#### **Research Article**



### Effect of dietary trypsin extracted from the viscera of yellowfin sea bream, (*Acanthopagrus latus*) on growth performance, body composition, and digestive trypsin activity in Sobaity sea bream (*Sparidentex hasta*) larvae

#### Namjou F.<sup>1</sup>; Yeganeh S.<sup>1\*</sup>; Madani R.<sup>2</sup>; Ouraji H.<sup>1</sup>

Received: July 2022

Accepted: April 2023

#### Abstract

In this study, trypsin was extracted from the viscera of yellowfin sea bream (Acanthopagrus latus) and used as an additive in Sobaity sea bream, Sparidentex hasta, larvae microdiet. The microdiet was supplemented with the extracted enzyme and its efficiency was evaluated in the growth performance of S. hasta larvae. There were five treatments containing zero (control), 1000, 1500, and 2000 IU/kg of the extracted trypsin with 500 IU/kg of a commercial diet of porcine trypsin. S. hasta larvae (mean initial weight of 0.021±0.001 g) were fed with experimental diets for 28 days. Fifteen round polyethylene tanks (300 L, a water volume of 100 L) equipped with a water circulation system and an air stone were used for the treatments. The highest body weight gain (BWG), specific growth rate (SGR), daily growth, survival rate, and protein efficiency ratio (PER) were observed in the larvae fed the diet containing 2000 IU/kg trypsin (p < 0.05). In addition, the lowest and the highest values of the feed conversion ratio (FCR) vs daily feed intake and trypsin activity were significantly recorded in 2000 IU/kg trypsin and the control groups, respectively (p < 0.05). A positive correlation was observed between the increasing levels of trypsin in the microdiets and growth performance (*i.e.* BWG, SGR, daily growth rate, survival rate, and PER). Besides, the dietary addition of external trypsin reduced the values of FCR, daily feed intake, and trypsin activity in the larvae. Overall, trypsin at different doses, particularly at 2000 IU/kg, can significantly improve the performance and feed utilization of S. hasta larvae.

Keywords: Sparidentex hasta, Weight gain, Protease enzyme, Microdiet, Correlation coefficient.

<sup>1-</sup>Department of Fisheries, Faculty of Animal Science and Fisheries, Sari Agricultural Sciences and Natural Resources University, Sari, Iran

<sup>2-</sup>Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

<sup>\*</sup>Corresponding author's Email: skyeganeh@gmail.com; s.yeganeh@sanru.ac.ir

#### Introduction

The development of aquaculture industry, especially marine aquaculture operations, has led to advances in marine fish farming based on the production of high-quality resistant and larvae (Alvarez- Gonzáles et al., 2010). An important factor affecting larval quality and survival is the supply of sufficient and essential nutrients. Diets that are used as live or formulated feeds should be able to meet the essential nutritional needs for proper larval growth and to develop maximum physicochemical properties related to digestion and absorption (Kolkovski et al., 1993; Yılmaz and İkiz, 2006; Javahery et al., 2019; Rachmawati et al., 2020; Imaniy et al., 2022). In the larval stage of marine fish, the internal organs, in particular the gastrointestinal (GI) tract, do not develop completely at the time of hatching, and it develops during the development period (Nazemroaya et al., 2015b). Hence, initial feeding with microdites requires a high nutrient bioavailability during the early life stages in marine fish larvae, which depends on the proportion and formulation of the microdiets for larval growth and physiological development. In most marine fish larvae, enzymatic activities are significantly lower than the juvenile stage (Hamre et al., 2013), resulting in lower larval growth and survival at the onset of feeding with formulated diets (Munilla-Moran et al., 1990). Therefore. the complete replacement of live feed with microdiets is ineffective because of problems with the inability of digestion and absorption

in the first-feeding stage (Walford et al., 1991; Hamre et al., 2013). Dependence on live feed is a major problem in the intensive culture of most larval marine fish species (Kolkovski, 2001). This is because the production of live feed requires more than half of the nursery facilities and laborious work as it is an unpredictable process that can be affected by changes in water parameters and does not provide sustainable production (Marte and Toledo, 2015). The onset of external feeding in fish larvae is considered a critical stage associated with high mortality in laboratory and wild populations. After the absorption of the yolk sac, the lack of nutrients in natural conditions reduces larval survival in the first days and weeks of life, which corresponds to the lack of high-quality feed and a proper feeding process in farming systems (Yufera and Darias, 2007). The addition of digestive enzymes as a supplement was reported to result in a 30% increase of the absorption microdiets in (Kolkovski et al., 1993).

Fish are processed before their supply for human consumption. Two types of solid and liquid wastes are produced during the processing procedures. The solid waste includes the head, skeleton, fins, tail, skin, and GI tract, and the liquid waste comprises seafood processing wastewater. These wastes are rich in valuable microand macronutrients such as proteins, amino acids, bioactive peptides, collagen, gelatin, oils, calcium, and enzymes. Fish waste usually contains 58% protein and 19% ether extract or fat. Moreover,

minerals such as calcium, phosphorus, potassium, sodium, magnesium, iron, zinc, manganese, and copper are found in their compounds (Ramakrishnan *et al.*, 2013). The internal organs of aquatic animals are rich in enzymes, exhibiting a high catalytic activity at relatively low concentrations. Enzymes found in fish include pepsin, trypsin, chymotrypsin, and collagenase, which are extracted commercially from the viscera of aquatic animals (Prasertsan *et al.*, 2003; Zhao *et al.*, 2011).

The reduction of using live feeds during the larval stage is economically important and should be in line with maintaining proper larval survival and growth. Besides, using enzyme supplements in microdiets results in the improved nutrition of marine fish larvae. Some publications reported the effects of diet supplementation with dietary digestive enzymes in yellow perch (Perca flavescens) (Kolkovski et al., 2000), hybrid tilapia (Oreochromis niloticus x O. aureus) (Lin et al., 2007), Japanese seabass (Lateolabrax japonicas) (Ai et al., 2007), African catfish (Clarias gariepinus) (Yildirim and Turan, 2010), and Red sea bream (Pagrus major) (López-Alvarado, 2013). Enzyme-rich sources are also produced by the aquaculture industry waste. For instance, trypsin was purified from the waste of brown stripe red snapper (Lutjanus vitta) (Khantaphant and Benjakul, 2010), silver mojarra (Diapterus rhombeus) (Silva et al., 2011), zebra blenny (Salaria basilisca) (Ktari et al., 2012), olive flounder (Paralichthys olivaceus) (Kim and

Jeong, 2013), merigal carp (Cirrhinus mrigala) (Khangembam and Chakrabarti, 2015), and oil sardine (Sardinella longiceps) (Khandagale et al., 2017). Due to the alkalinity of the larval digestive environment in the early days of hatching, it is possible to increase the activity of alkaline proteases bv adding enzymatic supplements. Therefore, the growth and survival of S. hasta larvae fed with microdiets supplemented with trypsin extracted from the viscera of A. latus were investigated in the present study to evaluate the growth performance, trypsin activity and the regression between supplement enzyme and those parameters.

#### Materials and methods

#### Supply of larvae

The larvae were obtained through healthy and wild three-year-old breeders caught from the Persian Gulf. Female and male S. hasta broodstocks (mean weights of 2.5±0.98 and 1±0.10 kg, respectively) were spawned at 18°C. The fish were then undergone HCG (human chorionic gonadotropin) hormone therapy for two days in the afternoon to morning in the marine fish research station of Bandar Imam (Mahshahr, Khuzestan, Iran). Active feeding began two days after hatching. On the first day after hatching, the larvae were kept in water containing the microalga, Nannochloropsis sp. After active feeding began from the second day until the 8<sup>th</sup> day after hatching, larvae were fed with a combination of algae and small-size or the S-type rotifer

660

(Brachionus plicatiliss) at a rate of 500,000 cells/mL of the algae and 20 rotifers/mL. From days 9 to 11, 500,000 cells/mL of the algae and 30 large-size L-type rotifers/mL were added to the previous food composition (Nazemroaya et al., 2015a, b). To prepare the larvae for manual feeding, microplates without enzvme supplementation with live feed were added to the diet according to the cofeeding protocol (Kolkovski, 2008) from days 12 to 15. The culture facility was equipped with a chlorination system in the water inlet, ventilation, aeration, and central drainage for larval treatments. Fifteen round polyethylene tanks with a capacity of 300 L and a water volume of 100 L were equipped with a water circulation system (from 10-day larvae to the end of the experiment, water exchange was 25% with a flow rate of 850 mL/min, but water was not exchanged before 10 days because of larval size), and an air stone was used for each tank. Seawater with a salinity of 39 ‰ was passed through a sand filter before entry into the chlorination system. A total of 15,000 15-day-old larvae (15 days after hatch: DAH) was randomLy transferred into the tanks (1000 larvae for each replicate) and fed with experimental diets for 4 weeks. The water quality parameters including temperature (22±0.98°C), pН  $(7.9\pm0.02),$ and dissolved oxygen  $(7.7\pm0.05 \text{ mg/L})$  were determined daily during the trial. The larvae were randomLy sampled from all replications and treatments at the end of each week, and each tank was daily siphoned to

remove all uneaten feeds (if available) and feces. Dead fish were also removed and discarded at each observation.

#### Preparation of experimental diets

Since micron-sized food preparation facilities are not available in Iran. the experimental feed sample for feeding the larvae was procured from Coppens Co. (The Netherlands) (Advance type, 200-300 microns, Altek Koppens). This feed was specific to marine fish larvae and contained no enzymatic additives, which was selected with a size of 200-300 microns to be suitable for feeding marine fish larvae in the first weeks of active feeding. Protein, fat, fiber, ash, and total phosphorus contents in the prepared food were 56, 15, 0.3, 11, and 1.85%, respectively. The feed also contained stable amounts of such vitamins as A (14000 IE kg<sup>-1</sup>), D (1300 IE kg<sup>-1</sup>), E (280 mg kg<sup>-1</sup>), and C (350 mg kg<sup>-1</sup>). To add trypsin enzyme supplement extracted from the viscera of yellowfin sea bream, A. latus, the dry enzyme powder was mixed with edible oil (sunflower oil) and sprayed on the feed (Kazerani and Shahsavani, 2011). To prevent the adhesion of very fine feed particles, the oil-added feed was air-dried for 2-3 h, powdered again using a stirrer, and then passed through a screen (with mesh size in microns). The same amount of oil without the enzyme supplement was added to the control diet.

Accordingly, experimental dietary treatments (T) consisted of T1 (control) with 30 rotifers/mL without extracted trypsin, T2 containing 500 IU of commercial trypsin (derived from the The enzyme supplement was a lyophilized powder, with a specific activity of 56.6 IU/mg protein, obtained from trypsin extracted from 15 *A. latus* viscera (with a mean weight of  $179.93\pm93$  g and a mean length of  $213.67\pm29$  cm) and used in the experimental treatments (Namjou *et al.*, 2019). T2 contained a commercial porcine pancreatic trypsin supplement obtained from Sigma-Aldrich (product code T4799).

Fish were fed manually in the tanks 12 h a day every 2 h based on apparent satiety but not during dark hours. The feed was sprayed on the tanks to spread all over the tank surface.

#### Growth parameters

After recording the weight and mortality of larvae, growth performance was measured using the following formulas:

Body weight gain (BWG, g) =  $Wt_2$  (g)- $Wt_1$  (g)

Where, Wt<sub>1</sub> and Wt<sub>2</sub> are initial and final weights, respectively.

Specific growth rate (SGR, %/day) =  $[(LnWt_2-LnWt_1)/t_2-t_1] \times 100$ 

Where,  $LnWt_1$ = the normal logarithm of the initial weight,  $LnWt_2$ = the normal logarithm of the final weight and t<sub>2</sub>- t<sub>1</sub>= the length of the test period

Daily Growth Rate (GR, g/day) = [Wt<sub>2</sub> (g)-Wt<sub>1</sub> (g)/(t<sub>2</sub>-t<sub>1</sub>)]

Where,  $Wt_1$ = Initial weight,  $Wt_2$ = The final weight, and  $t_2$ -  $t_1$ = the length of the test period

Feed conversion ratio (FCR) = F(g)/WG(g)

WG= weight gain and F= the amount of food given

Feed intake (FI, %/day) =100×I (g)/[(Wt<sub>2</sub> (g) + Wt<sub>1</sub> (g))/2× (t<sub>2</sub>- t<sub>1</sub>)]

Where, I= total food eaten and  $t_2$ -  $t_1$ = the length of the test period

Survival rate (SR, %) =  $(N_t - N_0) \times 100$  (Ai *et al.*, 2007)

Where,  $N_0$ =Number of fish at the beginning of the period) and  $N_t$  = Number of fish at the end of the period

Protein Efficiency Ratio (PER) = wet weight gain (g) / protein fed (g)

## *Trypsin activity (in the extracted enzyme and fish larvae)*

It should be noted that about 1 g of the sample was needed for the enzyme analysis (Ramzanzadeh *et al.*, 2016). Based on the final weight of the fish, therefore, all fish from each treatment were collected for the enzyme assay and the body composition analysis. To determine trypsin activity, enzymeextracted samples were first prepared by the defatting process and preparation of a dry powder, followed by preparing the enzyme crude extract and then purifying the enzyme from the crude extract (Namjou *et al.*, 2019). To determine the trypsin activity of larvae at the end of the 4-week experimental period, sampling time was scheduled in the morning prior to feeding, and no feed was added to the rearing tank at night on the day prior to sampling. The small size of larvae makes it difficult to cut the head or spinal cord or to kill them with a sharp blow to the head. Therefore, they were anesthetized moderately with clove powder (200 ppm) (Mirali et al., 2013) and then immediately submerged in liquid nitrogen at -196°C. Trypsin (EC 3.4.21.4) activity was measured with N--benzoyldlarginine-p-nitroanilide (BAPNA) as the substrate. BAPNA (1 mmol/L in 50 mmol/L Tris-HCl, pH 7.5, 20 mmol/L CaCl<sub>2</sub>) was incubated with the enzyme extract at 37°C. Absorbance was read at 410 nm. After the preparation of samples, the enzyme activity was examined using the following formula (Erlanger et al. 1961):

Trypsin activity  $(U/mL) = \frac{\text{light absorption at 410 nm *1000* the reaction mixture}(ml)}{8800* \text{ reaction time }(min)* \text{ sample volume }(ml)}$ 

Where, 8800 (cm<sup>2</sup>/mg) is the molar extinction coefficient for  $\rho$ -nitroaniline (Erlanger *et al.*, 1961).

#### Carcass analysis

At the end of the fourth week, samples taken randomLy from were all treatments for the carcass analysis. To this aim, the whole larval body was macerated and homogenized due to its small size. Carcass constituents. including protein, moisture, fat, and ash, were analyzed according to the AOAC method (AOAC, 1995).

#### Data analysis

This experiment was performed based on a completely randomized design with five treatments and three replications. Data normality and homogeneity of variances were examined by Kolmogorov-Smirnov and Levene's tests, respectively. The effects of enzyme supplementation on growth parameters, trypsin activity, and carcass composition in *S. hasta* larvae were evaluated by the one-way analysis of variance (ANOVA) using SPSS 21 software. Significant differences between the means were determined with Duncan's test at a significance level of 5%. Microsoft EXCEL 2016 was also used to determine Pearson's correlation coefficient between data and to draw figures.

#### Results

According to the results of larval BWG (Table 1), a significantly higher BWG was observed in the treatments fed with trypsin supplement (p<0.05). T5 (0.121±0.003 g) and the control treatment (0.09±0.00 g) presented the highest and the lowest BWG values, respectively. In addition, a positive correlation coefficient was obtained

between increasing trypsin levels and BWG in the experimental treatments (Fig. 1; p<0.05, r=0.90). SGR (%day<sup>-1</sup>) was higher in treatments containing the extracted trypsin than in commercial trypsin and diets without enzyme supplements (p<0.05). The highest (7.523±0.79% day<sup>-1</sup>) and the lowest (6.540±0.002% day<sup>-1</sup>) SGR values

belonged to T5 and the control treatment, respectively. A positive correlation coefficient was found between increasing the extracted trypsin and the increase in SGR (Fig 2; p<0.05, r=0.89) (Table 2).

 Table 1: Growth performance and survival of Sparidentex hasta larvae fed with diets containing different levels of trypsin (commercial trypsin and trypsin extracted from Acanthopagrus latus viscera).

unus viscera).					
Parameter	<b>T1</b>	Τ2	Т3	<b>T4</b>	Т5
Initial weight (g)	$0.021 \pm 0.001$ <sup>a</sup>	$0.021{\pm}0.001$ a	$0.021{\pm}0.001$ <sup>a</sup>	0.021±0/001 <sup>a</sup>	$0.021{\pm}0.001$ <sup>a</sup>
Final weight (g) 41 (DAH*)	$0.110 \pm 0.000$ <sup>c</sup>	0.128±0.003 <sup>b</sup>	0.123±0.006 <sup>b</sup>	0.132±0.003 <sup>b</sup>	0.142±0.003 ª
Weight gain (g)	$0.09 \pm 0.00^{\text{ d}}$	$0.108{\pm}0.003^{\rm \ bc}$	$0.103 \pm 0.006$ °	$0.112{\pm}0.002^{\text{ b}}$	$0.121{\pm}0.003$ a
Specific growth rate (%/day)	$6.540{\pm}0.002^{d}$	$7.148{\pm}0.079^{bc}$	6.989±0.174 °	$7.247 \pm 0/072^{b}$	7.523±0.079 ª
Daily growth Rate (g/day)	$0.0034{\pm}0.0000^{d}$	$0.0042{\pm}0.0001^{\text{ bc}}$	0.0040±0.0002 c	$0.0043 \pm 0.0001$ <sup>b</sup>	0.0047±0.0001 a
Feed conversion ratio	1.11±0.003 <sup>a</sup>	$0.91{\pm}0.018^{bc}$	$0.95{\pm}0.052^{b}$	$0.88 \pm 0.019$ °	$0.80{\pm}0.020^{d}$
Daily feed intake (%/day)	5.79±0.01 <sup>a</sup>	$5.03 \pm 0.07$ bc	5.21±0.21 <sup>b</sup>	$4.91{\pm}0.08^{\circ}$	$4.60\pm0.08^{d}$
Survival rate (%)	95.23±0.21 °	95.83±0.42 <sup>b</sup>	$96.07{\pm}0.23$ <sup>ab</sup>	96.10±0.17 <sup>ab</sup>	$96.37{\pm}0.06^{a}$
Protein Efficiency Ratio	$0.0017{\pm}0.000^{\rm \ d}$	$0.0021{\pm}0.000^{\ bc}$	$0.0020\pm0.000$ <sup>c</sup>	$0.0021 \pm 0.000^{b}$	0.0023±0.000 ª

Values are represented as mean $\pm$ STD. Different letters in each row indicate a significant difference among treatments (*p*<0.05). T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively. DAH: days after hatch.



# Figure 1: The positive correlation between dietary trypsin levels and body weight gain in *Sparidentex* hasta larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively

Daily growth rates were uppermost in treatments containing enzyme supplements, with T5 (0.0047±0.0001 g day<sup>-1</sup>) and T1 (0.0034±0.001 g day<sup>-1</sup>) showing the highest and lowest rates, respectively (p<0.05). There was also a positive correlation coefficient between increasing the trypsin supplement and the daily growth rate of larvae (Fig. 3; p<0.05, r=0.90). The examination of

FCR graphs indicated that treatments fed with diets containing the trypsin supplement had significantly lower FCR, with values of 0.8 and 1.1 in T5 and T1, respectively (p<0.05). There was also a negative correlation between FCR and the trypsin increase (Fig. 4; p<0.05, r=-0.89).



Pearson Correlation = 0.89, p<0.05

Figure 2: The positive correlation between dietary trypsin levels and specific growth rate in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

 Table 2: Trypsin activity of Sparidentex hasta larvae samples fed with diets containing different levels of trypsin (commercial trypsin and trypsin extracted from Acanthopagrus latus viscera).

Parameter	T1	T2	Т3	<b>T4</b>	Т5
Total activity (IU)	9.80±0.006 °	$10.61 \pm 0.012$ a	10.30±0.010 <sup>b</sup>	$2.72{\pm}0.006^{d}$	0.224±0.001 °
Specific activity (IU/mg protein)	0.08±0.001 <sup>a</sup>	0.06±0.002 °	$0.07 \pm 0.002^{b}$	$0.05 {\pm} 0.001$ <sup>d</sup>	0.01±0.001 <sup>e</sup>
Protein content (mg/mL)	2.60±0.006 °	3.90±0.010 <sup>a</sup>	3.20±0.010 <sup>b</sup>	$1.14 \pm 0.001$ <sup>d</sup>	0.48±0.001 <sup>e</sup>

Values are represented as mean $\pm$ STD. Different letters in each row have a significant difference among treatments (p < 0.05). T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

The feed intake levels in T5 and T1 were respectively equal to  $4.0\pm0.08\%$  d<sup>-1</sup> and  $5.79\pm0.01$  % d<sup>-1</sup> (*p*<0.05), with a negative correlation between the increasing use of trypsin in the diets of experimental treatments and feed intake levels (Fig 5; *p*<0.05, r= -0.89). The survival rate was also significantly affected by the dietary trypsin level. Survival rates of 96.37%±0.06 and 95.23%±0.21 were recorded in T5 and T1, respectively, at the end of the experimental period (p < 0.05), with a positive correlation coefficient between these two parameters (Fig. 6; p < 0.05, r=0.93). Similar results were observed for PER so that T5 with the highest enzyme supplementation had a better PER  $(0.0023\pm0.00)$  than that of the treatment  $(0.0017 \pm 0.00)$ control (*p*<0.05). Accordingly, a positive correlation coefficient was obtained between PER and the trypsin increase (Fig 7; *p*<0.05, r=0.90).

666



Figure 3: The positive correlation between dietary trypsin levels and daily growth rate in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.



Figure 4: The negative correlation between dietary trypsin levels and feed conversion ratio in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.



Figure 5: The negative correlation between dietary trypsin levels and feed intake in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.







Pearson Correlation = 0.90, p<0.05

Figure 7: The positive correlation between dietary trypsin levels and protein efficiency ratio in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

Measurements of the total activity and specific activity of trypsin in the larval body revealed the lowest levels of  $0.224\pm0.001$  and  $0.01\pm0.01$  IU/mg

protein, respectively, in T5 (p<0.05). Among the other treatments, T2 presented the highest enzyme activity (p<0.05). Similar to the results of enzyme activity, protein content was lowermost (0.48±0.001 mg/mL) in T5 (p < 0.05), with a negative correlation coefficient between total enzyme activity and increasing dietary trypsin supplementation (p < 0.05, r = -0.87). Similar results were observed for specific enzyme activity (p < 0.05, r= -0.88). In addition, a negative correlation coefficient was observed between dietary trypsin supplementation and protein content in the larval body (p < 0.05, r = -0.77).

Based on the results of larval carcass analysis at the end of the 4-week experiment (Table 3), carcass protein contents were different in all treatments (p<0.05), with higher levels in trypsincontaining treatments than in the control fish (p < 0.05), as well as in T3, T4, and T5 than those of T1 and T2. T5 and the control treatment contained higher and lower protein levels, respectively than the other treatments (p < 0.05). The carcass fat content of S. hasta larvae was significantly different in all treatments, with significantly lower levels in trypsin-fed fish than in the control treatment. The highest and lowest fat levels were observed in the control treatment and T4 and T2, respectively (p < 0.05). There was no significant difference between T2 and T4 (p>0.05), but these two treatments differed significantly from the other treatments (*p*<0.05).

 Table 3: Carcass analysis of Sparidentex hasta larvae fed with diets containing different levels of trypsin (commercial trypsin and trypsin extracted from Acanthopagrus latus viscera).

Parameter (% in dry basis)	<b>T1</b>	T2	Т3	Т4	Т5
Crude protein	$13.42 \pm 0.09^{e}$	15.42±0.04 c	15.90±0.04 b	14.54±0.04 d	16.11±0.01 a
Fat	5.03±0.2 ª	$4.01 \pm 0.10^{d}$	$4.24{\pm}0.06^{\circ}$	$3.90{\pm}0.02^{d}$	$4.42{\pm}0.05^{b}$
Ash	$4.84{\pm}0.06^{a}$	$4.43 \pm 0.01$ <sup>c</sup>	$4.30{\pm}0.02^{d}$	$4.63 \pm 0.03^{b}$	$4.12 \pm 0.06^{e}$
Moisture	76.72±0.10 b	76.15±0.06 c	75.55±0.01	76.93±0.08 ª	75.35±0.10 e

Values are represented as mean $\pm$ STD. Different letters in each row indicate significant differences among treatments (*p*<0.05). T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

Carcass ash contents were significantly lower in all treatments containing the extracted trypsin than in the control fish. T5 and the control treatment contained the lowest and the highest ash levels, respectively, among the other treatments. There were significant differences between the results of all treatments (p<0.05). The examination of carcass moisture contents showed that T4 and T5 contained the highest and the lowest levels, respectively. The results of carcass moisture content differed significantly in all treatments (p<0.05).

#### Discussion

Understanding the condition and function of the GI tract is as important as the determination of nutritional and

environmental needs to specify the hormonal function, food digestion, and absorption processes in the critical stage of larval growth and development. The main feature of this stage is the sources of materials and energy required for larval growth from the yolk sac absorption to external feeding stages. To successfully pass through this stage, all organs related to food capture, digestion, and absorption must be prepared in larvae, and proper food must be available (Yufera and Darias, 2007). Most marine fish larvae within the second and third weeks of life have insufficient digestive capacity for using formulated feed because of their GI tract fully developed not considering structure and function (Nolting et al., 2001). Accordingly, the beginning of manual feeding in marine fish larvae is considered after the metamorphic cycle (Kolkovski, 2008). In the present study, adding the extracted trypsin to the diets of larvae resulted in acceptable effects on improvements in growth parameters at the end of the experimental period, in addition to the possibility of feeding after the 15<sup>th</sup> day of hatching. Moreover, there was also a positive correlation between increasing external trypsin to microdiets and BWG, SGR, daily growth rate, survival rate, and PER, and a negative correlation was found between increasing external trypsin to microdiets, FCR, and daily feed intake. In a study on S. hasta larvae for the early replacement of live feed with dry feed, the initiation of feeding with manual feed from day 18 did not negatively affect the growth compared with day 25

(Nazemroaya *et al.*, 2015a). It seems that the use of the diet supplemented with extracted trypsin in the present study could help to initiate feeding with manual feed 3 days earlier compared with the last study without negative effects.

In a previous study, the onset of early external feeding with manual feed reduced the growth and quality of larvae and increased the risk of developing abnormal skeletons (Kolkovski, 2008). Although there were adequate levels of digestive enzymes for the digestion of live feed in larvae, these amounts were not sufficient for the digestion of microdites as they contained a higher protein content (60-90% dry matter) while this is only 10% in zooplankton; thus, a high protein content makes the digestion of microdiets difficult for larvae (Kolkovski, 2001). Therefore, the initiation of active feeding with manual food or microdiets was examined with a simultaneous feeding protocol using manual and live feeds concurrently, which enabled the larvae to change faster and more efficiently from live feed to feeding on microdiets. This method leads to more growth and better survival than the method of using only live feed or microdiets. Early feeding of larvae with suitable microdiets provides better nutritional status and acceptance of microdiets by larvae (Kolkovski, 2008).

The use of external enzymes has been suggested as a method for the improvement of larval growth when using microdiets. Different results were observed in some studies by utilizing trypsin and other proteases extracted from mammals (e.g., pigs and cattle) (Yildirim and Turan, 2010; Kazerani and Shahsavani, 2011).

People Le Ruyet et al. (1993) suggested that if manual feeding could be started 15 days earlier in larval sea bass. Dicentrarchw labrax. it could replace 80% of Artemia. According to previous studies, however, the early use of manual food led to a weight loss of up to 30% and produced low-quality and poor larvae with skeletal problems. In the present study, the results of trypsin use showed that BWG was higher in all enzyme-fed treatments than in the control, and T5 with the highest trypsin supplementation and the trypsin-free control treatment attained the highest and the lowest BWG, respectively. There was also a significant difference between the results of T5 and the other treatments.

López-ALvarado et al. (2013) used pig pancreatin enzyme as the extracted trypsin in larval red sea bream, Pagrus major, and found that treatment with the highest level of the enzyme supplement (0.1% of diet) presented the highest SGR and survival rates. In a study on African catfish, Clarias gariepinus, increasing extracted trypsin by 0.75 g/kg of the diet increased SGR and PER and produced better FCR in large fish (Yildirim and Turan, 2010). Using commercial complex enzymes containing protease, lipase, and amylase could improve the growth performance of giant gourami (Osphronemus goramy) (Imaniy et al., 2022).

In general, the function of enzyme supplements can improve growth

parameters in two ways. This function can be considered both a supplement to digestive enzymes in larval bodies and an activator of zymogens (Kolkovski et al., 1993). Some researchers presented evidence that the addition of external enzymes to fish diets reduced the effect of anti-nutritional factors and improved the yield of dietary amino acids and energy use, thereby improving the growth performance of fish (Lin et al., 2007; Soltan, 2009). To explain the relationship between increasing dietary trypsin and elevated survival, increasing the enzyme in the GI tract simplifies the structure of nutrients and provides favorable conditions for increasing the beneficial microbial flora and thus the reduction of pathogens. Enzyme activity in the GI tract produces organic acids, including lactic acid, which enter the structure of pathogenic microbial flora and reduce their development (Ghomi et al., 2010). Finally, it should be borne in mind that food efficiency in the growth process of marine fish larvae depends on many internal and external factors. Moreover, the access, identification, and capture of food are influenced by many physicochemical factors, including color, shape, size, movements, and olfactory stimulation at the molecular level (Kolkovski, 2008).

Measurement of trypsin enzyme activity (after day 41 of hatching) revealed that the effect of the commercial enzyme with higher purity and activity was less than the enzyme extracted from the viscera of *A. latus*, which can be due to a variety of reasons. Better growth performance in treatments receiving extracted trypsin more indicated that trypsin functioned well and improved the digestive process in larvae by binding to available substrates, and therefore its activity was minimal at the end of the experimental period (Caruso et al., 2009). Additionally, develop enzymes during the developmental period in marine fish larvae depending on the species, temperature, and the type of available food (Kolkovski, 2008). In S. hasta larvae, a continuous change in the relative activity of proteases from alkaline to acidic during larval development indicates the fact that alkaline proteases are not the main enzymes of protein digestion, and trypsin levels will decrease from day 40 onwards with the development of the GI tract by the presence of pepsin (Nazemroaya et al., 2015b). It can, therefore. be concluded that the decreased trypsin enzyme activity in the larvae that received more extracted trypsin resulted in an elevated larval development and growth process, as well as a decrease in trypsin levels with the stomach development. The results of the correlation between enzyme activity in the larval bodies and increasing the extracted trypsin cannot be considered a negative function of the extracted trypsin. The negative correlation between the total and specific activities of trypsin in larval bodies with increasing the extracted trypsin might indicate the increased gastric development in the larvae.

In a similar study on juvenile yellow perch (*Perca flavescens*) fed with diets

containing 1% of enzyme supplements (bombesin and pancreatin), the specific activities of trypsin and chymotrypsin enzymes did not change significantly compared to other treatments, which was attributed to the development of the GI tract and the enzyme secretion process (Kolkovski *et al.*, 2000).

The use of enzymes in fish food can not only affect growth and reduce nutritional costs but also increase protein levels in the carcasses of produced fish. In vertebrates, digestion generally takes place in two ways, through enzymes produced by the food host and the other through enzymes secreted by the bacterial flora in the GI tract (Kar and Gosh, 2008). Besides improving diet efficiency, the secretion of enzymes facilitates the digestion process and ultimately increases protein retention, leading to increases in nutrients, including carcass protein. In addition to improving the digestion of protein compounds. the activity of such enzymes as protease leads to the better absorption of these compounds and thereby increases the carcass crude protein content (Ghobadi et al., 2009).

The carcass chemical composition indicated that the protein content was significantly higher in the body of larvae that received the extracted trypsin than in the extract-free treatment, which increased with rising dietary levels of the extracted trypsin. Using the extracted enzyme yielded better results even at low levels than the commercial enzyme in terms of carcass protein content in larvae. In contrast, fat and ash contents were higher in the bodies of larvae with no trypsin supplementation and even in those fed the commercial enzyme than in the other treatments.

The elevated carcass protein was in line with the improvement of growth parameters, which might eventually be due to the increased levels of enzymes responsible for dietary protein digestion, resulting in more protein retention in larval carcasses. In a study on the African catfish, C. gariepinus, protein content was measured at 21.75% by increasing the enzyme supplementation by 0.75 g/kg diet in large fish, which was higher than treatments with less and enzyme supplementation without (Yildirim and Turan, 2010).

In conclusion, utilizing enzymes extracted from the viscera of the marine fish, A. latus, at 1000 and 1500 IU/kg of the diet yielded similar results to using the commercial enzyme at a level of 500 IU/kg of the diet. In addition, better results can be achieved by increasing 2000 IU/kg of the diet, thus producing S. hasta larvae with better growth, reduced production costs, and larval survival improvement. Therefore, this study demonstrates that unvalued marine fish waste can be used to produce valuable products with proper management to improve the growth of fish larvae and reduce environmental pollution by affecting feeding efficacy. It can be concluded that the supplementation of manual feed with enzymes can be used to start manual feeding at a younger age and reduce the period of feeding with live feeds.

#### References

Ai, Q.H., Mai, K., Zhang, W., Xu, W., Tan, B., Zhang, C. and Li, H., 2007. Effects of exogenous enzymes (phytase, non-starch polysaccharide enzyme) in diets on growth, feed utilization, nitrogen and phosphorus excretion of Japanese seabass. Lateolabrax japonicas. Comparative Biochemistry and Physiology A, 1, 502-508. DOI:

10.1016/j.cbpa.2007.01.026

- Alvarez-González, C.A., Movano-López, F.J., Civera-Cerecedo, R., Carrasco-Chávez, V., Ortíz Galindo, J.L., Nolasco-Soria, H., Tovar-Ramírez, D. and Dumas, S., 2010. Development of digestive enzyme activity in larvae of spotted sand bass Paralabrax maculatofasciatus II: electrophoretic analysis. Fish Physiology and *Biochemistry*, 36(1), 29–37. DOI: 10.1007/s10695-008-9276-4
- AOAC (Association of Official Analytical Chemists), 1995. Official methods of analysis of the association official analytical chemists. 16th edition. AOAC, Inc., Arlington, Virginia, USA.
- Caruso, G., Denaro, M.G. and Genovese, L., 2009. Digestive Enzymes in Some Teleost Species of Interest for Mediterranean Aquaculture. *Open Fish Science Journal*, 2, 74-86. DOI: 10.2174/1874401X00902010074
- Erlanger, B.F., Kokowsky, N. and Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin.

*Archives* of *Biochemistry and Biophysics*, 95, 271–278. DOI: 10.1016/0003-9861(61)90145-X

- Ghobadi, Sh., MatinFar, A., Nezami, Sh.A. and Soltani, M., 2009. Influence of supplementary enzymes Avizyme on fishmeal replacement by soybean meal and its effects on growth performance and survival rate of rainbow trout (*Oncorhynchus mykiss*). New Technologies in Aquaculture Development, 2, 11-22.
- Ghomi, MR., Heshmatipour, Z., Nazari, R.M., Sohrabnezhad, M., Zarei, M., Nikoo, M., Ovissipour, M. and EsmaeiliMolla, A., 2010. Intestinal micofora of Kutum *Rutilus frisii kutum* under dietary supplementation with probiotic and vitamin C. *Bulgarian Journal of Agricultural Science*, 16(5), 635-642.
- Hamre, K., Yúfera, M., Ronnestad, I. and Boglione, C., 2013. Fish larval nutrition and feed formulation: Knowledge gaps and bottlenecks for advances in larval rearing. *Reviews in Aquaculture*, 5, S26–S58. DOI: 10.1111/j.1753-5131.2012.01086.x
- Imaniy, A.N., amid, M.L. and Budi, D.S., 2022. The effects of exogenous dietary enzyme on the growth of giant gourami (Osphronemus goramy). IOP Conf. Series: Earth and Environmental Science, 1036. 012108 DOI: 10.1088/1755-1315/1036/1/012108
- Javahery, S., Noori, A. and Hoseinifar, S.H., 2019. Growth performance, immune response, and digestive enzyme activity in Pacific white shrimp, *Penaeus vannamei*

Boone, 1931, fed dietary microbial lysozyme. *Fish and Shellfish Immunology*, 92, 528–535. DOI: 10.1016/j.fsi.2019.06.049

- Kar, N. and Ghosh, K., 2008. Isolation and Characterization of Extracellular Enzyme Producing Bacilli in the Digestive Tracts of *Rohu, Labeo rohita* (Hamilton) and Murrel, *Channa punctatus* (Bloch). *Asian Fisheries Science*. 21, 421-434.
- Kazerani, H.R. and Shahsavani, D., 2011. The effect of supplementation of feed with exogenous enzymes on the growth of common carp (*Cyprinus carpio*). Iranian Journal of Veterinary Research, 12(2), 31-35.
- Khandagale, A.S., Mundodi, L. and Sarojini, B.K., 2017. Isolation and characterizati on of trypsin from fish viscera of Oil Sardine (*Sardinella longiceps*). *International Journal of Fisheries and Aquatic Studies*, 5, 33-37.
- Khangembam, B.K. and Chakrabarti, R., 2015. Trypsin from the digestive system of carp *Cirrhinus mrigala*: purification, characterization and its potential application. *Food Chemistry*, 175, 386-394. DOI: 10.1016/j.foodchem.2014.11.140
- Khantaphant, S. and Benjakul, S., 2010. Purification and characterization of trypsin from the pyloric caeca of brownstripe red snapper (*Lutjanus vitta*). Food Chemistry, 120, 658-664. DOI: 10.1016/j.foodchem.2009.09.098
- Kim, M. and Jeong, Y., 2013. Purification and Characterization of a Trypsin-Like Protease from Flatfish

(*Paralichthys olivaceus*) Intestine. Journal of Food Biochemistry, 37, 732-741. DOI: 10.1111/j.1745-4514.2012.00672.x

- Kolkovski, S., Tandler, A., Kissil, G.W. and Gertler A., 1993. The effect of dietary exogenous digestive enzymes on ingestion, assimilation, growth and survival of gilthead seabream (*Sparus aurata*, Sparidae, Linnaeus) larvae. *Fish Physiology* and *Biochemistry*, 12, 203-209. DOI: 10.1007/BF00004368
- Kolkovski, S., Yackey, C. and Czesny, S., 2000. The effect of microdiet supplementation of dietary digestive enzymes and a hormone on growth and enzyme activity in Yellow Perch juveniles. *North American Journal of Aquaculture*, 62,130–134. DOI: 10.1577/1548-

8454(2000)062<0130:TEOMSO>2.0 .CO;2

- Kolkovski, S., 2001. Digestive enzymes in fish larvae and juveniles implications and applications to formulated diets. *Aquaculture*, 200(1-2), 181-201. DOI: 10.1016/S0044-8486(01)00700-1
- Kolkovski, S. 2008. Advances in marine fish larvae diets. Editores: L. Elizabeth Cruz Suárez, Denis Ricque Marie, Mireya Tapia Salazar, Martha G. Nieto López, David A. Villarreal Cavazos, Juan Pablo Lazo y Ma. Teresa Viana. Avances en Nutrición Acuícola IX. IX Simposio Internacional de Nutrición Acuícola. 24-27 Noviembre. Universidad Autónoma Nuevo de León,

Monterrey, Nuevo León, México. pp 20-45.

Ktari, N., Ben Khaled, H., Nasri, R., Jellouli, K., Ghorbel, S and, Nasri, M., 2012. Trypsin from zebra blenny (*Salaria basilisca*) viscera: Purification, characterisation and potential application as a detergent additive. *Food Chemistry*, 30, 467-474. DOI:

10.1016/j.foodchem.2011.07.015

- Lin, S., Mai, K. and Tan, B., 2007. Effects of exogenous enzyme supplementation in diets on growth and feed utilization in tilapia (Oreochromis niloticus x O. aureus). Aquaculture Research, 38, 1645-1653. DOI: 10.1111/j.1365-2109.2007.01825.x
- López-Alvarado, J., 2013. Effects of dietary enzymes on the larval performance of the Red sea bream (*Pagrus major*). International Journal of Science and Research. 4(4), 24-27.
- Marte, C.L. and Toledo J.D., 2015. Marine fish hatchery: developments and future trends. Proceedings of the International Workshop on Resource Enhancement and Sustainable Aquaculture Practices in Southeast Asia 2014(RESA). Philippines. pp. 189-200.
- Mirali, A., Movahedinia, A., Abdi, R. and Salati, A., 2013. Histological structure of kidney in Sobaity, *Sparidentex hasta. Journal of Marine Biology.* 5(2), 71-80
- Munilla-Moran, R., Stark, J.R. and Barbour A., 1990. The role of exogenous enzymes on the digestion

of the cultured turbot larvae, Scophthdmus mclximus L. Aquaculture, 88, 337-350. DOI: 10.1016/0044-8486(90)90159-K

- Namjou, F., Yeganeh, S., Madani, R. and Ouraji, H., 2019. Extraction, Purification, and Characterization of Trypsin obtained from the digestive system of Yellowfin Seabream (*Acanthopagrus latus*). Archives of Razi Institute, 74(4), 405-411. DOI: 10.22092/ari.2018.122854.1229
- Nazemroaya, S., Nematollahi, M., Yazdanparast, R., Farahmand, H. and Mirzadeh, Q., 2015a. Effect of early weaning on Sobaity sea bream larvae performance (*Sparidentex hasta*). Journal of Fisheries (Iranian Journal of Natural Resources), 86, 310-328. [in Farsi]
- Nazemroaya, S., Yazdanparast, R., Ali Nematollahi, M., Farahmand, H. and Mirzadeh, Q., 2015b. Ontogenetic development of digestive enzymes in Sobaity sea bream *Sparidentex hasta* larvae under culture condition. *Aquaculture*, 448, 545–551. DOI: 10.1016/j.aquaculture.2015.06.038
- Nolting, M., Ueberschär, B. and Rosenthal, H., 2001. Trypsin activity and physiological aspects in larval rearing of European sea bass (*Dicentrarchus labrax*) using live prey and compound diets. *Journal of Applied Ichthyology*, 15, 138–142. DOI: 10.1046/j.1439-0426.1999.00138.x
- People-le Ruyet, J., Alexandre, J.C.,Thebaud, L. and Mugnier, C.,1993. Marine fish larvae feeding:

Formulated diets or live prey. *Journal* of *the World Aquaculture Society*, 24(2), 211–224. DOI: 10.1111/j.1749-7345.1993.tb00010.x

- Prasertsan, P., Jitbunjerdkul, S. and
  Prachumratana T., 2003.
  Production of Enzyme and Protein
  Hydrolysate from Fish Processing
  Waste. In: Roussos S., Soccol C.R.,
  Pandey A. and Augur C. (eds) New
  Horizons in Biotechnology. Springer,
  Dordrecht, Netherlands. pp 63-71.
  DOI: 10.1007/978-94-017-0203-4\_7
- Rachmawati, D., Hutabarat, J., Samidjan, I. and Windarto, S., 2020. Utilization of papain as feed additive in the fish feed on activity of digestive enzymes, contents of nutrient and minerals of Sangkuriang catfish (*Clarias gariepinus* var. *Sangkuriang*). AACL Bioflux, 13(5), 2738-2744.
- Ramakrishnan, V.V., Ghaly, A.E.,
  Brooks, M.S. and Budge, S.M.,
  2013. Enzymatic extraction of amino acids from fish waste for possible use as a substrate for production of Jadomycin. *Enzyme Engineering*, 2(2), 1-9. DOI: 10.4172/2329-6674.1000112
- Ramzanzadeh, F., Yeganeh, S., JaniKhalili, Kh. and Babaei, S.S., 2016. Effects of different photoperiods on digestive enzyme activities in rainbow trout (Oncorhynchus mykiss) alevin and fry. Canadian Journal of Zoology, 94, 435-442. DOI: 10.1139/cjz-2015-0180
- Silva, J.F., Espósito, T.S., Marcuschi, M., Ribeiro, K., Cavalli,

**R.O., Oliveira, V. and Bezerra, R.S., 2011.** Purification and partial characterisation of a trypsin from the processing waste of the silver mojarra (*Diapterus rhombeus*). *Food Chemistry*, 129, 777-782. DOI: 10.1016/j.foodchem.2011.05.019

- Soltan, M.A., 2009. Effect of dietary fishmeal replacement by poultry byproduct meal with different grain source and enzyme supplementation on performance, faeces recovery, body composition and nutrient balance of Nile tilapia. *Pakistan Journal of Nutrition*, 8, 395–407. DOI: 10.3923/pjn.2009.395.407
- Walford, J., Lam, T.M. and Lam, T.J., 1991. Replacing live feeds with microencapsulated diets in the rearing of seabass (*Lates calcarifer*) larvae: do the larvae ingest and digest protein-membrane microcapsules? *Aquaculture*, 92, 225–235. DOI: 10.1016/0044-8486(91)90024-2
- Yılmaz, M. and İkiz, R., 2006. Effects of Dietary Enzyme Supplement on

Growth of Gilthead Sea Bream (Sparus aurata L., 1758). E.U. Journal of Fisheries and Aquatic Sciences. 23 (1/2), 323-326.

Yildirim, Y.B. and Turan, F., 2010. Effects of exogenous enzyme supplementation in diets on growth and feed utilization in African catfish, *Clarias gariepinus. Journal* of *Animal Veterinary Advances*, 9(2), 327-331. DOI: 10.3923/javaa.2010.327.331

Yufera, M. and Darias M.J., 2007. The onset of exogenous feeding in marine fish larvae. *Aquaculture*, 268, 53–63. DOI:

10.1016/j.aquaculture.2007.04.050

Zhao, L.S., Budge, S.M., Ghaly, A.E. and Brooks, M.S., 2011. Extraction, purification and characterization of fish pepsin: a critical review. *Journal* of Food Processing and Technology, 2(6), 126 ref.129. DOI: 10.4172/2157-7110.1000126