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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.[1]; Yeganeh S.[1*]; Madani R.[2]; Ouraji H.[1]

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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.

1-Department of Fisheries, Faculty of Animal Science and Fisheries, Sari Agricultural Sciences and Natural Resources University, Sari, Iran
2-Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
*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 resistant and high-quality 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 in the absorption of microdiets (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 micro- and 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 by 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 8th day after hatching, larvae were fed with a combination of algae and small-size or the S-type rotifer

(*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 enzyme supplementation with live feed were added to the diet according to the co-feeding 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), pH (7.9±0.02), and dissolved oxygen (7.7±0.05 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^{-1}), D (1300 IE kg^{-1}), E (280 mg kg^{-1}), and C (350 mg kg^{-1}). 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

porcine pancreas) per kg of diet, and T3, T4, and T5 each containing 1000, 1500, and 2000 IU/kg, respectively, of dietary trypsin extracted from *A. latus* viscera.

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±93 g and a mean length of 213.67±29 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_1 and Wt_2 are initial and final weights, respectively.

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

Where, $LnWt_1$= the normal logarithm of the initial weight, $LnWt_2$= the normal logarithm of the final weight and $t_2\text{-}t_1$= the length of the test period

Daily Growth Rate (GR, g/day) = [Wt_2 (g)-Wt_1 (g)/ ($t_2\text{-}t_1$)]

Where, Wt_1= Initial weight, Wt_2= The final weight, and $t_2\text{-}t_1$= 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) =$100 \times$I (g)/[(Wt_2 (g) + Wt_1 (g))/2$\times$ ($t_2\text{-}t_1$)]

Where, I= total food eaten and $t_2\text{-}t_1$= 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, enzyme-extracted 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 $CaCl_2$) 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):

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

Where, 8800 (cm^2/mg) is the molar extinction coefficient for ρ-nitroaniline (Erlanger *et al*., 1961).

Carcass analysis

At the end of the fourth week, samples were taken randomLy from 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^{-1}) 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^{-1}) and the lowest (6.540±0.002% day^{-1}) 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).

Parameter	T1	T2	T3	T4	T5
Initial weight (g)	0.021±0.001 [a]	0.021±0.001 [a]	0.021±0.001 [a]	0.021±0/001 [a]	0.021±0.001 [a]
Final weight (g) 41 (DAH*)	0.110±0.000 [c]	0.128±0.003 [b]	0.123±0.006 [b]	0.132±0.003 [b]	0.142±0.003 [a]
Weight gain (g)	0.09±0.00 [d]	0.108±0.003 [bc]	0.103±0.006 [c]	0.112±0.002 [b]	0.121±0.003 [a]
Specific growth rate (%/day)	6.540±0.002 [d]	7.148±0.079 [bc]	6.989±0.174 [c]	7.247±0/072 [b]	7.523±0.079 [a]
Daily growth Rate (g/day)	0.0034±0.0000 [d]	0.0042±0.0001 [bc]	0.0040±0.0002 [c]	0.0043±0.0001 [b]	0.0047±0.0001 [a]
Feed conversion ratio	1.11±0.003 [a]	0.91±0.018 [bc]	0.95±0.052 [b]	0.88±0.019 [c]	0.80±0.020 [d]
Daily feed intake (%/day)	5.79±0.01 [a]	5.03±0.07 [bc]	5.21±0.21 [b]	4.91±0.08 [c]	4.60±0.08 [d]
Survival rate (%)	95.23±0.21 [c]	95.83±0.42 [b]	96.07±0.23 [ab]	96.10±0.17 [ab]	96.37±0.06 [a]
Protein Efficiency Ratio	0.0017±0.000 [d]	0.0021±0.000 [bc]	0.0020±0.000 [c]	0.0021±0.000 [b]	0.0023±0.000 [a]

Values are represented as mean±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.

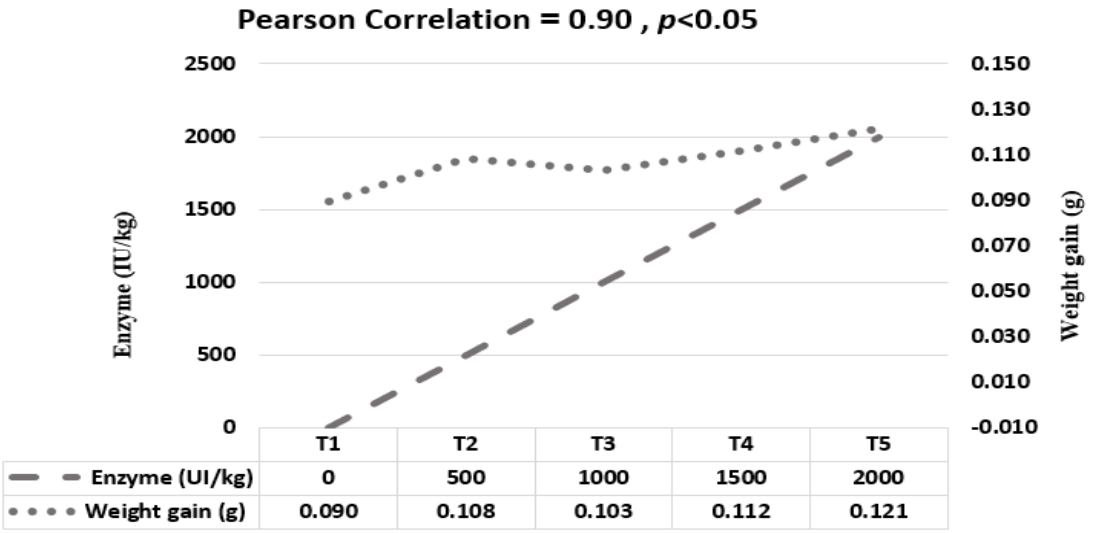

Figure 1: The positive correlation between dietary trypsin levels and body weight gain in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted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

Daily growth rates were uppermost in treatments containing enzyme supplements, with T5 (0.0047±0.0001 g day^{-1}) and T1 (0.0034±0.001 g day^{-1}) 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).

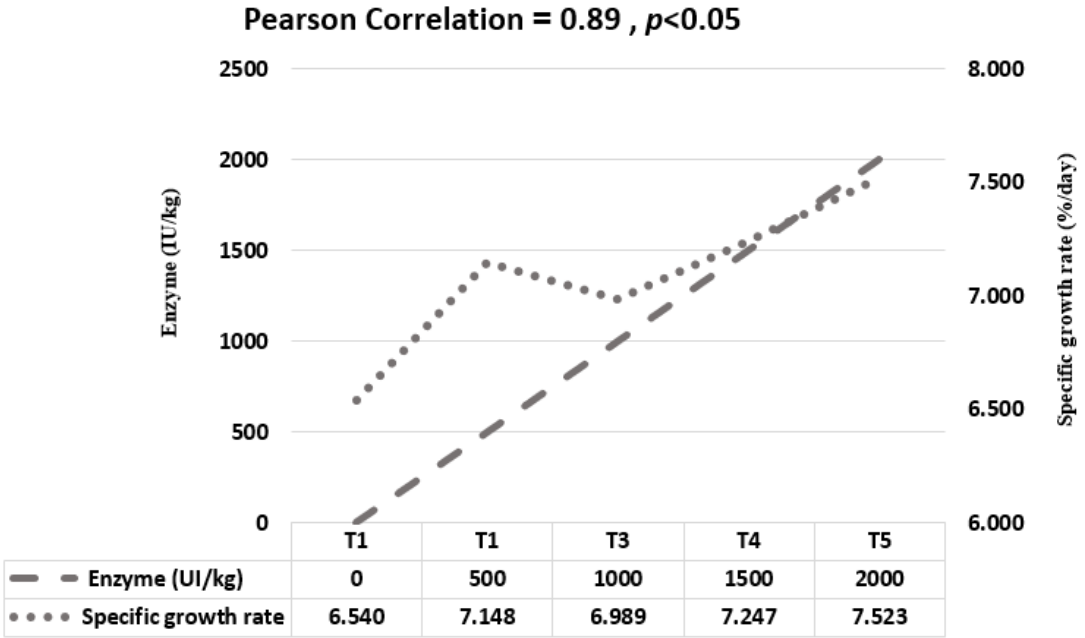

Figure 2: The positive correlation between dietary trypsin levels and specific growth rate in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted 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	T3	T4	T5
Total activity (IU)	9.80±0.006 ^{c}	10.61±0.012 ^{a}	10.30±0.010 ^{b}	2.72±0.006 ^{d}	0.224±0.001 ^{e}
Specific activity (IU/mg protein)	0.08±0.001 ^{a}	0.06±0.002 ^{c}	0.07±0.002 ^{b}	0.05±0.001 ^{d}	0.01±0.001 ^{e}
Protein content (mg/mL)	2.60±0.006 ^{c}	3.90±0.010 ^{a}	3.20±0.010 ^{b}	1.14±0.001 ^{d}	0.48±0.001 ^{e}

Values are represented as mean±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±0.08% d^{-1} and 5.79±0.01 % d^{-1} (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±0.00) than that of the control treatment (0.0017±0.00) (p<0.05). Accordingly, a positive correlation coefficient was obtained between PER and the trypsin increase (Fig 7; p<0.05, r=0.90).

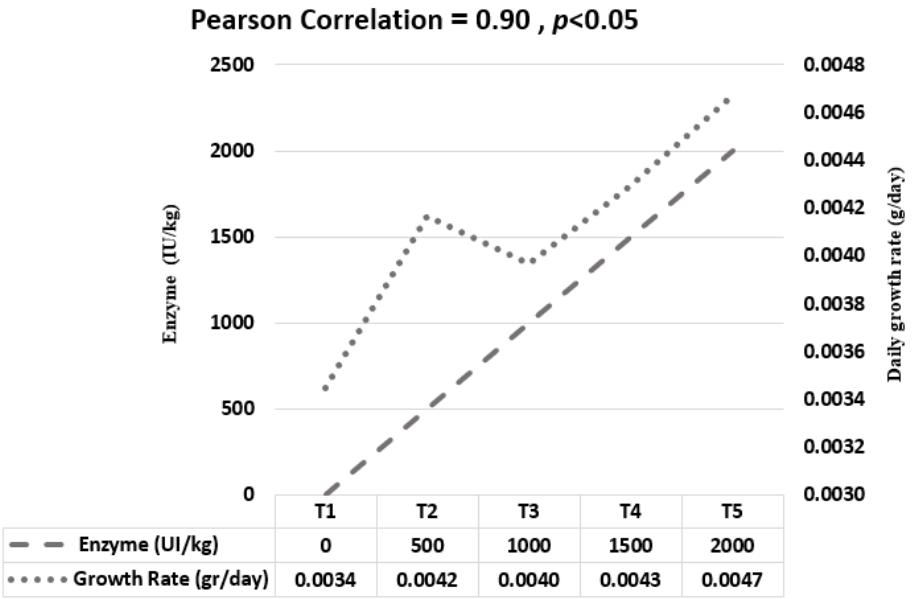

Figure 3: The positive correlation between dietary trypsin levels and daily growth rate in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted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

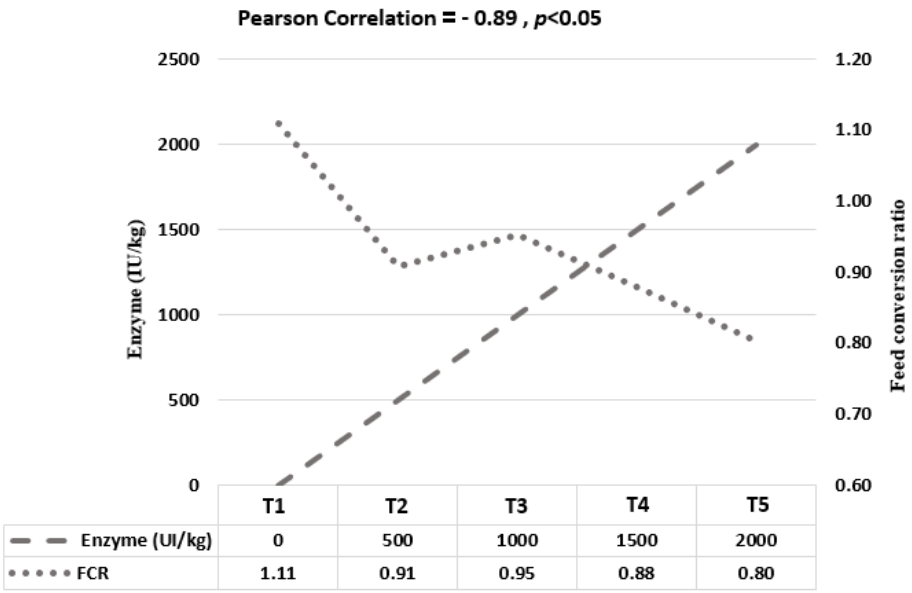

Figure 4: The negative correlation between dietary trypsin levels and feed conversion ratio in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted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

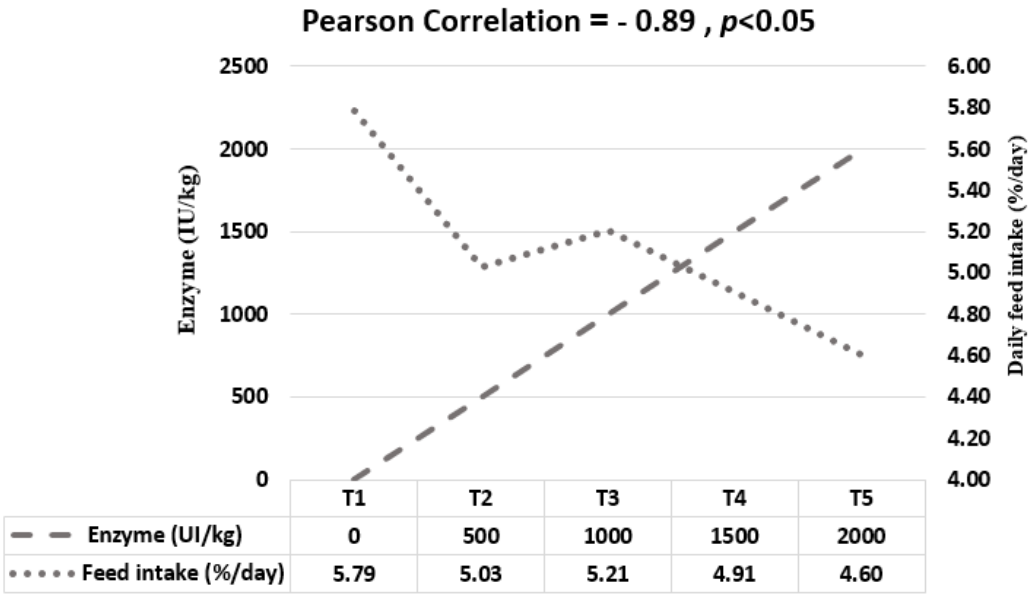

Figure 5: The negative correlation between dietary trypsin levels and feed intake in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from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

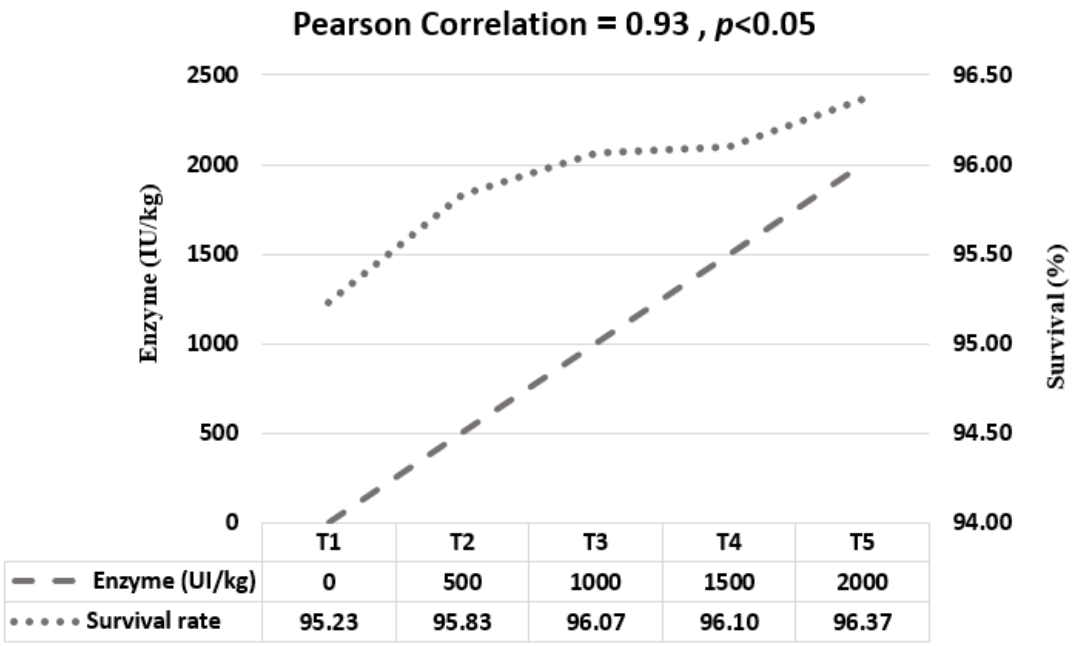

Figure 6: The positive correlation between dietary trypsin levels and survival rate in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin extracted from *Acanthopagrus latus* viscera, respectively.

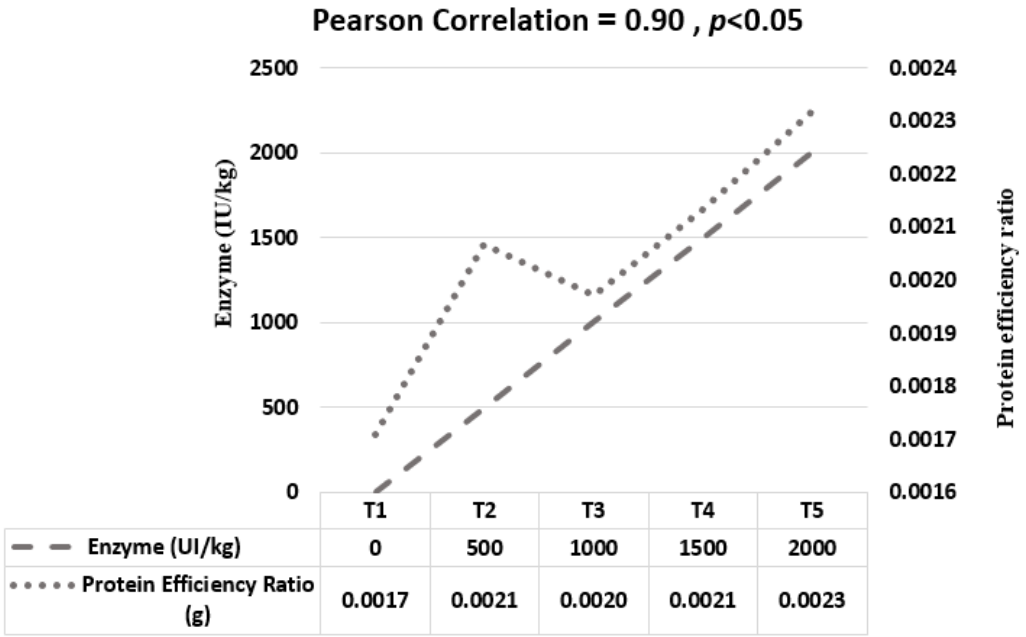

Figure 7: The positive correlation between dietary trypsin levels and protein efficiency ratio in *Sparidentex hasta* larvae. T1 (control): 30 rotifers/mL without trypsin, T2: 500 IU/kg of a commercial trypsin (derived from the porcine pancreas), and T3, T4, and T5: 1000, 1500, and 2000 IU/kg trypsin 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±0.001 and 0.01±0.01 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 trypsin-containing 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)	T1	T2	T3	T4	T5
Crude protein	13.42±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±0.10 d	4.24±0.06 c	3.90±0.02 d	4.42±0.05 b
Ash	4.84±0.06 a	4.43±0.01 c	4.30±0.02 d	4.63±0.03 b	4.12±0.06 e
Moisture	76.72±0.10 b	76.15±0.06 c	75.55±0.01 d	76.93±0.08 a	75.35±0.10 e

Values are represented as mean±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 not fully developed 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 15th 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 more extracted trypsin 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, enzymes develop 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 without enzyme supplementation (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.

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