Larval rearing and ontogeny of digestive enzyme activities in yellowfin seabream (*Acanthopagrus latus*, Houttuyn 1782)

Vahid Morshedi[1*], Shirin Hamedi[1], Fatemeh Pourkhazaei[2], Mansour Torfi Mozanzadeh[3*], Rezvan Tamadoni[4], Mina Ebadi[4], Afsane Esmaili[4], Maryam Azodi [1], Enric Gisbert[5]

[1] Department of Fisheries and Biology, Persian Gulf Research Institute, Persian Gulf University, Bushehr, Iran

[2] Department of Natural Resources, Isfahan University of Technology, Isfahan, Iran

[3] South Iran Aquaculture Research Centre, Iranian Fisheries Science Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Ahwaz, Iran

[4]Department of Fisheries and Aquaculture, Faculty of Marine Science and Technology, Persian Gulf University, 7516913798, Bushehr, Iran

[5] IRTA, Centre de Sant Carles de la Rápita (IRTA-SCR), Aquaculture program, Crta. del Poble Nou Km 5.5, 43540 Sant Carles de la Rápita, Spain

Running title: Ontogeny of digestive enzymes in yellowfin seabream larvae

*Both authors equally contributed to the study: v.morshedi@pgu.ac.ir; v.morshedi@gmail.com, mansour.torfi@gmail.com

Abstract

The present research was conducted to provide insight into digestive larval capacity in *Acanthopagrus latus* larvae from hatching up to 30 days after hatching (DAH). Newly hatched larvae were stocked into six 300-L cylindrical polyethylene tanks at a density of larvae 50 larvae/L and reared by means of the green water system using *Nannochloropsis oculata* (0.5×10^6/mL). After mouth opening, larvae were fed with rotifers (5-16 individual/mL) from 2 to 20 DAH; then, *Artemia* nauplii (0.5-3.0 individuals/mL) were offered to larvae from 18 to 30 DAH, meanwhile a commercial microdiet was offered to larvae from 25 to 30 DAH. Larval performance in terms of growth and survival, and the assessment of the activity of selected digestive enzymes ontogeny of digestive enzymes activities was evaluated in larvae sampled at 0 (hatching), 7, 15, 22 and 30 DAH. Larvae showed an exponential growth characterized by two different growth stanzas, a first one characterized by slow growth rates comprised between hatching to 15 DAH (4.7 ± 0.2 mm), followed by a period of faster growth rates between 16 to 30 DAH (7.5 ± 0.6 mm). The activities of the brush border (alkaline phosphatase, ALP) and cytosolic (leucine-alanine peptidase, LAP) enzymes, as well as those of the pancreatic ones like total alkaline proteases, bile salt-activated lipase and α-amylase were detected from the mouth opening stage. Total activities of pancreatic and gastric enzymes increased with larval growth showing an enhancement of digestive capacities with larval age and size. The intestinal maturation in *A. latus* as assessed by the ratio of AP to LAP did not occur as expected by end of the first month of life suggesting the complete establishment of digestive luminal processes may take place at older ages. This study related to the growth patterns and ontogenic changes in activity of pancreatic, gastric and intestinal enzymes in *A. latus* and their nutritional regulation may be considered as the first step for improving the larviculture, as well as assessing and refining the nutritional requirements during the larval and early juvenile stages of this sparid species.

Keywords: Alkaline phosphatase, cytosolic enzymes, marine fish larvae, pancreatic enzymes, pepsin, yellowfin seabream.

1. Introduction

Larviculture of marine fish species is considered as the main bottleneck for their successful propagation due to the low larval survival rates (Gisbert et al., 2008; Mai et al., 2005; Morioka et al., 2010; Rotenstein et al., 2009). There are several critical periods during the development of marine fish larvae. For example, the transition from endogenous to exogenous feeding at early larval development, as well as weaning from live food to compound feeds are considered two of the main critical periods of fish larviculture, which may result in mass mortality or cause deformities as a consequence of nutritional deficiencies or unsuitable zootechnical rearing conditions (Hamre et al., 2013). Furthermore, it has been proved that the production of high-quality marine fish larvae largely depends on their nutritional condition (Alvarez-González et al., 2010). Thus, the knowledge of ontogeny and development of the gastrointestinal tract of a new candidate species for aquaculture can provide valuable data about its digestion capacity, nutritional requirements and recognition of an appropriate time for proper nutrition and weaning strategies (Gisbert et al., 2008; 2009). This information can also be applied for the formulation of microdiets according to the nutritional needs and digestive capacity of marine fish larvae as well as for establishing feeding protocols that eventually improve larval mass production in hatcheries (Kolkovski, 2001; Faulk et al., 2007; Hamre et al., 2013). Regardless of species-specific differences in terms of biological and ecological guilds, a plethora of studies focused on studying larval digestive physiology have indicated a similar pattern with regard to changes of digestive enzyme activities during their early larval stages and their dietary modulation (Rønnestad et al., 2013; Yúfera et al., 2018). Generally, at early life stages, pancreatic enzymes and especially alkaline proteases (*e.g.* trypsin and chymotrypsin) produced by the exocrine pancreas along with cytosolic enzymes (*e.g.* leucine-alanine peptidase) in enterocytes are the main proteolytic enzymes involved in protein digestion due to the lack of a functional stomach (Zambonino-Infante & Cahu 2001; Rønnestad et al., 2013). In addition, the brush

border (BB) membrane of enterocytes develops during larval ontogeny, which enhances the activities of BB-associated enzymes (*e.g.* alkaline phosphatase, aminopeptidase-N and maltase) (Zambonino-Infante and Cahu, 2001). Towards larval metamorphosis, the stomach becomes functional and acid digestion increases larval capability to digest more complex proteins (Zambonino-Infante and Cahu, 2001, Rønnestad et al., 2013; Yúfera et al., 2018). This change in digestive mechanism has been empirically considered the proper time for weaning early juveniles onto dry microdiets, since it is associated to digestive maturation).

Yellowfin seabream (*Acanthopagrus latus*) is an important carnivorous sparid species from the coasts of Japan, southern Korea, Taiwan, China, northern Vietnam and the Indo-West Pacific Ocean. This species has several characteristics that makes it a preferable candidate for mariculture such as spawning in captivity, wide tolerance to rearing conditions, especially high water temperatures, reasonable growth rate and good feed conversion ratio (Karimi et al., 2013). The dietary macronutrients (*i.e.* energy, protein, lipid, carbohydrate, protein to energy and lipid to carbohydrate ratios) requirements also determined and reported for grow-out phase of this species (Mozanzadeh et al., 2017). However, the mass production of yellowfin seabream fry still has some bottlenecks such as low survival rates and cannibalistic behavior during larval stages, which jeopardizes the expansion of its commercial production. Feeding practices of *A. latus* larviculture is mainly based on the use conventional live preys, including rotifers and *Artemia* nauplii and metanauplii. However, the nutritional composition of live food can vary and it may not meet all nutritional requirements of larvae due to some deficiencies on important nutrients like long-chain polyunsaturated fatty acids, phospholipids, water-soluble nutrients, vitamins, trace elements and free amino acids (Hamre et al., 2013). Thus, the enrichment of live preys is necessary for meeting larval nutritional requirements (Kandathil Radhakrishnan et al., 2020). Previous studies demonstrated anatomical changes during larval stages of *A. latus* (Akazaki and Tokito, 1982; Abol-Munafi and Umeda, 1993;

Sarvi et al., 2019) conducted basic studies on the differentiation of the gastrointestinal tract of the *A. latus* by histological and microscopic studies; that, their result with our study about digestive enzymes provide good results in the field of larval rearing of this fish. In this sense, the knowledge of changes in digestive enzyme activities and morphological development of the alimentary tract along larval ontogeny is of importance. Therefore, the present study aimed to determine the ontogeny of several digestive enzymes in *A. latus* from hatching to 30 days after hatching (DAH) reared at 23 ºC.

2. Materials and methods

2.1. Rearing conditions

The present research was carried out at the laboratory of the Aquatic Research, Persian Gulf University, Bushehr, Iran. Larvae used in the present study were obtained from a group of wild-caught *A. latus* broodfish (n = 14 males, average body weight = 0.25 kg; n = 28 females, average body weight = 0.45 kg) that were stocked in a 4000-L circular fiberglass tank in a flow-through system (5 L/min), supplied sand-filtered, chlorinated and UV-treated seawater. Water temperature was 20°C and the photoperiod was set up at 12 h light (L): 12 h dark (D). Broodfish were fed with chopped squid and fish meat every day. Fish naturally spawned (February, 2018) and floating eggs were collected with a funnel net (200 µm). Fertilized eggs were transferred into 300-L cylindroconical polyethylene tanks filled with 220 L of seawater at a density of 100 eggs/L. Incubation tanks were equipped with air stones for providing gentle aeration during the incubation period. The percentage of egg fertilization and hatching rates were *ca.* 70 and 85%, respectively. After 36 h, newly hatched larvae were stocked into six 300-L cylindrical polyethylene tanks that filled with 100 L of seawater. The initial density of larvae was 50 larvae/L, and the number of larvae in each replicate (tank) was about 5,000.

The green water system was used for *A. latus* larviculture by daily addition of microalgae (*Nannochloropsis oculata*, 0.5×10^6/mL) into water. After mouth opening at 2 days after hatching (DAH) until 20 DAH, larvae were fed with rotifer (*Brachionus rotundiformis*) at increasing densities (5-10 rotifers/mL from 2 to 10 DAH and 10-16 rotiters/mL from 11 to 20 DAH). *Artemia* nauplii (*Artemia franciscana*) were offered to larvae from 18 to 30 DAH at a density of 0.5-3.0 individuals/mL, meanwhile formulated diet was offered to larvae from 25 to 30 DAH (GemmaMicro®, Skretting; Netherlands, particle size: 200–300 μm. Rpximate composition: 55% crude proteins, 15% crude lipids, 13.5% ash and 5% fiber) was supplied until the end of experiment. Water exchange in the rearing tanks began at 7 DAH and gradually increased (*ca.* 10% in each day) up to the end of the husbandry trial (ca. 100% in each day). The bottom of rearing the tanks was siphoned daily from 7 DAH to remove dead larvae and wastes. Water temperature (23.0 ± 1.0 ºC), dissolved oxygen (6.8 ± 0.5 mg/L), pH (7.9 ± 0.2) and salinity (40.0 ± 1.0 ppt) were measured two times daily along the trial.

2.2. Sampling

For evaluating changes in digestive enzyme activities along larval ontogeny, larvae were randomly sampled from the rearing tanks at 0 (hatching), 7, 15, 22 and 30 DAH. Sampling took place in the early morning before the addition of live prey into the tanks in order to minimize the potential effects of exogenous enzymes from undigested prey in larval fish gut. Different quantities of larvae, from 1,000 larvae at hatching to 20 individuals at 30 DAH (number of samples specimens needed for reaching a minimum body mass of 150-200 mg for biochemical enzyme quantification), were collected with a net (100 μm of mesh size). Larvae were then euthanized with 2-phenoxyethanol (300 ppm), rinsed with distilled water and kept in a −80 °C for further analyses. Additionally, for evaluating growth performance in terms of total length (TL) and wet weight (WW), 20 larvae of each tank were caught at 0 (hatching), 7,

15, 22 and 30 DAH, anaesthetized with 2-phenoxyethanol and fixed in 4% neutral buffered formalin. Total length was measured by a stereomicroscope (Leica, M50, Bloomington, United States) equipped with a versatile digital microscope camera (Dino-Lite, AM4113T-1.3MP, Almere, Netherland), followed by DinoCapture 2.0 software (version 1.4.5.B) using an image analyzing system with 0.001 mm precision. Larval WW were determined with a digital balance (Sartorius, Gottingen, Germany; precision of 0.01 mg). Larval specific growth rate (SGR) and survival rate were calculated using the following standard equations: SGR (% WW/day) = [(ln final WW – ln initial WW)/30] × 100, survival (%) = [(number of larvae at 30 DAH – number of sampled larvae)/initial number of larvae)] × 100.

2.3. Quantification of digestive enzyme activities

For the determination of digestive enzyme activities, the whole body of larvae younger than 30 DAH was completely homogenized, but the gut of larvae aged 30 DAH was dissected on a precooled glass plate maintained at 0-4 °C. Samples were homogenized for 30s in 30 volumes (v/w) of ice-cold Tris-Mannitol buffer (50 mM Mannitol, 2 mM Tris-HCl; pH 7.5). After homogenization, 0.1 M $CaCl_2$ was added to the homogenate and the extract was subjected to sonication for 60 s. The homogenized samples were centrifuged at 9,000 x *g* (10 min at 4 °C) and the supernatant was extracted. One mL of the supernantant was used for quantification of pancreatic enzymes. Then, the supernatant was centrifuged at 34,000 x *g* (30 min at 4 °C) and the pellet, containing the brush border (BB) of enterocytes, was re-suspended in 1 mL of buffer (0.1 M KCl, 5mM Tris-Hepes, 1mM DTT; pH 7.5) and kept at −80 °C (Gisbert et al., 2018). Total alkaline proteases (TAP) were assayed according to García-Carreño and Haard (1993) using azo-casein (catalogue number, CN: A2765; Sigma-Aldrich, Spain) as substrate and 50 mM Tris-HCl (pH 9.0) as buffer. In brief, 20 µL of enzyme extract was incubated with 500 µL substrate for 10 min at 25 °C, the reaction was

stopped with 500 μL 20% TCA (trichloroacetic acid) and centrifuged at 10,000 x *g* for 5 min and absorbance of the supernatant was measured at λ = 366 nm. Leucine-alanine peptidase (LAP) was performed using leucine-alanine (CN: L9250, Sigma-Aldrich) as substrate in 50mM Tris-HCl buffer (pH 8.0); one unit of enzyme activity (U) was defined as 1 nmol of the hydrolyzed substrate per min and ml of homogenate at 25 °C and measured at λ = 530 nm (Nicholson and Kim, 1975). Alkaline phosphatase (AP) activity was quantified using PNPP (4-nitrophenyl phosphate; CN: 34045, Thermo Scientific™, Spain) as substrate in 30mM Na2CO3 buffer (pH 9.8); one unit of enzyme activity (U) was defined as 1 μg nitrophenol released per min and ml of BB homogenate at 25 °C and measured at λ = 407 nm (Bessey et al., 1946). Activity of total acid proteases activity was determined using hemoglobin (CN: H4131, Sigma-Aldrich) as substrate (Worthington, 1991). In brief, the extract was mixed with the substrate (2% hemoglobin solution in 0.3 N HCl, pH 2.0) and incubated for 10 min. The reaction was stopped with 5% trichloroacetic acid and assay tubes were centrifuged at 4,000 x *g* for 6 min at 4 °C. The absorbance of the supernatant was read at λ =280 nm. One unit of pepsin was defined as the μg of tyrosine released at 25 °C per min and mL. The activity of bile salt-activated lipase was measured using 4 p-nitrophenyl myristate (CN: 70124, Sigma-Aldrich) as substrate dissolved in 0.25mM Tris-HCl (pH 9.0), 0.25mM 2-methoxyethanol and 5mM sodium cholate buffer. The reaction was stopped with a mixture of acetone:n-heptane (5:2), the extract centrifuged (6,080 x *g*, 2 min at 4 °C) and the absorbance of the supernatant read at room temperature at λ = 405 nm (Ijima et al., 1995). Bile salt-activated lipase activity was defined as the μmol of substrate hydrolyzed per min and mL of homogenate. The activity of α-amylase activity was evaluated at λ = 580 nm using soluble starch (0.3%) (CN: 03967, Sigma-Aldrich) dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate (Metais and Bieth, 1968), and its activity was defined as the mg of starch hydrolyzed during 30 min per mL of homogenate at 25 °C. All spectrophotometric analyses were performed as

recommended by Solovyev and Gisbert (2016) in order to prevent sample deterioration. Enzymatic activities were measured using a microplate scanning spectrophotometer (Synergy HT, Bio-Tech, Germany) and expressed as specific (U/mg protein) and total (U/larva) enzyme activities. Soluble protein in enzyme extracts was quantified using the Bradford method using bovine serum albumin as a standard (Bradford, 1976). All the assays were made in triplicate (methodological replicates).

2.4. Statistics

In this study, all the data are presented as means ± standard error of the mean calculated from six replicates (n = 6). Changes in activity of digestive enzymes along larval development were assessed by means of a one-way ANOVA after confirming the normality (Kolmogorov-Smirnov test) and homogeneity of variance (Leven's test) of all data. When significant differences were found between sampling times, the Tukey's post hoc test was run. The level of significance considered was $P < 0.05$. All statistical methods were conducted using SPSS ver.22.0 (IBM, USA).

3. Results

3.1. *Growth*

Growth of *A. latus* in WW and TL was exponential from hatching until 30 DAH, following these regression equations: $WW = 0.1804\ e^{0.1156\ DAH}$ ($R^2 = 0.99$) and $TL = 2.3536\ e^{0.0416\ DAH}$ ($R^2 = 0.96$) (Fig. 1). Notably, the mean larval WW (mg) increased from 0.17 ± 0.02 mg (newly hatched larvae) to 5.92 ± 0.3 mg (30 DAH) and the mean larval TL (mm) elevated from 2.6 ± 0.04 mm (newly hatched larvae) to 7.46 ± 0.6 mm (30 DAH). As shown in Figure 1, growth in WW could be depicted into two phases; a first one comprised between hatching (0.17 ± 0.00 mg) and 15 DAH (0.77 ± 0.21 mg) characterized by a progressive but slight

growth in WW, and a second phase characterized by marked increase in WW to the end of the experiment at 30 DAH. The SGR and the survival rates were estimated at 11.8 ± 1.0 7% and 49.9 ± 0.4 %, respectively.

3.2. *Digestive enzyme activities*

The specific activity of TAP sharply increased from 33.23 ± 7.39 mU/mg protein to 625.83 ± 43.34 mU/mg protein at 7 DAH then it gradually decreased to 145.81 ± 6.39 mU/mg protein at the end of the trial. The total activity of TAP was detected at hatching day (4.31 ± 0.21 µU/larvae) and it gradually increased to 7 DAH (44.74 ± 5.51 µU/larvae) (Fig. 2a). Then, it sharply elevated to 351.40 ± 8.38 µU/larvae at 15 DAH and it continuously increased about three times to 1,013.81 ± 41.58 µU/larvae at the end of the trial ($P < 0.05$).

The specific activity of LAP elevated pronouncedly from the hatching day (861.56 ± 25.78U/mg protein) and to reach a maximum level at 7 DAH (3788.39 ± 673.33 U/mg protein), then its activity sharply dropped at 30 DAH (702.19 ± 21.85 U/mg protein) ($P < 0.05$) (Fig. 2b). Total activity of LAP gradually increased from hatching day (114.48 ± 15.80 mU/larvae) to 7 DAH (259.83 ± 4.75 mU/larvae), then its activity sharply elevated from 1737.66 ± 14.74 mU/larvae at 15 DAH to 4882.66 ± 138.13 mU/larvae at 30 DAH ($P < 0.05$).

Regarding intestinal enzymes, the specific activity of AP was detected at hatching day (0.14 ± 0.05 U/mg protein; 2.6 ± 0.04 mm TL) and its activity sharply increased up to 7 DAH (3.51 ± 0.1 mm TL) when it reached a maximum value of 0.88 ± 0.16 U/mg protein ($P < 0.05$). Afterwards, the specific activity of AP decreased to the minimum level (0.01 ± 0.00 U/mg protein) at 30 DAH (7.46 ± 0.6 mm TL) (Fig. 2c). Total activity of AP gradually increased from (0.75 ± 0.00 µU/larvae) in newly hatched larvae to 7 DAH (15.96 ± 2.71 µU/ larvae) ($P < 0.05$), then its activity sharply peaked at 15 DAH (4.72 ± 0.2 mm TL) (92.66 ± 17.51 µU/larvae). From 15 DAH to the end of the experiment total activity of AP remarkably

decreased to 57.29 ± 16.23 µU/larvae and 24.18 ± 0.89 µU/larvae at 22 (6.12 ± 0.3 mm TL) and 30 DAH, respectively ($P < 0.05$). The ratio AP to LAP activities was low in newly hatched larvae, then it pronouncedly increased at 7 DAH, but it gradually decreased up to 30 DAH (Fig. 2d).

The specific activity of acid proteases was detected at hatching day (0.15 ± 0.04 mU/mg protein) and it's dramatically increased at 7 DAH (1.54 ± 0.07 mU mg/ protein) (Fig. 2e). Afterward, the activity decreased sharply from 7 to 22 DAH (0.16 ± 0.06 mU mg/protein), then it increased again at 30 DAH (0.28 ± 0.03 mU mg/protein). Total activity of acid proteases showed fluctuations with two peaks at 15 DAH (1.04 ± 0.16 µU/larvae) and 30 DAH (1.88 ± 0.09 µU/larvae). Its total activity increased from hatching day (0.02 ± 0.008 µU/larvae) up to 15 DHA, then it gradually decreased at 22 DHA (0.25 ± 0.01 µU/larvae) and pronouncedly increased again at 30 DAH ($P < 0.05$).

The specific activity of bile-salt dependent lipase was detected from hatching day (0.21 ± 0.01 U/mg protein) and showed a bimodal shape in activity with two peaks at 7 DAH (3.10 ± 1.3 U/mg protein) and 22 DAH (2.06 ± 0.20 U/mg protein) and finally dropped to 0.66 ± 0.18 U/mg protein at 30 DAH ($P < 0.05$) (Fig. 2f). However, these patterns were not statistically significant when expressed in terms of specific activity. Total activity of bile-salt dependent lipase increased from 0.02 ± 0.00 (mU/larvae) in newly hatched larvae to 1.32 ± 0.46 mU/larvae at 15 DAH, then it decreased to 0.74 ± 0.09 mU/larvae at 22 DAH and at the end of the experiment it remarkably increased to 3.81 ± 0.26 mU/larvae at 30 DAH ($P < 0.05$).

The specific activity of α-amylase showed an increase from 7.42 ± 1.04 U/mg protein at hatching day (2.6 ± 0.04 mm TL) to 71.62 ± 23.78 U/mg protein at 7 DAH (3.51 ± 0.1 mm TL) (Fig. 2g). Its activity strikingly decreased at 15 DAH (4.72 ± 0.2 mm TL) (21.37± 1.95 U/mg protein); then, it increased about two times at 22 DAH (6.12 ± 0.3 mm TL) (44.88 ±

4.79 U/mg protein), and eventually dropped to 20.48 ± 1.66 U/mg protein at 30 DAH (7.46 ± 0.6 mm TL). Total activity of α-amylase gradually increased from 0.95 ± 0.02 mU/larvae at hatching day to 20.0 ± 1.82 mU/larvae at 15 DAH, then it pronouncedly increased to 72.78 ± 10.02 and 142.56 ± 11.98 m U/larva at 22 and 30 DAH, respectively ($P < 0.05$).

4. Discussion

4.1. Growth and survival

Growth of *A. latus* larvae had a similar pattern of exponential growth as reported in other sparid species such as common pandora (*Pagellus erythrinus,* Suzer et al., 2006), red seabream (*Pagrus pagrus,* Suzer et al., 2007b), blackspot seabream (*Pagellus bogaraveo,* Ribeiro et al., 2008), common dentex (*Dentex dentex,* Gisbert et al., 2009) and sobaity seabream (*Sparidentex hasta,* Nazemroaya et al., 2015). Yellowfin seabream larvae showed an initial smooth growth during the endo-exogenous feeding phase up to 15 DAH when larvae were 4.72 ± 0.2 mm, which may be attributed to dedication of most of the energy provided by the diet to the physiological development of larvae including the splachnocranium (Sarvi et al., 2019) and the gastrointestinal and other biological systems (Pittman et al., 2013), rather than somatic growth as it also reported in sobaity seabream (Nazemroaya et al., 2015). On the other hand, the increased growth performance of *A. latus* from 15 to 30 DAH was coincided with the flexion of the notochord occurring at 7 mm TL (22 DAH) (Akazaki and Tokito, 1982), which may enhance larval swimming capacity, as well as their foraging abilities (Osse and van den Boogaart, 1995). The above-mentioned changes coupled to a more developed digestive system may favor the digestion and absorption of dietary nutrients from live prey (Hamre et al., 2013). In addition, changes in the feeding protocol and the replacement of rotifers by *Artemia* may have also been responsible for such changes in larval growth performance, since *Artemia* nauplii have higher energy

content compared to rotifers (Darias et al., 2015). Higher concentrations of taurine in *Artemia* compared to rotifers (Aragão et al., 2004) may also explain the high growth rates of larvae observed from 15 to 30 DAH in comparison to earlier ages. In the present study, SGR values in *A. latus* larvae (11.8%/day, at 23°C) were higher than those reported in some seabream species (Suzer et al., 2007a; Guerreiro et al., 2010; Suzer et al., 2014), and similar to those reported for sobaity seabream (11.1 ± 1.1 %/day at 20 °C, Nazemroaya et al., 2015). These differences might be related to species-specific developmental patterns, and different larval husbandry practices, and feeding regimes among studies.

Larval performance depends on multiple factors and variables, and each species requieres its own larviculture protocol; thus, in general terms, results may not be directly comparable among different species. In the present study, survival rates of *A. latus* larvae was ca. 49.9% by 30 DAH, values that were comparable to those recently reported by Sarvi et al. (2019) in this species (43% at 42 DAH). These results found in *A. latus* larviculture are comparatively higher than those reported in other sparid species such as common pandora (*Pagellus erythrinus*, 21.2% by 40 DAH, Suzer et al., 2006), sharpsnout sea bream (21.7% by 50 DAH, Suzer et al., 2007a), white sea bream (6% by 48 DAH, Guerreiro et al., 2010), common dentex (16.2% by 45 DAH, Suzer et al., 2014), sobaity seabream (18.2% by 42 DAH, Nazemroaya et al., 2015) and red seabream (30.1% by 40 DAH, Khoa et al., 2019). These differences may be related to species-specific differences regarding larviculture practices and live prey quality, as well as difference in broodfish management (Hamre et al., 2013).

4.2. Digestive enzyme activities

As previously reported in other marine carnivorous fish species, the activity of pancreatic and intestinal digestive enzymes was already detected after hatching and before mouth opening in

A. latus larvae, which supported the bulk of literature indicating that this phenomenon is genetically programmed (Zambonino-Infante et al., 2008). Except for bile salt-activated lipase, the activity of the other pancreatic digestive enzymes in *A. latus* exhibited a similar ontogenetic pattern of specific activity, each beginning with a low level in activity during the lecitotrophic stage and peaking at 7 DAH (3.51 ± 0.1 mm SL); then, it progressively decreased and remained stable between 15 DAH (4.72 ± 0.2 mm SL) and 30 DAH (7.46 ± 0.6 mm SL). In spite of some fluctuations in the specific activity of bile salt-activated lipase, this enzyme did not show significant differences along larval ontogeny. In the case of total alkaline proteases, specific activity values decreased after 7 DAH, remained stable between 15 DAH and 22 DAH (6.12 ± 0.3 mm SL), while their further decreased at 30 DAH. The above-mentioned trends in specific activity of pancreatic digestive enzymes may be associated to an increase in the amount of soluble proteins in tissue extracts due to larval somatic growth (Lazo et al., 2007). In this sense, larval digestive capacities progressively increased along larval development as data of enzyme activity expressed as total activity units (U/larva) indicated. A similar pattern of digestive enzyme activities was reported in other sparid species like common dentex (Gisbert et al., 2009), sobaity seabream (Nazemroaya et al., 2015), meagre (*Argyrosomus regius*, Solovyev et al., 2016b) and longfin yellowtail (*Seriola rivoliana*, Teles et al., 2019). Previous studies also demonstrated that newly hatched *A. latus* has undifferentiated alimentary tract and it important digestive organs including the intestine, stomach and accessory digestive glands (liver and pancreas) are differentiated at *ca.* 4 mm TL during the first week after hatching (Abol-Munafi and Umeda, 1993; Sarvi et al., 2019). In addition, the increment of digestive enzymes activities during the first week after hatching coincided with the first feeding that facilitates food digestion and nutrient absorption, promoting larval growth at older ages (Hamre et al., 2013).

During the early stages of larval development, protein digestion takes place in an alkaline environment in the intestinal lumen by the action of alkaline proteases produced by the exocrine pancreas, while the complete protein digestion is accomplished within enterocytes with the action of intestinal cytosolic peptidases such as LAP (Zambonino-Infante and Cahu, 2001). In particular, TAP activity was detected at hatching and it progressively increased along larval development when expressed in total activity units, a common pattern found among fish species, which is generally associated with the development of the exocrine pancreas, as well as changes in the diet (Rønnestad et al., 2013). When considering TAP activity expressed in specific units we could observe two different developmental time points, one at 7 DAH (3.51 ± 0.1 mm TL) characterized by a peak in specific activity that may be associated with the complete transition to exogenous feeding and the functional development of the exocrine pancreas, and another one at 30 (7.46 ± 0.6 mm SL) DAH characterized by a drop in specific activity. This reduction in specific activity at this age might be mainly attributed to development of the digestive system in *A. latus* larvae and the establishment of protein digestion with the cooperation of gastric (pepsin-like) and pancreatic alkaline proteases (Rønnestad et al., 2013).

In the present study, the specific activities of AP and LAP peaked at 7 DAH (3.51 ± 0.1 mm SL) coinciding with the rest of assayed pancreatic enzymes (TAP, α-amylase and bile salt-activated lipase), which indicated an important period in terms of digestive system morphogenesis and functionality (Zambonino-Infante et al. 2008; Rønnestad et al., 2013). However, changes in AP and LAP in terms of specific and total activities indicated that intestinal maturation as assessed by the ratio of AP to LAP did not occur as expected by end of the first month of life in *A. latus* coinciding with the increase in acid proteases. Thus, the maturational features of the intestine with regard to AP and LAP activities were not achieved by the end of the current study at 30 DAH (7.46 ± 0.6 mm SL), indicating that the complete

establishment of digestive luminal processes may take place at older ages in *A. latus*. In this sense, high activities of LAP were found at 30 DAH, which indicated that intracellular digestion was still important at this age.

Regarding acid proteases, these were already detected after hatching, which might be attributed to the presence of lysosomal proteases like cathepsins involved in the intracellular digestion of proteins (Zambonino-Infante et al., 2008). In agreement with the results of the present study, Guerreiro et al. (2010) detected acid proteases activity from hatching in white seabream and its activity increased with fish larvae development. In contrast, the detection of acid proteases mainly pepsin-like ones occurred at different developmental stages such as sharpsnout seabream at 32 DAH (8 mm SL) (Suzer et al., 2007a), common dentex at 19 DAH (5.1 mm SL) (Gisbert et al., 2009), sobaity seabream at 14 DAH (7 mm SL) (Nazemroaya et al., 2015) and red seabream at 10 DAH (4 mm SL) (Khoa et al., 2019). The slight but statistically significant increase in the specific activity of acid proteases found at 30 DAH (7.46 ± 0.6 SL) might be linked to a decrease in specific activity of TAP, suggesting a transition of the larval digestive process based on alkaline proteolytic enzymes to a juvenile mode of digestion with a relevant contribution of acid digestive enzymes, However, further validation at older ages (beyond 30 DAH) is required in order to use such patterns in digestive enzyme activities for refining feeding protocols (Rønnestad et al., 2013). Furthermore, present results are in agreement with histological data that described that the stomach in *A. latus* is fully differentiated between 25 and 35 DAH (Sarvi et al., 2019). In addition, considering that the evaluation of pepsin-like acid proteases is considered as a valuable biomarker to apply a proper larviculture strategy and also as an optimal point for larval weaning onto microdiets in fish hatcheries (Nolasco-Soria et al., 2020), present results indicate that *A. latus* may start to be weaned at 30 DAH (7.46 ± 0.6 mm SL) when reared at 23 ºC.

Among lipolytic enzymes, bile salt-activated lipase is a key enzyme for fat digestion, especially for hydrolyzing triacylglycerides, in addition to phospholipids, esters of cholesterol, and lipid-soluble vitamins. Regardless of detecting this enzyme at hatching in *A. latus* larvae in the present study, it is doubtful that this lipolytic enzyme participates in the hydrolysis of lipids contained in yolk sac, since the exocrine pancreas is not fully differentiated and functional at hatching in altricial marine species (Rønnestad et al., 2013). These findings show that the spectrophotometric method used for measuring the total activity of this pancreatic enzyme, whose total activity is enhanced by means of bile salts (sodium cholate), is not specific (Nolasco-Soria et al., 2018), and it may also detect lipases hydrolysing triglycerides and wax esters contained in the yolk (Heming and Buddington, 1988). Furthermore, significant changes in total bile salt-activated lipase along ontogeny found in the present study could be associated with changes in the feeding protocol and variations in lipid content in live preys, since this enzyme is regulated by lipid composition (Morais et al., 2004). In particular, despite the incorporation of a commercial microdiet in the feeding schedule from 25 to 30 DAH, such increase found in total activity of bile salt-activated lipase between 22 and 30 DAH might be attributed to changes in lipid content of live prey, since *Artemia* nauplii contain higher lipid levels compared to rotifers (Evjemo and Olsen, 1997).

Regarding the activity profile of α-amylase, several studies have shown a close relationship of this pancreatic enzyme with the ability to digest dietary carbohydrates, the morphological changes in the digestive system during larval development and feeding habits (Gisbert et al., 2009 among others). In the current study, α-amylase activity was detected at hatching and two peaks in the specific α-amylase activity were observed at 7 and 22 DAH. The first peak in specific activity found at 7 DAH may be related to the proper establishment of exogenous feeding based on rotifers, whereas the second peak coincided with a diet shift

from rotifers to *Artemia* nauplii. Such increase in activity may be related to changes in the dietary carbohydrate levels between both preys, especially regarding their glycogen content (Ma et al., 2005; Gisbert et al., 2009). Although the early increase in α-amylase may be genetically programmed, its progressive increase with larval development when expressed in total activity units may be dietary induced. Similar results regarding changes in the activity of α-amylase with changes in the type of live prey have been reported in other marine fish species like large yellow croaker *Pseudociaena crocea* (Ma et al., 2005), totoaba *Totoaba macdonaldi* (Galaviz et al., 2015) and red seabream (Khoa et al., 2019).

Present results revealed that important physiological digestive developmental changes occurred during the first week of life in *A. latus,* which were in agreement with previous morphological and histological studies on this species (Abol-Munafi and Umeda, 1993; Sarvi et al., 2009), whereas the larval digestive function improved with age; nevertheless, the maturation of the intestine and the shift from the larval to the juvenile mode of digestion was not observed within the period studied (0-30 DAH), which indicated that this maturational process takes place at older ages in this species. Furthermore, this study related to the growth patterns and ontogenic changes in activity of pancreatic, gastric and intestinal enzymes in *A. latus* and their nutritional regulation under current rearing practices using the green water method may be considered as the first step for improving the larviculture, as well as assessing and refining the nutritional requirements during the larval and early juvenile stages of this sparid species.

Acknowledgments

This article is extracted from the project recorded under code number 95842262 and financially supported by Iran National Science Foundation. We are grateful to the director and staff of the Mariculture Research Station, Sarbandar, Iran and Persian Gulf Research

Institute, Persian Gulf University, Bushehr, Iran for providing the necessary facilities for the experiment. We thank to the director and staff of IRTA for the grant and support for visiting IRTA to learn the techniques and analyses the samples. Special thanks to Marta Sastre (IRTA, Spain) for their technical help in measuring the activity of digestive enzymes.

Ethical approval

This study was carried out in accordance with the principle of the Basel Declaration and recommendations of Iranian Fisheries Science Research Institute and the Faculty of Veterinary Medicine at University of Tabriz, the FVM.REC.1396.939.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Akazaki, M., Tokito, A., 1982. Studies on the seedling production of yellowfin porgy (Kichinu), *Acanthopagrus latus* Houttuyn—II. Aqua. Sci. 29, 218-228. https://doi.org/10.11233/aquaculturesci1953.29.218

Abol-Munafi, A.B., Umeda, S., 1993. Studies on the development and differentiation of the digestive system of Yellowfin porgy during larval and juvenile stages. Aquac. Sci. 41, 257-264.

Alvarez-González, C.A., Moyano-López, F.J., Civera-Cerecedo, R., Carrasco-Chávez, V., Ortíz-Galindo, J.L., Nolasco-Soria, H., Tovar-Ramírez, D., Dumas, S., 2010. Development of digestive enzyme activity in larvae of spotted sand bass *Paralabrax maculatofasciatus* II: Electrophoretic analysis. Fish Physiol. Biochem. 36, 29–37. https://doi.org/10.1007/s10695-008-9276-4

Aragão, C., Conceição, L.E.C., Dinis,M.T., Fyhn, H.-J., 2004. Amino acid Pools of rotifers and Artemia under different conditions: Nutritional implications for fish larvae. Aquaculture 234, 429–445.doi: 10.1016/j.aquaculture.2004.01.025

Bessey, O.A., Lowry, O.H., Brock, M.J., 1946. Rapid coloric method for determination of alkaline phosphatase in five cubic millimeters of serum. J. Biol. Chem. 164, 321–329.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Darias, M.J., Castro-Ruiz, D., Estivals, G., Quazuguel, P., Fernández-Méndez, C., Núñez-Rodríguez, J., Clota, F., Gilles, S., García-Dávila, C., Gisbert, E., Cahu, C., 2015. Influence of dietary protein and lipid levels on growth performance and the incidence of cannibalism in *Pseudoplatystoma punctifer* (Castelnau, 1855) larvae and early juveniles. J. Appl. Ichthyol. 31, 74–82. https://doi.org/10.1111/jai.12978

Evjemo, J.O., Olsen, Y., 1997. Lipid and fatty acid content in cultivated live feed organismscompared to marine copepods. Hydrobiologia 358, 159-162.

Faulk, C.K., Benninghoff, A.D., Holt, G.J., 2007. Ontogeny of the gastrointestinal tract and selected digestive enzymes in cobia *Rachycentron canadum* (L.). J. Fish Biol. 70, 567–583. https://doi.org/10.1111/j.1095-8649.2007.01330.x

Galaviz, M.A., López, L.M., García Gasca, A., Álvarez González, C.A., True, C.D., Gisbert, E., 2015. Digestive system development and study of acid and alkaline protease digestive capacities using biochemical and molecular approaches in totoaba (*Totoaba macdonaldi*) larvae. Fish Physiol. Biochem. 41, 1117–1130. https://doi.org/10.1007/s10695-015-0073-6

García-Careño, F.L., Haard, N.F., 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. J. Food Biochem

17, 97–113. https://doi.org/10.1111/j.1745-4514.1993.tb00864.x

Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y., Estévez, A., 2009. Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. Aquaculture 287, 381–387. https://doi.org/10.1016/j.aquaculture.2008.10.039

Gisbert, E., Ortiz-Delgado, J.B., Sarasquete, C., 2008. Nutritional cellular biomarkers in early life stages of fish. Histol. Histopathol. https://doi.org/10.14670/HH-23.1525

Gisbert, E., Nolasco, H., Solovyev, M., 2018. Towards the standardization of brush border purification and intestinal alkaline phosphatase quantification in fish with notes on other digestive enzymes. Aquaculture 487, 102-108. https://doi.org/10.1016/j.aquaculture.2018.01.004

Guerreiro, I., de Vareilles, M., Pousão-Ferreira, P., Rodrigues, V., Dinis, M.T., Ribeiro, L., 2010. Effect of age-at-weaning on digestive capacity of white seabream (*Diplodus sargus*). Aquaculture 300, 194–205. https://doi.org/10.1016/j.aquaculture.2009.11.019

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L.E.C., Izquierdo, M., 2013. Fish larval nutrition and feed formulation: Knowledge gaps and bottlenecks for advances in larval rearing. Rev. Aquacult. 5, 26–58. doi: 10.1111/j.1753-5131.2012.01086.x

Heming, T. A., Buddington, R. K., 1988. Yolk absorption in embryonic and larval fishes. In W. S. Hoar, & D. J. Randall (Eds.), *Fish Physiology* 11, 407–446. NY: Academic Press

Iijima, N., Tanaka, S., Ota, Y., 1995. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiol. Biochem. 18, 59–69. https://doi.org/10.1023/A:1007725513389

Kandathil Radhakrishnan, D., AkbarAli, I., Schmidt, B.V., John, E.M., Sivanpillai, S., Thazhakot Vasunambesan, S., 2020. Improvement of nutritional quality of live feed for aquaculture: An overview. Aquac. Res. 51, 1-17. https://doi.org/10.1111/are.14357

Karimi, S., Kochinian, P., Salati, A.P., 2013. The effect of sexuality on some haematological parameters of the yellowfin seabream, *Acanthopagrus latus* in Persian Gulf. Iran. J. Vet. Res. 14, 65–68.

Khoa, T.N.D., Waqalevu, V., Honda, A., Shiozaki, K., Kotani, T., 2019. Early ontogenetic development, digestive enzymatic activity and gene expression in red sea bream (*Pagrus major*). Aquaculture 512, 734283. https://doi.org/10.1016/j.aquaculture.2019.734283

Kolkovski, S., 2001. Digestive enzymes in fish larvae and juveniles - Implications and applications to formulated diets. Aquaculture 200, 181–201. https://doi.org/10.1016/S0044-8486(01)00700-1

Lazo, J.P., Mendoza, R., Holt, G.J., Aguilera, C., Arnold, C.R., 2007. Characterization of digestive enzymes during larval development of red drum (*Sciaenops ocellatus*). Aquaculture 265, 194–205. https://doi.org/10.1016/j.aquaculture.2007.01.043

Ma, H., Cahu, C., Zambonino, J., Yu, H., Duan, Q., Le Gall, M.M., Mai, K., 2005. Activities of selected digestive enzymes during larval development of large yellow croaker (*Pseudosciaena crocea*). Aquaculture 245, 239–248. https://doi.org/10.1016/j.aquaculture.2004.11.032

Mai, K., Yu, H., Ma, H., Duan, Q., Gisbert, E., Infante, J.L.Z., Cahu, C.L., 2005. A histological study on the development of the digestive system of *Pseudosciaena crocea* larvae and juveniles. J. Fish Biol. 67, 1094–1106. https://doi.org/10.1111/j.0022-1112.2005.00812.x

Métais, P., Bieth, J., 1968. Détermination de l'alpha-amylase par une microtechnique. Ann. Biol. Clin. (Paris) 26, 133–142.

Morais, S., Cahu, C., Zambonino-Infante, J.L., Robin, J., Rønnestad, I., Dinis, M.T., Conceição, L.E.C., 2004. Dietary TAG source and level affect performance and lipase expression in larval sea bass (*Dicentrarchus labrax*). Lipids 39, 449-458.

https://doi.org/10.1007/s11745-004-1250-2

Morioka, S., Sano, K., Phommachan, P., Vongvichith, B., 2010. Growth and morphological development of laboratory-reared larval and juvenile *Pangasianodon hypophthalmus*. Ichthyol. Res. 57, 139–147. https://doi.org/10.1007/s10228-009-0140-z

Mozanzadeh, M.T., Marammazi, J.G., Yaghoubi, M., Agh, N., Pagheh, E., Gisbert, E., 2017. Macronutrient requirements of silveryblack porgy (*Sparidentex hasta*): a comparison with other farmed sparid species. Aust Fish 2:5. https://10.3390/fishes2020005

Nazemroaya, S., Yazdanparast, R., Nematollahi, M.A., Farahmand, H., Mirzadeh, Q., 2015. Ontogenetic development of digestive enzymes in Sobaity sea bream *Sparidentex hasta* larvae under culture condition. Aquaculture 448, 545–551. https://10.1016/j.aquaculture.2015.06.038

Nicholson, J.A., Kim, Y.S., 1975. A one-step l-amino acid oxidase assay for intestinal peptide hydrolase activity. Anal. Biochem. 63, 110–117. https://doi.org/10.1016/0003-2697(75)90194-3

Nolasco-Soria, H., Moyano-López, F., Vega-Villasante, F., del Monte- Martínez, A., Espinosa-Chaurand, D., Gisbert, E., & Nolasco-Alzaga, H. R., 2018. Lipase and phospholipase activity methods for marine organisms. In G. Sandoval (Ed.), *Lipases and Phospholipases. Methods in Molecular Biology* 1835, 139–167. New York, NY: Humana Press.

Nolasco-Soria, H., Nolasco-Alzaga, H-R., Gisbert, E., 2020. The importance of pepsin-like acid protease quantification in aquaculture studies: a revision of available procedures and presentation of a new protocol for its assessment. Rev. Aquac. 12, 1928-1943. https://doi.org/10.1111/raq.12417

Osse, J. W. M.; van den Boogaart, J. G. M., 1995: Fish larvae, development, allometric growth, and the aquatic environment. ICES Mar. Sci. Symp. 201, 21–34.

Pittman, K., Yúfera, M., Pavlidis, M., Geffen, A.J., Koven, W., Ribeiro, L., Zambonino-Infante, J.L., Tandler, A., 2013. Fantastically plastic: fish larvae equipped for a new world. Rev. Aquac. 5, S224-S267. https://doi.org/10.1111/raq.12034.

Ribeiro, L., Couto, A., Olmedo, M., Álvarez-Blázquez, B., Linares, F., Valente, L.M.P., 2008. Digestive enzyme activity at different developmental stages of blackspot seabream, *Pagellus bogaraveo* (Brunnich 1768). Aquac. Res. 39, 339–346. https://doi.org/10.1111/j.1365-2109.2007.01684.x

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: Current knowledge, and gaps and bottlenecks in research. Rev. Aquac. 5, 59-98. https://doi.org/10.1111/raq.12010

Rotenstein, L., Milanes, A., Juarez, M., Reyes, M., de Bellard, M.E., 2009. Embryonic development of glial cells and myelin in the shark, *Chiloscyllium punctatum*. Gene Expr. Patterns 9, 572–585. https://doi.org/10.1016/j.gep.2009.09.001

Sarvi, B., Rafiee, G.R., Marammazi, G.J., Najafabadi, M.Z., SeyedAlhosseini, S.H., 2019. Investigation into reproductive biology and larval rearing technique of *Acanthopagrus latus* in captivity. Iranian Scientific Fisheries Journal 28: 1-12 (in Persian with English abstract). https://doi.org/119519.2019.ISFJ/22092.1

Solovyev, M., Gisbert, E., 2016a. Influence of time, storage temperature and freeze/thaw cycles on the activity of digestive enzymes from gilthead sea bream (*Sparus aurata*). Fish Physiol. Biochem. 42, 1383–1394. https://doi.org/10.1007/s10695-016-0226-2

Solovyev, M.M., Campoverde, C., Öztürk, S., Moreira, C., Diaz, M., Moyano, F.J., Estévez, A., Gisbert, E., 2016b. Morphological and functional description of the development of the digestive system in meagre (*Argyrosomus regius*): An integrative approach. Aquaculture 464, 381–391. https://doi.org/10.1016/j.aquaculture.2016.07.008

Suzer, C., Aktülün, S., Çoban, D., Okan Kamaci, H., Saka, Ş., Firat, K., Alpbaz, A., 2007a.

Digestive enzyme activities in larvae of sharpsnout seabream (*Diplodus puntazzo*). Comp. Biochem. Physiol. 148A, 470–477. https://doi.org/10.1016/j.cbpa.2007.06.418

Suzer, C., Firat, K., Saka, Ş., 2006. Ontogenic development of the digestive enzymes in common pandora, *Pagellus erythrinus*, L. larvae. Aquac. Res. 37, 1565–1571. https://doi.org/10.1111/j.1365-2109.2006.01598.x

Suzer, C., Kamaci, H.O., Çoban, D., Saka, Ş., Firat, K., Özkara, B., Özkara, A., 2007b. Digestive enzyme activity of the red porgy (*Pagrus pagrus*, L.) during larval development under culture conditions. Aquac. Res. 38, 1778–1785. https://doi.org/10.1111/j.1365-2109.2007.01841.x

Suzer, C., Çoban, D., Yildirim, S., Hekimoğlu, M., Kamacı, O.H., Firat, K., Saka, S., 2014. Stage-Specific Ontogeny of Digestive Enzymes in the Cultured CommonDentex (*Dentex dentex*) Larvae. Turk. J. Fish. Aquat. Sci. 14, 759-768. 10.4194/1303-2712-v14_3_18

Teles, A., Salas-Leiva, J., Alvarez-González, C.A., Tovar-Ramírez, D., 2019. Changes in digestive enzyme activities during early ontogeny of *Seriola rivoliana*. Fish Physiol. Biochem. 45, 733–742. https://doi.org/10.1007/s10695-018-0598-6

Worthington Biochemical Corporation, 1991. Worthington Enzyme Manual: Enzymes, Enzyme Reagents, Related Biochemicals; Worthington Biochemical Corp.: Freehold, New Jersey.

Yúfera, M., Moyano, F.J., Martínez-Rodríguez, G., 2018. The digestive function in developing fish larvae and fry. From molecular gene expression to enzymatic activity. In: Yúfera, M. (Ed.), Emerging issues in fish larvae research, Springer Publishing, Berlin, pp. 51–86. https://doi.org/10.1007/978-3-319-73244-2_3

Zambonino-Infante, J., Gisbert, E., Sarasquete, C., Navarro, I., Gutiérrez, J., Cahu, C., 2008. Ontogeny and physiology of the digestive system of marine fish larvae. In: Cyrino, J. E.

P., Bureau, D.P., Kapoor, B.G. (Eds.), Feeding and digestive functions of fishes, CRC Press, USA, pp. 281–348. https://doi.org/10.1201/b10749-8

Zambonino Infante, J.L., Cahu, C.L., 2007. Dietary modulation of some digestive enzymes and metabolic processes in developing marine fish: applications to diet formulation. Aquaculture 268, 98-105. https://doi.org/10.1016/j.aquaculture.2007.04.032

Zambonino Infante, J.L., Cahu, C.L., 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comp. Biochem. Physiol. 130C, 477–487. https://doi.org/10.1016/S1532-0456(01)00274-5

Figure captions

Fig. 1. Larval growth of *A. latus* in total length (mm) and wet body weight (mg) from 0 to 30 days after hatching (DAH). Results are presented as mean ± standard error of the mean (N=6).

Fig. 2. Specific (mU mg^{-1} protein) and total (mU $larva^{-1}$) activities of pancreatic (total alkaline proteases, a; bile salt-activated lipase, f; α-amylase, g), gastric (Acid protease, e) and intestinal (alkaline phosphatase, c; leucine alanine peptidase, b) enzymes and the alkaline phosphatase to leucine aminopeptidase ratio (d) during the larval development of *A. latus*. Results are expressed as mean ± standard error of the mean (N=6).

Different letters indicate significant differences among different larval ages (ANOVA, $P < 0.05$).

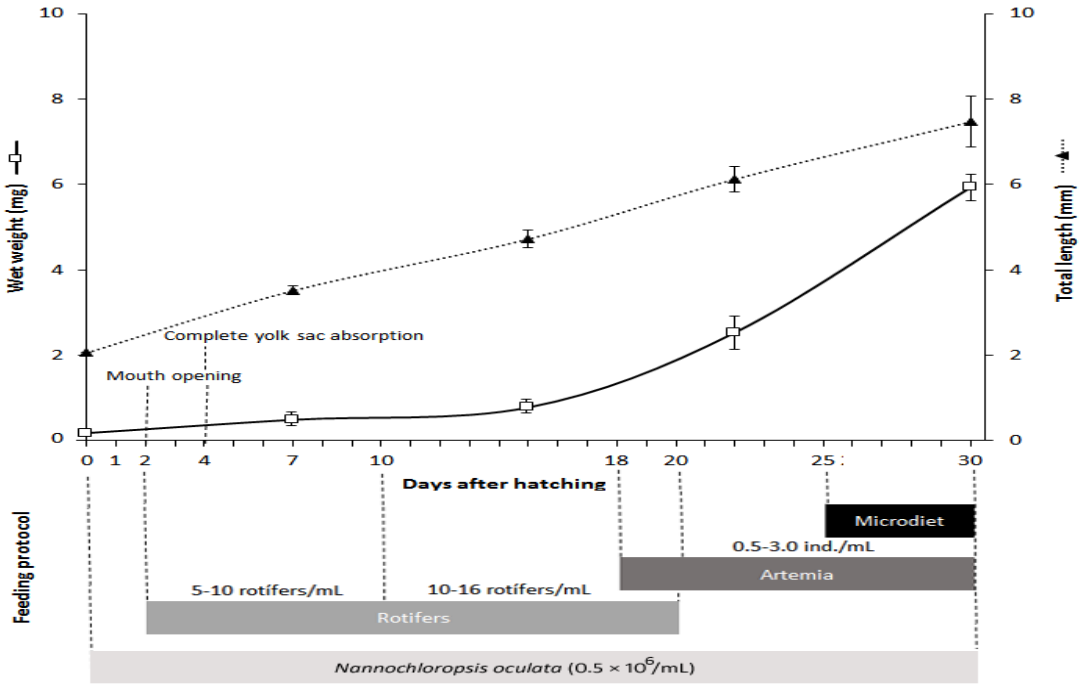
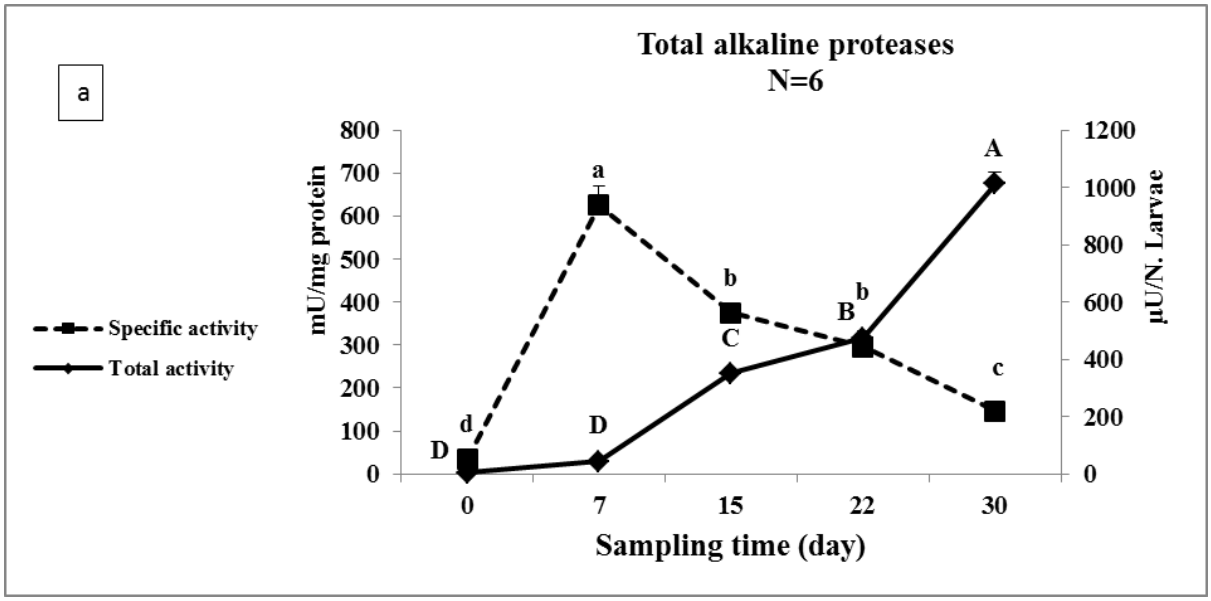
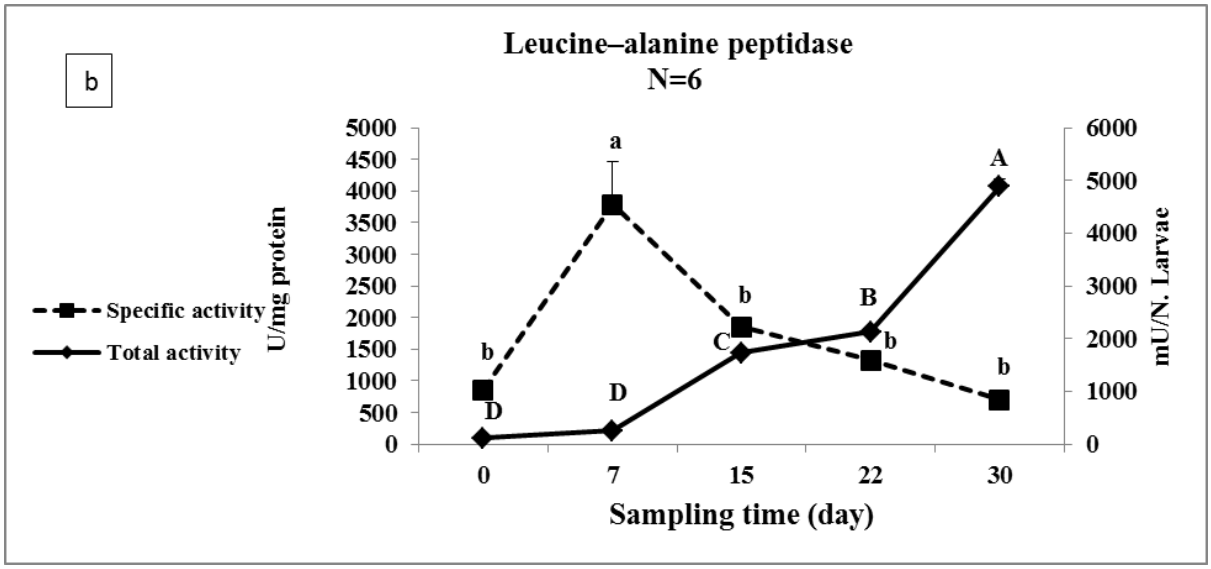
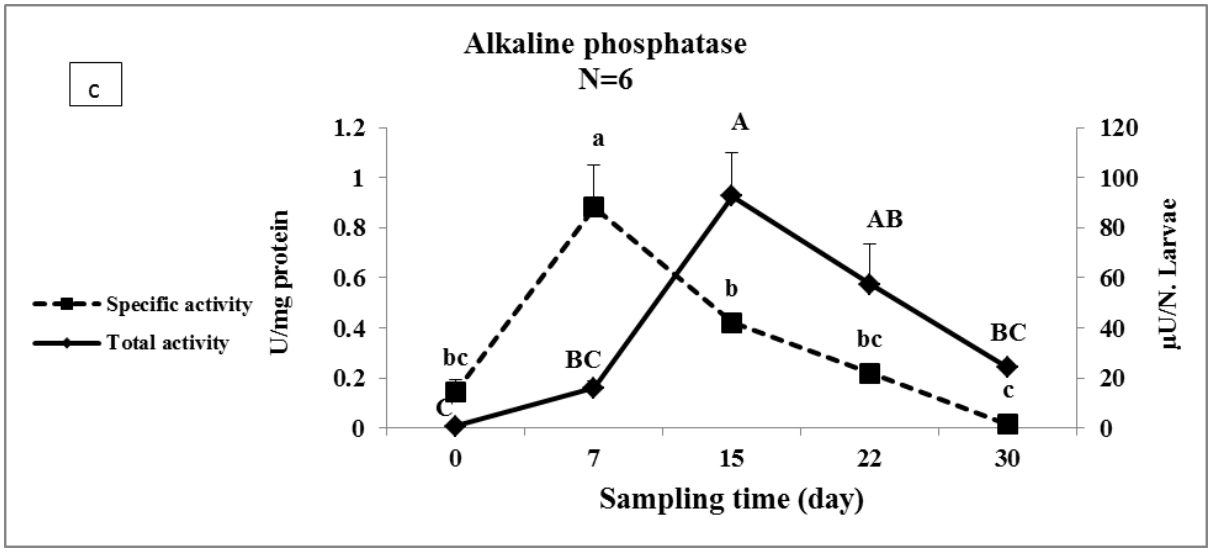
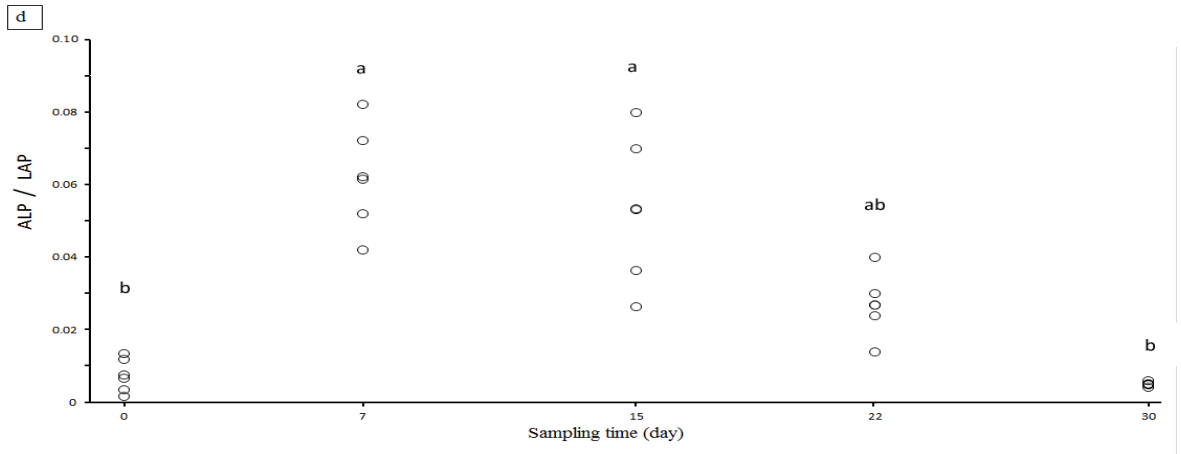

Wet weight (mg)
Total length (mm)
Complete yolk sac absorption
Mouth opening
Days after hatching
Feeding protocol
Microdiet
0.5-3.0 ind./mL
Artemia
5-10 rotifers/mL
10-16 rotifers/mL
Rotifers
Nannochloropsis oculata (0.5 × 10⁶/mL)

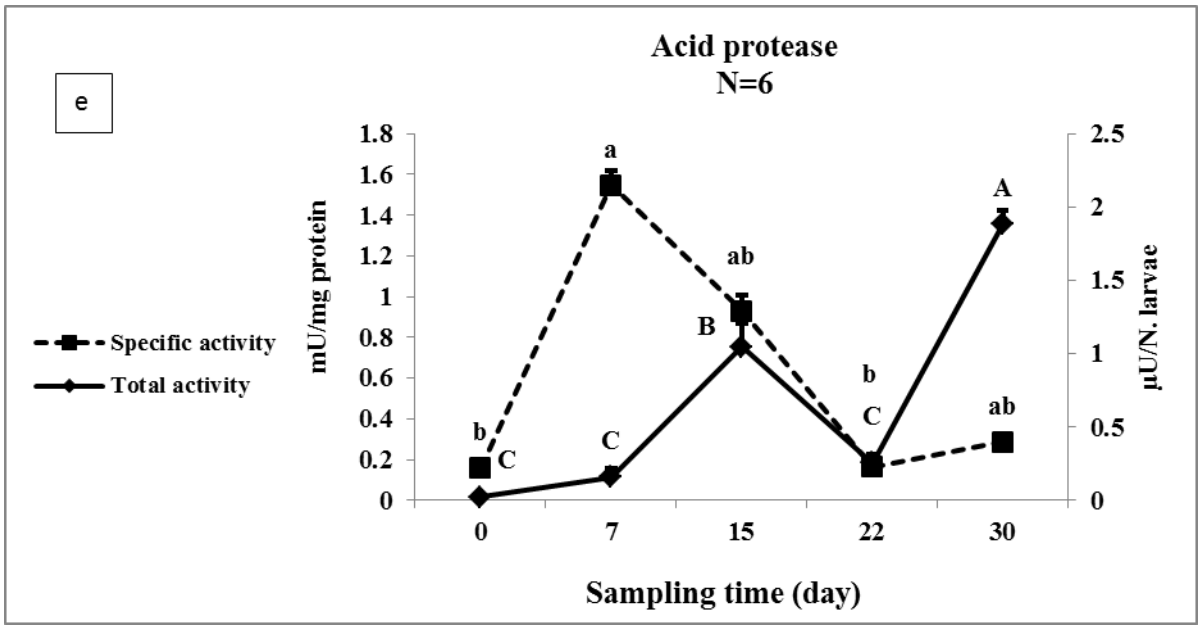
Figure 1


a
Total alkaline proteases
N=6
Specific activity
Total activity
mU/mg protein
µU/N. Larvae
Sampling time (day)
0
7
15
22
30
800
700
600
500
400
300
200
100
0
1200
1000
800
600
400
200
0
a
b
b
c
d
A
B
C
D
D

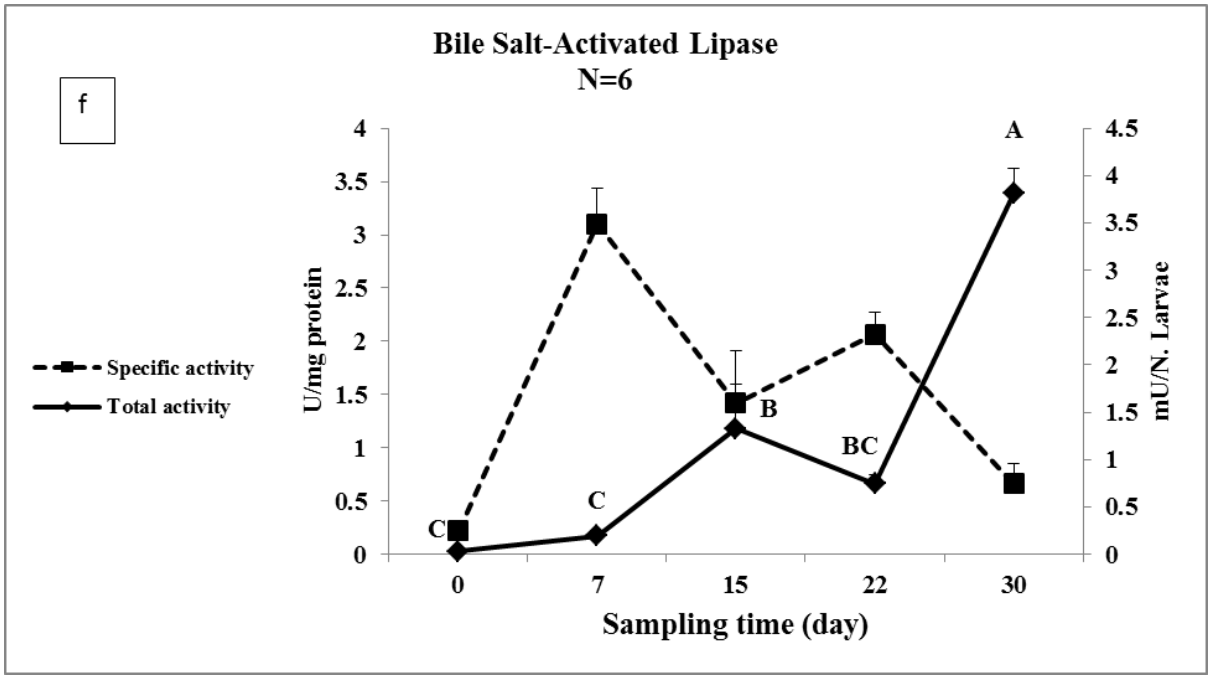

b
Leucine–alanine peptidase
N=6
Specific activity
Total activity
U/mg protein
mU/N. Larvae
Sampling time (day)
0
7
15
22
30
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
6000
5000
4000
3000
2000
1000
0
a
b
b
b
b
A
B
C
D
D


c
Alkaline phosphatase
N=6
Specific activity
Total activity
U/mg protein
µU/N. Larvae
Sampling time (day)
a
A
AB
b
bc
BC
bc
BC
c
C
0
7
15
22
30
1.2
1
0.8
0.6
0.4
0.2
0
120
100
80
60
40
20
0


d
ALP / LAP
Sampling time (day)
a
a
ab
b
b
0.10
0.08
0.06
0.04
0.02
0
0
7
15
22
30



709
710
711
712
713



714
715
716

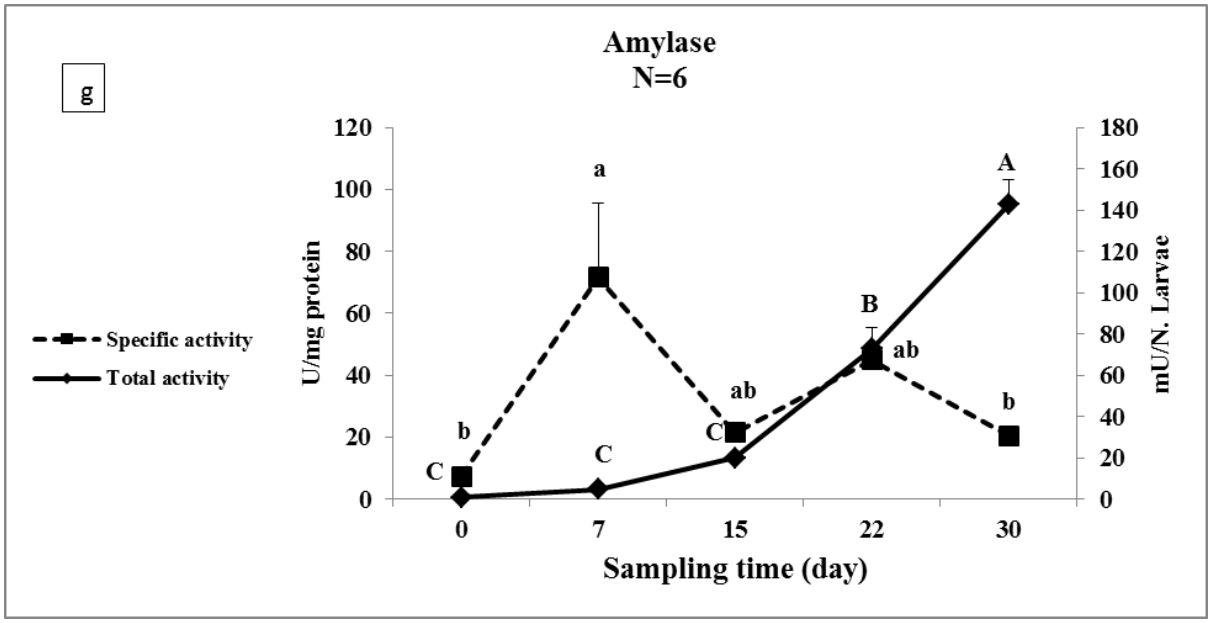

g
Amylase
N=6
Specific activity
Total activity
U/mg protein
mU/N. Larvae
Sampling time (day)
0
7
15
22
30
a
b
C
C
ab
C
B
ab
A
b

Figure 2