and larvae started exogenous feeding (Figure 1d). On 2<sup>nd</sup> and 3<sup>rd</sup> day, the rudimentary gill arches and the external gill slit began to form in the posterior buccopharyngeal region. By 3 dph, the digestive tract

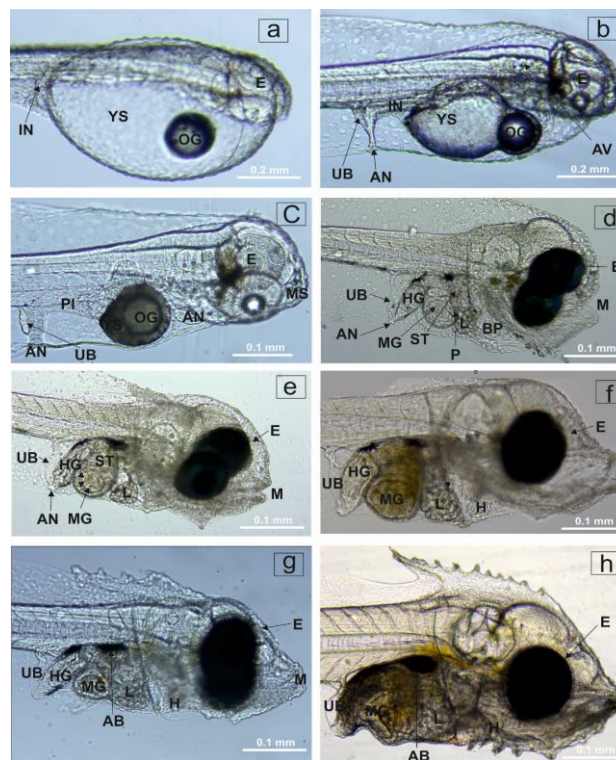
was fully opened from mouth to anus (Figure 2e and 3c). The tongue was developed on 7 dph (Figure 2g).

**Oesophagus**

In newly hatched larvae, oesophagus appeared as simple tube similar to the incipient gut and was very difficult to differentiate the developing intestine till the formation of buccopharyngeal cavity. Before the formation of buccopharyngeal cavity, the digestive system tract was closed at the oesophagus. On 3 dph, oesophagus was visible in the anterior part of foregut as a short and narrow lumen which connects buccopharyngeal cavity with intestine (Figure 2e and 3c). Then the oesophagus expanded posteriorly to form stomach (Figure 2d and 3c). Oesophageal mucous cells and microvilli were observed from 10 dph onwards (Figure 3f). Mucous or goblet cell numbers increased as the days progressed.

**Stomach**

At 2 dph, the developing stomach appeared as a bulge at the posterior end of oesophagus (Figure 2d). On 3 dph, a rudimentary stomach appeared between the oesophagus and intestine (Figure 2e and 3c). Stomach started to elongate and fold from 7 dph onwards, and the wall of stomach became thicker (Figure 3e).



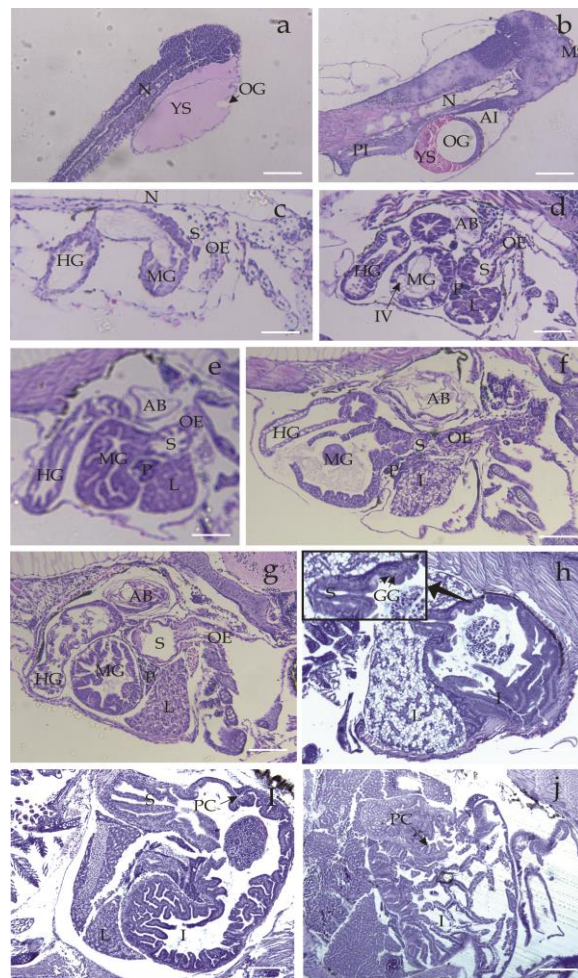
**Figure 2.** Morphological observations of the digestive tract during the development of *L. lentjan* larvae. (a) Newly hatched larva, (b) 12 hph larvae, c) 1 dph larvae, Note the mouth slits, (d) 2 dph larvae. Note the mouth opening (arrowhead) and pigmented eye, (e) 3 dph larvae. Note food particles-copepod (arrowheads), (f) 5 dph larvae, (g) 7 dph, (h) 10 dph larvae. Abbreviations: AB, air bladder; AI, anterior intestine; AN, anus; AV, autic vesicle; BP, buccopharynx; E, eye; H, heart; HG, hindgut; IN, incipient intestine; L, liver; MG, midgut; MS, mouth slit; N, notochord; OE, oesophagus; OG, oil globule; P, pancreas; PI, posterior intestine; ST, stomach; UB, urine bladder; YS, yolk sac.

Longitudinal folds developed on 10 dph and the pyloric sphincter, which separated the stomach from the midgut appeared on 12 dph (Figure 3g). Stomach with cardiac and pyloric regions were evident on 12 dph (Figure 3g), which later divided into cardiac, fundic and pyloric regions. The well-developed fundic region became a prominent part and primary food storage site of stomach. Gastric glands were observed from 15 dph onwards between the cardiac and pyloric stomach (Figure 3h). The number and size of gastric glands increased along with the progression of digestive tract development.

**Intestine and Pyloric Caeca**

Upon hatching, the incipient intestine was a narrow straight tube (Figure 2a and 3a), lined with a single layer of columnar cells. At 12 hph, developing intestine was clearly visible at the posterior of yolk (Figure 2b). On 1 dph, anterior and posterior intestines got well-differentiated and the posterior intestine

appeared as an L-shaped organ with a bent of 90° (Figure 2c and 3b). At 2 dph, rectum opened, and the lumen of intestine became visible by the increased numbers of epithelial cell, and the intestine started to open by 2 to 3 dph (Figure 2d and 3c). At 3 dph, early convolution of gut with folding of intestine was observed (Figure 3c) and within 2 to 3 days intestine was divided into midgut and hindgut sections. With the complete separation of the rectum by the rectal valve, structural changes occurred in the intestine between 5 and 6 dph (Figure 2f and 3d). The appearance of empty infranuclear vesicles (IV) or neutral lipids on 5 dph denoted the first evident sign of intestinal lipid absorption (Figure 3d). On 7 dph, intestine became coiled with lipid vacuoles (Figure 3e). A more coiled intestine was observed on 12 dph. The pyloric sphincter which controls the food movement at the anterior part of intestine appeared on 12 dph (Figure 3g). Goblet cells appeared from 15 dph onwards and increased later on. Numerous lipid vacuoles and acidophilic supranuclear vacuoles developed on intestinal folds till the end of the experiment (Figure 3j).



**Figure 3.** Histological sections of the *L. lentjan* digestive tract. (a) at hatchling, (b) 1 dph larvae with anterior and posterior intestine, (c) 3 dph larvae with development of incipient stomach, (d) 5 dph larvae, (e) 7 dph larvae, (f) 10 dph larvae, (g) 12 dph larvae, (h) 15 dph larvae, (i) 20 dph larvae, (j) 27 dph larvae. Abbreviations: AB, air bladder; AI, anterior intestine; AN, anus; BP, buccopharynx; E, eye; GG, gastric gland; HG, hindgut; IN, incipient intestine; L, liver; MG, midgut; MS, mouth slit; N, notochord; OE, oesophagus; OG, oil globule; P, pancreas; PC, pyloric caeca; PI, posterior intestine; PS-, pyloric caeca, ST, stomach; YS, yolk sac.

In *L. lentjan*, pyloric caeca which increase the surface area and nutrient absorption in the gut were developed after 20 dph, later completely formed and visible by 27 dph (Figure 3j).

#### Liver and pancreas

Liver, and pancreas were not formed at hatching. Primordial liver and exocrine pancreas were observed between the developing digestive tract and yolk sac on 1 to 2 dph. On 2 dph, incipient liver was visible (Figure 2d). Both liver and pancreas tissues started to differentiate by 2 to 3 dph (Figure 2d and 2e), and both hepatocytes and pancreatic cells became easily distinguishable on 5 dph (Figure 3d). The hepatic sinusoids, with blood cells were visible at 5 dph and proliferated as the size, cell numbers and vacuolization of liver increased. On 10 dph, hepatocytes were spherical in shape and more defined, and their numbers increased by 12 dph (Figure 3g). On 15 dph, the liver enlarged with more hepatocytes and vacuoles for glycogen and lipid storage (Figure 3h) probably due to ingestion of *Artemia* by the larvae. After the initial feeding of larvae, the size of pancreas and the number of acini and zymogen granules in pancreas increased. From 10 to 12 dph, exocrine pancreas in the gut loop got diffused to abdominal cavity and the size of pancreas also increased as fish grew (Figure 3g).

#### Gut Content Analysis

We identified only algal concentration in the larval gut on 2 and 3 dph and complete absence of copepods (Figure 2d and 4a). On 4 dph onwards, algal mixture together with partially digested copepod nauplii were identified inside the gut of larvae (Figure 2e, 2f and 4b).

Both copepod and rotifer shell parts were identified from 7 to 8 dph onward. Digested materials of *Artemia* were identified on 15 dph in the gut of larvae.

#### Enzyme Development

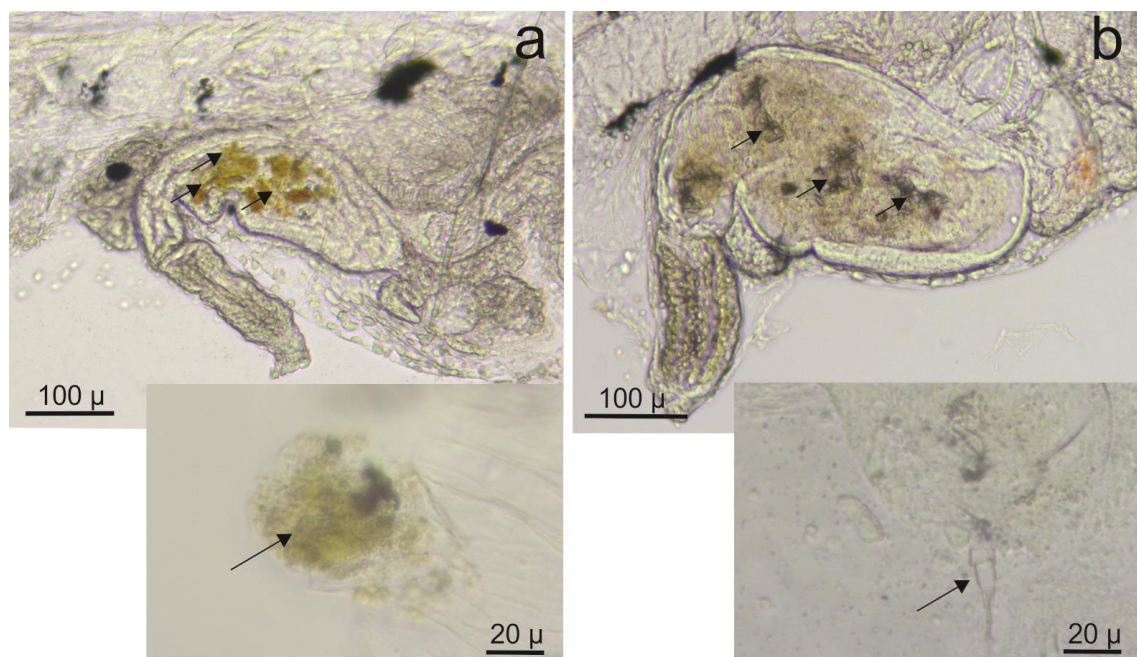
The specific digestive enzyme activity of amylase, protease, lipase, trypsin, chymotrypsin and pepsin were evaluated from 1 to 40 dph. Significant differences in digestive enzyme activity were detected among the sampling days.

#### Amylase

The specific activity of amylase was detected as early as hatching. At 1 dph, specific activity was  $0.079 \pm 0.009$  U mg<sup>-1</sup> protein, which gradually increased and peaked at 7 dph ( $P < 0.05$ ). After that it showed up and down trend till 40 dph ( $0.1 \pm 0.01$  U mg<sup>-1</sup> protein). However, there was no significant difference ( $P > 0.05$ ) observed between these two sampling days of 1 and 40 dph (Figure 5a).

#### Protease

Protease activity was observed well before exogenous feeding. On 1 dph, about  $2.65 \pm 0.7$  U mg<sup>-1</sup> protein of protease activity was detected and reached a peak ( $P < 0.05$ ) at 13 dph ( $19.958 \pm 0.66$  U mg<sup>-1</sup> protein). Afterwards, a sharp decline in activity was observed till 19 dph ( $6.436 \pm 1.558$  U mg<sup>-1</sup> protein), which was not significantly differed ( $P > 0.05$ ) from 1 dph (Figure 5b). After 19 dph, the specific activity showed an increasing trend, and by 40 dph the activity detected was around  $15.059 \pm 1.85$  U mg<sup>-1</sup> protein.



**Figure 4.** Gut content of *L. lentjan* larvae. (a) 2 dph larvae (arrow mark represents only algal matters), (b) 4 dph larvae (arrow mark represents remnants of copepods).

**Lipase**

Specific lipase activity on 1 dph was  $0.071 \pm 0.002$  U  $\text{mg}^{-1}$  protein. After a slight reduction on 5 dph, it increased steadily ( $P < 0.05$ ) till 13 dph ( $0.16 \pm 0.014$  U  $\text{mg}^{-1}$  protein), which then reduced slightly and remained the same ( $P > 0.05$ ) till the end of the experiment (Figure 5c).

**Trypsin**

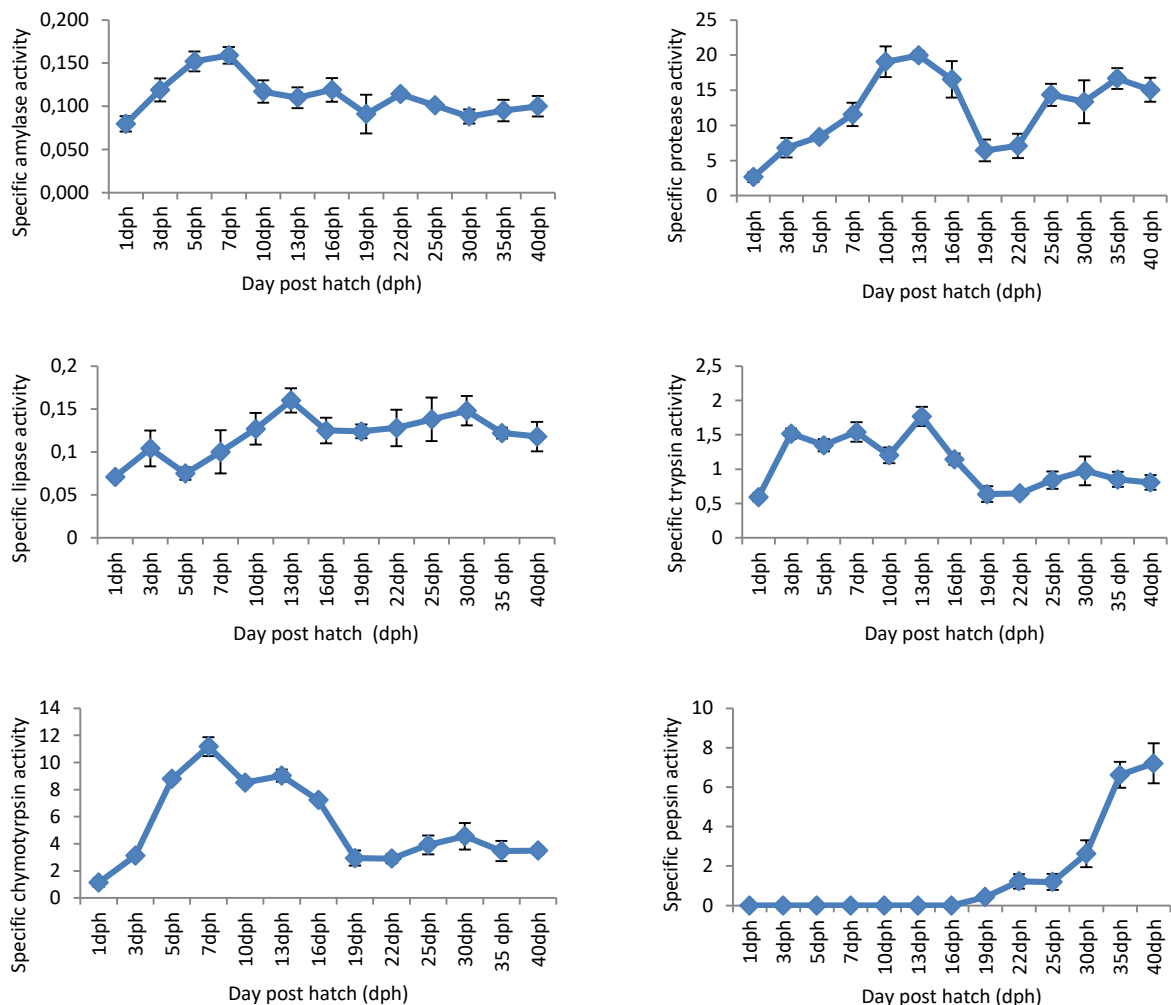
The specific trypsin activity on 1 dph was  $0.591 \pm 0.057$  U  $\text{mg}^{-1}$  protein, which significantly increased to  $1.516 \pm 0.076$  U  $\text{mg}^{-1}$  protein on 3 dph and remained constant and peaked at 13 dph ( $1.767 \pm 0.139$  U  $\text{mg}^{-1}$  protein). Rapid decline was observed as days progressed, and the lowest value was detected on 19 dph ( $0.636 \pm 0.114$  U  $\text{mg}^{-1}$  protein), which was similar to 1 dph. No significant differences ( $P > 0.05$ ) of trypsin activity was observed from 19 dph to 40 dph of larval rearing (Figure 5d).

**Chymotrypsin**

The specific activity of chymotrypsin is similar to trypsin. A sharp increase was detected from 1 dph ( $1.138 \pm 0.33$  U  $\text{mg}^{-1}$  protein) to 7 dph ( $11.177 \pm 0.70$  U  $\text{mg}^{-1}$  protein), which was significantly higher ( $P < 0.05$ ) than all other sampling days. Then, a declining trend was observed till 22 dph and the activity slightly increased after 22 dph and the activity slightly increased after 22 dph. There was no significant differences ( $P > 0.05$ ) from 19 dph to 40 dph (Figure 5e).

**Pepsin**

Pepsin specific activity was detected for the first time on 19 dph ( $0.425 \pm 0.062$  U  $\text{mg}^{-1}$  protein) and there was no significance difference ( $P > 0.05$ ) in its specific activity till 25 dph. After that, a sudden increase was noted and higher specific activity values were detected on 35 and 40 dph, which were significantly higher ( $P < 0.05$ ) than other sampling days (Figure 5f).



**Figure 5.** The specific activity of digestive enzymes of *L. lentjan* larvae. (a) amylase, (b) protease, (c) lipase, (d) trypsin, (e) chymotrypsin, (f) pepsin. Values in mU  $\text{mg}^{-1}$  protein from 1 to 40 days post hatch (dph). Values are mean  $\pm$  SE with the same superscript letter is not significantly different in multiple comparisons ( $P < 0.05$ ).



## Discussion

### Development of Digestive System in *L. lentjan*

Like most of the marine teleost hatchlings, *L. lentjan* had an undifferentiated straight digestive tract on the dorsal side of the yolk sac. The yolk sac absorption was completed by 2 to 3 dph followed by mouth and anus opening on 2 dph. Similar observations were reported in other marine species like tiger grouper (*Mycteroperca tigris*), humpback grouper (*Cromileptes altivelis*), Dover sole (*Solea solea*) and crimson snapper (*Lutjanus erythropterus*) (Aiemsomboon et al., 2017; Abol-Munafi et al., 2011; Boulhic and Gabaudan, 1992; Chen et al., 2006; Cui et al., 2017), but a longer endogenous nutrition of 4 days was observed in sparid fish of *Dentex dentex* (Santamaria et al., 2004). Similar to most marine fishes, mouth opening and eye pigmentation were observed within a few days of hatching in *L. lentjan* (Micale et al., 2006; Kvenseth et al., 1996; Kawamura et al., 2003).

The oesophagus appeared as a simple tube at hatchling, which then developed into an irregular lumen with a short and narrow duct. Incipient stomach appeared at 2 dph, which then elongated from 7 dph. The cardiac and pyloric stomach were evident in *L. lentjan* by 12 dph. A more or less similar development was reported in sea bass larvae, in which the differentiation of oesophagus and stomach was not evident till 7 dph and development of stomach was nearly completed at 15 dph (Tan-Tue, 1976; Walford and Lam, 1993) and in crimson snapper (*L. erythropterus*) stomach differentiation was observed between 16 and 17 dph (Cui et al., 2017).

The formation of gastric glands widely varies among species. Gastric gland forms earlier on 11 dph in Pacific bluefin tuna (Kaji et al., 1996), on 12 dph in yellowtail amberjack (Umeda et al., 1987), on 14 to 15 dph in the yellowtail and crimson snapper (Chen et al., 2006; Cui et al., 2017). In groupers, gastric glands are usually detected at 20-30 dph of larval rearing (Chen et al., 2006; Abol-Munafiet et al., 2011; Kato et al., 2004; Aiemsomboon et al., 2017). However, in gilthead seabream, gastric gland formation completed on 60 dph (Elbal et al., 2004). But *L. lentjan* larvae, stomach differentiation and gastric gland formation occurred in between 12 and 15 dph. According to Strobant and Dabrowski, (1981) and Tanaka (1971), gastric gland formation marked the functional stomach development, which begins at the juvenile stage. This indicate the improvement in extracellular protein digestion of the larvae (Chen et al., 2006; Qu et al., 2012). Formation of gastric gland, fundic stomach, and pyloric caeca in grouper species, considered as the suitable time for weaning (Abol-Munafiet et al., 2011; Kato et al., 2014; Aiemsomboon et al., 2017).

Narrow straight intestine in hatchling, differentiated to straight anterior intestine and L-shaped posterior intestine at 1 dph. Structural changes

of the intestine with the well-developed rectal valve was observed after 5 dph in *L. lentjan*. In most of the marine fish larvae, undifferentiated narrow straight intestine was observed during hatching. The first evident sign of lipid absorption observed after 5 dph in *L. lentjan*. Related to this, lipid digestion starts with the appearance of the lipid vacuoles in the intestine at 5 dph in tiger grouper (*M. tigris*) and orange spotted grouper (*E. coioides*) (Aiemsomboon et al., 2017; Qu et al., 2012). The last significant changes in the digestive system of fishes are pyloric caeca development, which increase the surface area and nutrients uptake in fish (Bisbal and Bengston, 1995; Hamlin et al., 2000; Buddington and Diamond, 1986). In *L. lentjan*, pyloric caeca start appeared from 20 dph and completely developed by 27 dph. Its development varies between species and in *Epinephelus fuscogutatus* pyloric caeca has appeared from 15 dph onwards and completely developed in 24 dph (Aiemsomboon et al., 2017), but in *Epinephelus bruneus*, pyloric caeca appeared on 24 dph. Caecal development in yellowtail flounder was on 29 dph (Baglole et al., 1997). Some studies indicated that the best time of weaning should coincide with the formation of the gastric gland and pyloric caeca (Chen et al., 2006; Qu et al., 2012; Segner et al., 1993).

Between 1 and 2 dph, the primordial liver and exocrine pancreas were developed. By 2 to 3 dph, both liver and pancreas started to differentiate with clearly distinguishable hepatocytes and pancreatic cells. The liver and pancreas appeared within 3 dph in *E. bruneus* (Kato et al., 2004) and in 2 dph in humpback grouper (Abol-Munafi et al., 2011). This early functioning of the liver and pancreas at yolk sac absorption would help the larvae to ingest and assimilate first exogenous food efficiently (Micale et al., 2006; Qu et al., 2012). On 15 dph, the liver enlarged, and the hepatocytes number increased rapidly. Increase in the size of pancreas after first feeding was observed, and similar reports of an increase in hepatocytes number and pancreas size were reported in yellowtail king fish (*Seriola lalandi*) larvae (Chen et al., 2006).

### Gut Content Analysis

The gut content of 2 dph larvae (after mouth opening) revealed the presence of only algal matter. In earlier study, only algal matter was reported in the gut of *L. lentjan* till 7 dph (Gomathi et al., 2021), but in the present study, the gut had a mixture of algae and copepod nauplii on 4 dph. The yolk exhausted completely on 2 dph, and the oil globule completely absorbed on 3 dph (Gomathi et al., 2021). Larvae didn't survive when fed with only rotifer in green water system. So the present study confirmed the first feeding is a mixture of algae and copepod nauplii that only helps the larvae to survive. The use of microalgae (green water technique) during the first-feeding process was believed to improve the nutritional conditions of the larvae, either directly (Moffatt, 1981) or indirectly

through improving the nutritional value of the rotifers (Howell, 1979; Scott and Middleton, 1979; Lubzens, 1987) and also by improving the water quality (Palmer et al., 2007, Brown and Blackburn, 2013). Small size and superior nutritional value of copepods with high PUFAs, free amino acids and antioxidant pigments (Coneicao et al., 2010) may be the reason for better feed acceptability and high survival compared to first feeding with rotifer. In later stages of larval rearing, rotifer and digested material of *Artemia* were identified. Compound feed was only taken after 20 dph even though we started giving it from 16 dph. Mortality was noticed in early weaning. This also confirms the weaning of larvae can be done only after 19-20 dph. Weaning time of larvae to artificial feed is important for better survival, which have been studied in several species like golden pompano (Ma et al., 2015), flounders (Orihuela et al., 2018; Geng et al., 2019) and maraena whitefish (Stejskal et al., 2021).

### Digestive Enzyme Development

Amylase, lipase, trypsin and chymotrypsin activity were detected before the onset of exogenous feeding in *L. lentjan*. Similarly, most of the digestive enzyme activity was detected before mouth opening and beginning of exogenous feeding, which indicate a precocious development of the digestive system as described in many other fishes (Faluk et al., 2007; Infante and Cahu, 2001; Oozeki and Bailey, 1995; Martinez et al., 1999; Ribeiro et al., 1999; Hoenhe-Reitan et al., 2001; Cara et al., 2003; Ma et al., 2005; Galaviz et al., 2006; Bolasina et al., 2006; Chen et al., 2006).

Detection of amylase activity in *L. lentjan* at early larval stage may be due to algal uptake of larvae. Similarly, the amylase activity has been detected in several marine species including *Dicentrarchus labrax* (Cahu and Zambonino- Infante, 1995), *S. aurata* (Moyano et al., 1996) and *S. senegalensis* (Martinez et al., 1999). The decrease in amylase activity after hatching could be due to the low carbohydrate content of live feeds (Cara et al., 2003). In later stages of development it may be influenced by carbohydrates in compound diets (Henning et al., 1994; Peres et al., 1998; Cara et al., 2003). Similar to this, variation in amylase activity with change in live feed have been reported in other marine species of yellow croaker (Ma et al., 2005), red sea bream (Khoa et al., 2019) and yellow sea bream (Morshedi et al., 2021).

Protease activity showed a marked increase after exogenous feeding and reached the maximum at 13 dph; after that sharp decrease was observed till 19 dph. A sudden increase in trypsin activity was recorded from 3 dph which peaked at 13 dph, and similarly, the specific activity of chymotrypsin increased after mouth opening to a maximum at 7 dph. The slight increase in proteolytic enzymes of trypsin and chymotrypsin may be due to initial ingestion of exogenous food by larvae (Ribeiro et

al., 1999; Lazo et al., 2000; Alvarez-Gonzalez et al., 2006). A declining and increasing trend of trypsin and chymotrypsin activity was observed as days progressed in *L. lentjan*. According to Infante and Cahu, (2001), the changes in trypsin and chymotrypsin pattern could be genetically controlled.

In *L. lentjan*, the specific lipase activity was detected in hatchling; after initial ups and down, it steadily increased till 13 dph; later on, a slight decline in the activity was seen. Lipase activity in the early stage is essential for proper larval development because lipids are energy sources during the endogenous feeding period in most of the marine species (Sargent et al., 1989; Sargent, 1999; Rainuzzo et al., 1997; Tocher and sergeant, 1984). Oozeki and Bailey, (1995) suggested the existence of two types of lipase, one in early yolk sac absorption and other in later stages of exogenous lipids digestion.

Pepsin specific activity was not detected till 19 dph. Like in other species, pepsin activity was detected just before metamorphosis with the formation of gastric gland and functional stomach and peaked at 40 dph (Segner et al., 1993; Zacarias et al., 2006; Lazo et al., 2007; Infante and Cahu, 2001; Walford and Lam, 1993). Pepsin activity was detected on 30 dph in *Pagrus pagrus* (Darias et al., 2007) and 41 dph in *Epinephelus coioides* (Feng et al., 2008). So in *L. lentjan*, enzymes activities of amylase, lipase, trypsin and chymotrypsin were detected before the onset of exogenous feeding and pepsin was detected from 19 dph onwards. The variations observed in the digestive enzyme activity can be correlated with the development of functional stomach, gastric glands and pyloric caeca, or may be due to a change in diet pattern. According to Khoa et al. (2021), the most of the digestive enzyme secretion is genetically programmed except pepsin production which coincide with the development of gastric gland in Japanese flounder. Further studies are needed in *L. lentjan* to detect the selective digestive enzyme gene expression by molecular approaches using specific primers.

### Conclusion

According to the present study, the ontogenetic development of the digestive system of *L. lentjan* can be divided into three major stages: first stage is yolk sac larvae (0 to 2 dph), second stage from exogenous feeding to the formation of the gastric gland (3 to 15 dph) and third stage till the completion of metamorphosis (16 to 35 dph). The enzymes activities of amylase, lipase, trypsin and chymotrypsin were detected before the onset of exogenous feeding and pepsin was detected from 19 dph in *L. lentjan*. So considering both ontogenic development and enzyme activity, weaning of *L. lentjan* can be practiced after 19-20 dph coinciding with pyloric caeca development (transition to juvenile mode of digestion) and pepsin production. By 30-35 dph, coinciding with

metamorphosis, the entire digestive system developed to accept artificial feeds. Further studies can be done by molecular techniques to detect the expression of the coding gene for different enzymes by using specific primers to supplement the present investigation.

### Ethical Statement

The authors followed all applicable international, national, and/or institutional guidelines for the care and use of animals.

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### Author Contribution

Siju. R conceived the study and wrote the manuscript, Anil M.K. has designed the rearing system (RAS) for broodstock development, Paramita Banerjee Sawant assisted in the histology part, Babitha Rani analysed the data and Ambarish P.Gop has maintained the broodstock of fishes. Gomathi. P and Surya. S assisted in larval rearing part and enzyme analysis of the study. All the authors read and approved the final manuscript.

### Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper

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