

Research Article

Changes in Digestive Enzyme Activities during Larval Development of Spotted Seatrout (*Cynoscion nebulosus*)

Martín Alberto Arenas-Pardo ¹, Martha Gabriela Gaxiola-Cortés ²,
Alvaro Fabricio Barreto-Altamirano ², Adriana del Carmen Paredes-Medina,²
Iveth Gabriela Palomino-Albarrán,² Patricia Margarita Balam-Uc,²
Juan Carlos Maldonado-Flores ², and Carlos Alfonso Álvarez-González ¹

¹Laboratorio de Fisiología en Recursos Acuáticos, División Académica de Ciencias Biológicas, Universidad Juárez Autónoma de Tabasco (UJAT), 0.5 km Carretera Villahermosa-Cárdenas, 86000, Villahermosa, Tabasco, Mexico

²Unidad Multidisciplinaria de Docencia e Investigación (UMDI) Sisal, Facultad de Ciencias,

Universidad Nacional Autónoma de México (UNAM), Puerto de Abrigo, 97356, Sisal, Yucatán, Mexico

Correspondence should be addressed to Martha Gabriela Gaxiola-Cortés; mggc@ciencias.unam.mx

Received 28 November 2023; Revised 8 April 2024; Accepted 15 April 2024; Published 8 May 2024

Academic Editor: Yanjiao Zhang

Copyright © 2024 Martín Alberto Arenas-Pardo et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The spotted seatrout (*Cynoscion nebulosus*)—an important commercial species—has a high potential for aquaculture in the Gulf of Mexico. To optimize its feeding during larval rearing, this study aims to evaluate the primary gastric (pepsin), intestinal (leucine aminopeptidase and alkaline phosphatase), and pancreatic (alkaline protease, trypsin, chymotrypsin, amylase, and lipase) enzyme activities from hatching to day 30. A multivariate analysis identified three digestive enzyme development stages during the spotted seatrout larval transformation. The first stage occurred between 1 (mean \pm standard error (SE) = 1.73 ± 0.14 millimeter (mm) standard length (SL)) and 3 (2.14 ± 0.07 mm SL) days after hatching (DAH); a period of digestive stability showed the highest activity in amylase and bile salt-dependent lipase. The second stage (from 4 (2.53 ± 0.09 mm SL) to 20 (10.92 ± 0.51 mm SL) DAH) was a period of digestive transition, during which leucine aminopeptidase, chymotrypsin, and alkaline proteases were identified as the predominant enzymes from 4 to 5 DAH. In the third stage—a period of digestive stability—pepsin was the major enzyme that occurred between 25 (16.51 ± 0.81 mm SL) and 30 (25.91 ± 0.82 mm SL) DAH. These results indicate that the spotted seatrout larvae have a digestive system adapted to lipids and carbohydrates at the onset of feeding, with an immediate transition to protein digestion when exogenous feeding begins. Additionally, the digestive system of the spotted seatrout may be considered mature at 25 DAH. Further research is needed to elucidate the mechanisms of digestive tract development in the spotted seatrout larvae.

1. Introduction

The larval stage is one of the most critical factors in marine fish culture. Fish larvae necessitate specific biotic and abiotic conditions to ensure their survival, proper development, and growth [1]. One of the major bottlenecks in larval fish culture is the high mortality during the transition from endogenous to exogenous feeding and the weaning period [2]. During the transition from larva to juvenile, the digestive tract undergoes major anatomical and functional changes from a short and straight tube (immature) to a segmented and histologically differentiated tract (mature) [3]. Simultaneously, there

exists a sequential chronology in the variation of gastric, pancreatic, and intestinal enzymes; nevertheless, the timing of these processes varies among species, dependent on their life histories [4]. Knowledge of the development of digestive enzymes during the transformation from larvae to juveniles provides information on the digestive process that can be synchronized with feeding and rearing protocols [5]. In this sense, numerous studies on ontogenetic changes in the digestive enzymes of fish larvae have been conducted to understand their digestive physiology [6–12]. Identifying physiological biomarkers of the digestive system in fish larvae has allowed

the appropriate design of compound diets and pertinent weaning during larviculture [5, 13].

The spotted seatrout (*Cynoscion nebulosus*, Sciaenidae) is a carnivorous marine fish distributed along the Western and Northwestern Atlantic coasts [14]. This species is of high value for commercial and recreational fishing in Mexico and the United States of America [15, 16]. The spotted seatrout is a euryhaline [17], eurythermal [18], fast-growing species with a low food conversion rate [19, 20]; its reproduction is possible in captivity [21], a characteristic that makes it suitable for aquaculture [22]. In this context, protocols have been developed to induce reproduction and spawning in captivity for the spotted seatrout, both naturally and by using exogenous hormone therapies [23–25]. However, to our knowledge, no studies have been available on the ontogenetic development of the digestive system in the spotted seatrout larvae. Thus, the present study aims to evaluate the development of digestive enzyme activity (stomach, pancreas, and intestine) in spotted seatrout larvae to provide basic knowledge on digestive physiology to optimize their feeding protocols and weaning processes.

2. Materials and Methods

2.1. Egg Production and Larviculture. Wild adults of the spotted seatrout were captured at Sisal Beach, Yucatán, during their breeding season in May 2023 and transported to the Unidad Multidisciplinaria de Docencia e Investigación (UMDI) Sisal, Universidad Nacional Autónoma de México (UNAM). Seven females (606.8 ± 137 g (mean \pm standard deviation (SD))), capable of spawning according to Lowerre-Barbieri et al. [26], and three males (463 ± 26.9 g), exhibiting the presence of milt upon light abdominal pressure, were induced to spawn with a single injection ($1,100$ UI kg^{-1} body weight) of human chorionic gonadotropin (CG5, SIGMA) [21]. The broodstock was placed in an 18-m^3 cylindrical fiberglass tank (2 m in diameter, 1.5 m in height) under natural temperature and photoperiod (outdoor), utilizing a continuous water flow system and aeration. Spontaneous spawning occurred after 37 hr of hormonal induction. The eggs were collected in a 200-L capacity cylindrical fiberglass tank equipped with 0.5-mm mesh screens, positioned under the outflow of the spawning tank.

The viability of the collected eggs was assessed by recording the percentage of floating eggs. To estimate the total number of fertilized eggs, three 0.1-mL samples of floating eggs were counted, and the mean value of these samples was extrapolated to the total volume of floating eggs. Additionally, the mean diameter of the fertilized eggs was calculated by measuring three samples, each consisting of 100 fertilized eggs. Larval rearing was conducted in three cylindrical fiberglass tanks, each with a capacity of 450 L (100 cm in diameter, 58 cm in height). The initial stocking density was 95 fertilized eggs per liter, with 734.5 ± 0.0 μm in diameter. Hatching occurred 15 hr after incubation, and the eggs that did not hatch were removed with a siphon to avoid contamination by organic matter decomposition. The larval stocking density after hatching was 93 organisms per liter. The larval stock density after hatching was estimated by incubating

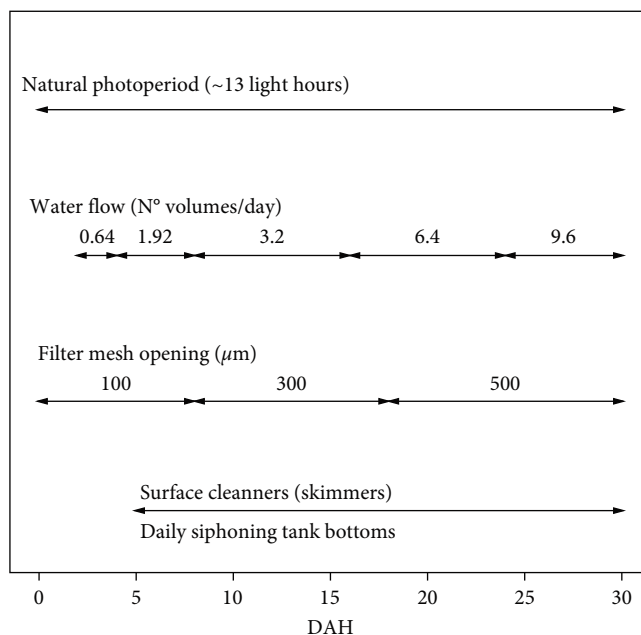


FIGURE 1: Water quality and environmental management protocols during larviculture of spotted seatrout (*C. nebulosus*).

three samples, each containing 100 fertilized eggs, in 2-L beakers.

A water quality and environmental management protocol for egg incubation and larval rearing is shown in Figure 1. The seawater used for larval rearing was treated in a pressurized sand filter (SD70, Pentair Inc., MN, USA), a multicartridge filter (P005 and P010, Filter Specialists, Inc., MI, USA), and an ultraviolet-visible (UV) lamp (R-pkbal.plat30, Purikor, NL, Mexico). Water quality during egg incubation and larval rearing were salinity (35 g/L), dissolved oxygen (4.8 ± 0.1 mg/L), temperature ($28.9 \pm 0.8^\circ\text{C}$), pH (7.8 ± 0.0), ammonia (0.3 ± 0.1 mg/L), nitrites (0 mg/L), and nitrates (7.2 ± 5.0 mg/L).

Table 1 shows the feeding regime used during the rearing period. The yolk was absorbed 15–24 hr after hatching, and then the larvae were fed four times a day (6:00, 10:00, 14:00, and 18:00 hr). The feeding regime was based on live food and commercial feeds; larvae were fed with rotifers (*Brachionus rotundiformis*) from 2 to 15 days after hatching (DAH) and nauplii and metanauplii of *Artemia franciscana* Biogrow[®] (Provedora de Insumos Acuicolas, S. A. de C.V. Mazatlán, SIN, MX; protein 70.2%, lipid 20.8%, and ash 6.1%) from 9 to 17 and 16–30 DAH, respectively. Rotifers were enriched with microalgae (*Nannochloropsis oculata*) and Origreen (Skretting, Stavanger, Norway; protein 43%, lipid 30%, and ash 12%), whereas *Artemia* metanauplii were enriched with Selco[®] (INVE Aquaculture, Belgium). Dry-formulated food Otohime[®] (Marubeni Nisshin Feed Co., Ltd., Japan; protein 56.3%, lipid 15.9%, ash 13.5%, and particle size 75–1,410 μm) was provided to larvae from 9 to 30 DAH.

2.2. Fish Sampling. According to Blaylock et al. [22], the larval rearing period of spotted seatrout was 30 days after hatching. During larval rearing, a variable number of larvae were collected for measurement of digestive enzyme

TABLE 1: Feeding schedule and food types during larval rearing of the spotted seatrout (*C. nebulosus*).

Food item	Type	Density	Period (DAH)
Microalgae	<i>Nanocloropsis oculata</i>	0.5–10 × 10 ⁶ cells/mL	1–15
Enriched rotifer	<i>B. rotundiformis</i>	7–17 rotifer/mL	1–15
<i>Artemia</i> nauplii	<i>A. franciscana</i>	1–2 nauplii/mL	9–17
Enriched <i>Artemia</i> metanauplii	<i>A. franciscana</i>	2–4 metanauplii/mL	16–30
Otohime™	A1 (75–150 μm), A2 (150–250 μm), B1 (250–360 μm), B2 (360–650 μm), C1 (580–840 μm) and C2 (840–1,410 μm)	Ad libitum	9–12, 11–16, 15–20, 19–24, 23–28, 27–30

activities. Larvae were collected 3.5 hr after the first feeding of the day (at 09:30hr) to ensure an empty digestive tract [27]. Twenty larvae were collected at 1 DAH (eleutheroembryo), and 10 larvae were collected at 3, 4, 5, 7, 10, 15, 20, 25, and 30 DAH per tank using a 250-μm net. Larvae were euthanized using eugenol overdose under cold water conditions (0–4°C), rinsed with distilled water, frozen in liquid nitrogen, and stored at –80°C until analysis. The official Mexican Norm (NOM-062-ZOO-1999 from Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación) was followed for animal welfare practices. The standard length (SL) of each larva was obtained by measuring five larva samples per tank from the lower jaw to the notochord with an optical microscope (VE-BC3 Plus, Velab). Larval growth was obtained from 10 pooled samples/tank using an analytical balance (Ohaus, NJ, USA; precision of 10^{–4} g). Thermal age units (cumulative degree DAH, CTU) were used to compare closely related species. The temperature units accumulated by day 0 embryos were zero; day 1 fish accumulated the mean temperature of day 0, day 2 fish accumulated the mean temperature of day 0 and day 1, and so on.

2.3. Biochemical Analysis. Whole tissues of embryos and larvae were homogenized in 30 volumes (v/w) [28] of ice-cold (0–4°C) universal buffer (25 mmol, pH 7) using pellet pestles and an Ultra Turrax IKA T18 homogenizer (IKA Works, Inc., NC, USA). Homogenates were centrifuged at 9,000g/10 min at 4°C; the precipitates were discarded, and the supernatants were again centrifuged at 30,000g/30 min at 4°C. The resulting supernatants were separated to measure pepsin, total alkaline proteases, trypsin, chymotrypsin, amylase, lipase, and leucine aminopeptidase. The pellets resulting were dissolved in tris-mannitol buffer (50 mmol/L mannitol, 2 mmol/L tris; pH 7) to measure enzyme alkaline phosphatase [29].

A Biotek Synergy 2 SL microplate reader (BioTek Instruments, Inc., VT, USA) was utilized to determine the activity of the gastric, pancreatic, cytosolic, and brush border enzymes. Pepsin (EC 3.4.11.1) activity was measured using hemoglobin: HCl 0.5% as substrate, pH 2 [30]. Alkaline protease activity was determined according to Nolasco-Soria [31] using casein 0.25% as substrate in a universal buffer (50 mmol/L, pH 8). The quantity of enzyme that releases 1 μmol of amino groups per minute was used to define one unit (U) of pepsin and alkaline protease activity, utilizing the *o*-phthalaldehyde (OPA)-dithiothreitol (DTT) reagent [31] and measured at

330 nm. An L-serine standard curve was used to determine the number of hydrolyzed peptide bonds.

Trypsin (EC 3.4.21.4) activity was quantified using 1 mmol/L N-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA) as substrate in 50 mmol/L Tris-HCl and 10 mmol/L CaCl₂ at pH 8 [32]. Leucine aminopeptidase (EC 3.4.11.1) was assayed using 0.1 mmol/L leucine p-nitroanilide as substrate in 50 mmol/L sodium phosphate at pH 7.2, according to Maroux et al. [33]. Chymotrypsin (EC 3.4.21.1) was determined using 0.1 mmol/L N-succinyl-ala-ala-pro-phe p-nitroanilide (SAAPNA) as substrate in 50 mmol/L Tris-HCl and 10 mmol/L CaCl₂ at pH 7.8 [34]. One unit of enzymatic activity was defined as 1 μmol p-nitroanilide released per minute and measured at 410 nm. A p-nitroanilide curve was used to determine the hydrolyzed number of micromoles of p-nitroanilide.

Alkaline phosphatase (EC 3.1.3.1) activity was quantified using as substrate 1 mmol/L 4-nitrophenyl phosphate in 50 mmol/L Tris-HCl at pH 8.5 [29]. Bile salt-dependent lipase (EC 3.1.1.1) was determined according to Gjellesvik et al. [35], using as substrate 0.4 mmol/L 4-nitrophenyl caprylate in 50 mmol/L Tris and 6 mmol/L sodium taurocholate at pH 7.4. One unit of enzymatic activity was defined as 1 μmol p-nitrophenol released per minute and measured at 405 nm. A p-nitrophenol curve was used to determine the number of p-nitrophenol hydrolyzed micromoles.

Alpha amylase (EC 3.2.1.1) was performed using starch 1% as substrate in Tris-HCl 100 mmol/L at pH 8, utilizing the dinitrosalicylic (DNS) reagent [36]. One unit of enzymatic activity was defined as 1 μmol of glucose released per minute and measured at 540 nm. A glucose curve was used to determine the number of glucose hydrolyzed micromoles. The Bradford [37] technique was used to determine the soluble protein concentration in the samples using bovine serum albumin as a standard. All enzymatic measurements were performed at 37°C and made in triplicate. Calculation of the specific activity of the extracts was performed according to Nolasco-Soria [31].

2.4. Statistical Analysis. Data are presented as the mean ± standard error of the mean (SE) and standard deviation (SD). Statistical analyses and graphical visualizations of the enzymatic activity were conducted in R software version 4.2.2 [38]. A linear model was fitted, and data of digestive enzyme activities were modeled as a function of DAH. The Pearson residuals were plotted against each explanatory variable, and observed data versus fitted values were used to validate this

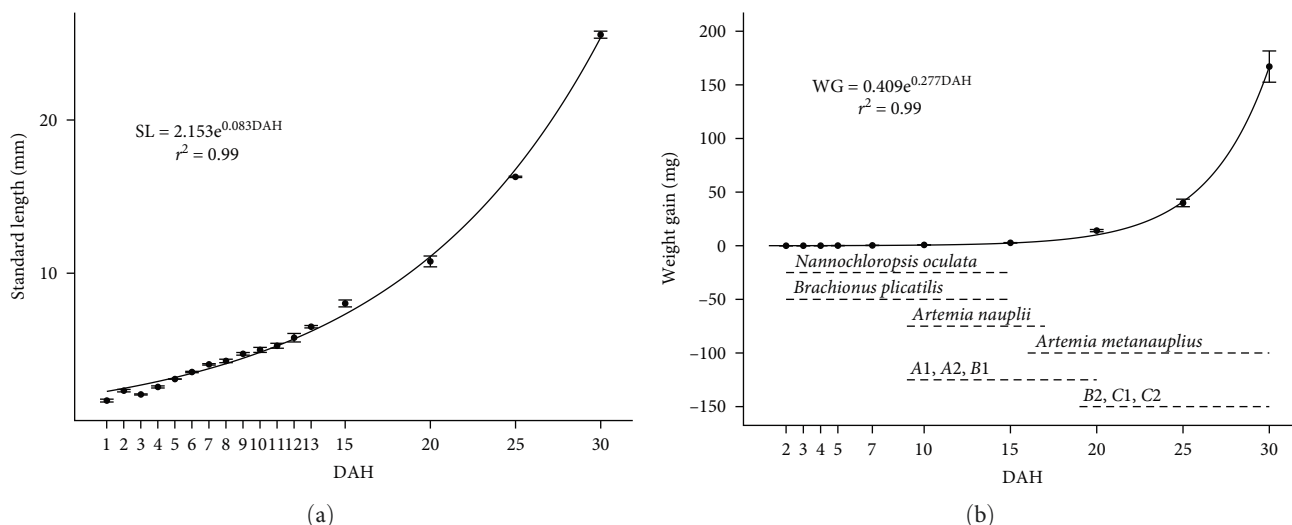


FIGURE 2: (a and b) Larval growth of the spotted seatrout (*C. nebulosus*) in terms of (a) standard length (mm) and (b) weight (mg) and feeding schedule with food type during the first 30 days after hatching (DAH). The results are expressed as mean \pm standard error (SE) ($n = 3$).

model. A p value < 0.05 was considered to be significant. When significant differences were observed, post hoc tests were performed using Tukey's adjustment from the Emmeans package [39]. Additionally, the patterns of variation in all the digestive enzymes for each DAH were evaluated using a multivariate analysis of variances (ANOVA) based on distances and permutations. Before the analysis, the data were scaled to zero mean and unit variance. The Euclidean distance between each pair of replicates was calculated. The total variation in this distance matrix was partitioned using a permutational multivariate analysis of variance (PERMANOVA) [40]. The null hypothesis for the DAH term in the model was created with 9,999 permutations of residuals under the reduced model. A matrix of centroids was calculated and projected in an ordination using the first two components of the principal component analysis (PCA) to visualize the pattern of similarity among DAH. Multivariate analyses were performed using the software PRIMER v7 and PERMANOVA [41].

3. Results

3.1. Larval Growth and Survival Rate. The spotted seatrout larvae showed a standard length (SL) of 2.39 ± 0.06 mm (mean \pm standard error (SE)) and a weight of 0.016 ± 0.00 mg at first feeding (86.7 CTU; 2 DAH); after 30 DAH (895.9 CTU), they reached a SL of 25.90 ± 0.37 mm and a weight of 167.04 ± 2.52 mg (Figure 2). During larval development, growth in SL and weight followed an exponential curve according to the following equations: $SL = 2.153e^{0.083DAH}$ ($r = 0.99$; $n = 3$) and $weight = 0.409e^{0.277DAH}$ ($r = 0.99$; $n = 3$). Survival at the end of the rearing period was 7.8%.

3.2. Digestive Enzyme Activities. The specific activities of pepsin and alkaline protease are shown in Figure 3. The specific activity of pepsin was first detected at high levels at 5 DAH (144.5 UTC; 10.56 ± 1.48 mU/mg protein) and decreased at 10 DAH (289 CTU; 4.11 ± 0.84 mU/mg protein) ($p < 0.05$); then, its activity gradually increased until 30 DAH (867 UTC;

12.4 ± 1.62 mU/mg protein) (Figure 3(a)). Specific alkaline protease activity was detected at low levels at 3 DAH (86.7 UTC; 4.43 ± 1.30 mU/mg protein) at the beginning of exogenous feeding and increased abruptly at 4 DAH (115.6 UTC; 63.41 ± 1.60 mU/mg protein) ($p < 0.05$), following a progressive decrease until 10 DAH (289 UTC; 5.33 ± 0.43 mU/mg protein) ($p > 0.05$), to remain constant until the end of the study (Figure 3(b)).

Changes in the specific activities of trypsin and chymotrypsin are shown in Figure 4. The specific activity of trypsin was detected at hatching (28.9 UTC; 5.81 ± 1.48 mU/mg protein). At 3 (86.7 UTC; 10.20 ± 1.29 mU/mg protein) and 7 DAH (202.3 UTC; 15.74 ± 2.41 mU/mg protein) ($p < 0.05$), trypsin showed maximum peaks in its specific activity. After 7 DAH, a decrease in specific activity was observed at 25 (722.5 UTC) and 30 DAH (867 UTC) with 1.19 ± 0.02 and 1.48 ± 0.08 mU/mg protein, respectively ($p < 0.05$). Chymotrypsin-specific activity started with low levels from hatching (28.9 UTC; 2.52 ± 0.69 mU/mg protein), and its activity increased 28-fold at 4 DAH (115.6 UTC; 71.17 ± 6.73 mU/mg protein) ($p < 0.05$) but abruptly decreased at 15 DAH (433.5 UTC; 2.69 ± 0.29 mU/mg protein) to remain constant until the end of the study ($p > 0.05$).

The specific activities of leucine aminopeptidase and alkaline phosphatase are shown in Figure 5. The specific activity of leucine aminopeptidase was detected from hatching (28.9 UTC; 4.05 ± 0.69 mU/mg protein) and increased until 4 DAH (115.6 UTC; 5.82 ± 0.30 mU/mg protein) ($p < 0.05$); then, its activity gradually decreased until 20 DAH (578 UTC; 1.85 ± 0.02 mU/mg protein). Alkaline phosphatase-specific activity was detected at low levels at hatching (28.9 UTC; 1.12 ± 0.10 mU/mg protein) and increased constant until 15 DAH (867 UTC; 3.78 ± 0.08 mU/mg protein) ($p < 0.05$) and then decreased abruptly to remain stable until the end of culture (1.62 ± 0.09 mU/mg protein).

The specific activities of amylase and bile salt-dependent (BSD) lipase are shown in Figure 6. The specific activity of amylase was the highest at hatching (28.9 UTC; 3.71 ± 0.63 mU/mg protein) ($p < 0.05$), but its activity gradually decreased until

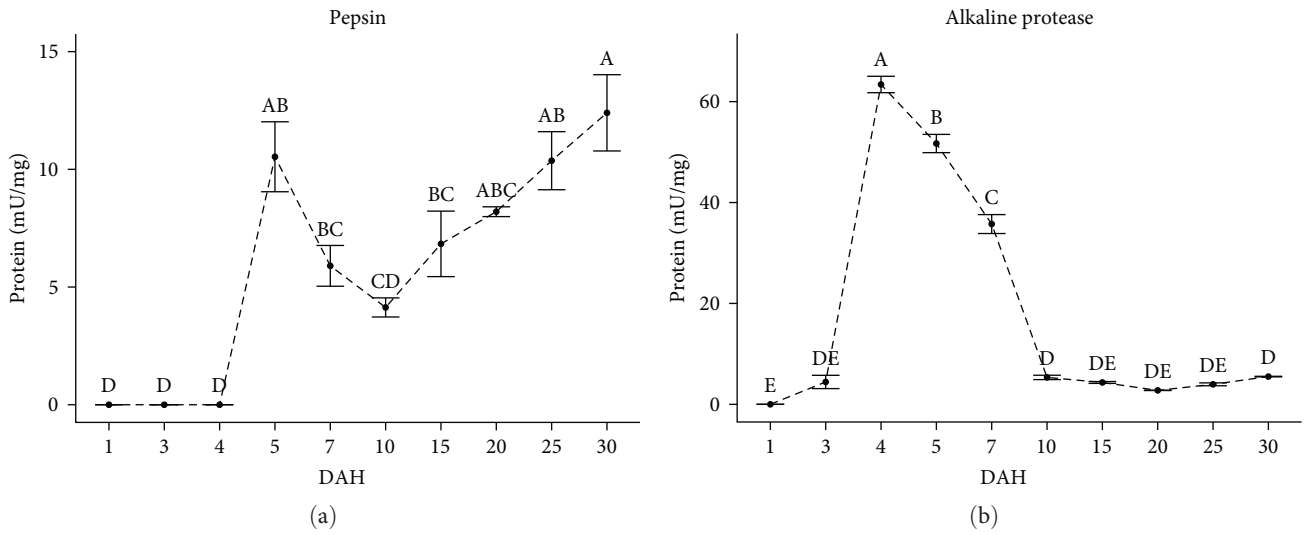


FIGURE 3: (a and b) Specific digestive enzyme activity (mU/mg protein) during larval development of spotted seatrout (*C. nebulosus*); superscript letters indicate statistical differences among ages. The results are expressed as mean \pm standard error (SE) ($n = 3$ pooled samples).

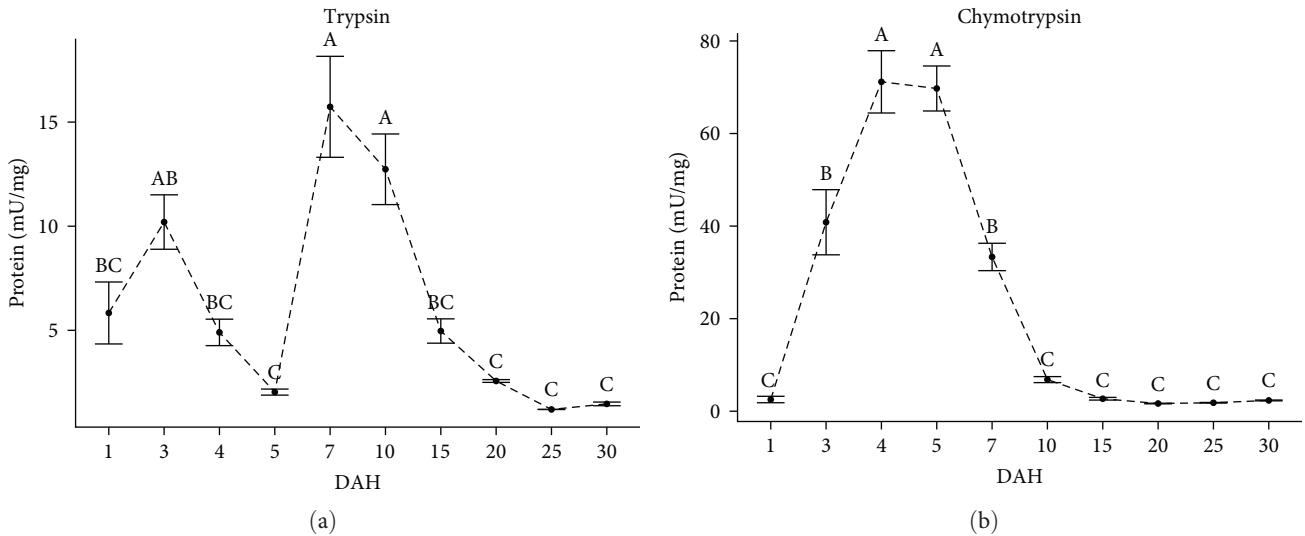


FIGURE 4: (a and b) Specific digestive enzyme activity (mU/mg protein) during larval development of the spotted seatrout (*C. nebulosus*); superscript letters indicate statistical differences among ages. The results are expressed as mean \pm standard error (SE) ($n = 3$ pooled samples).

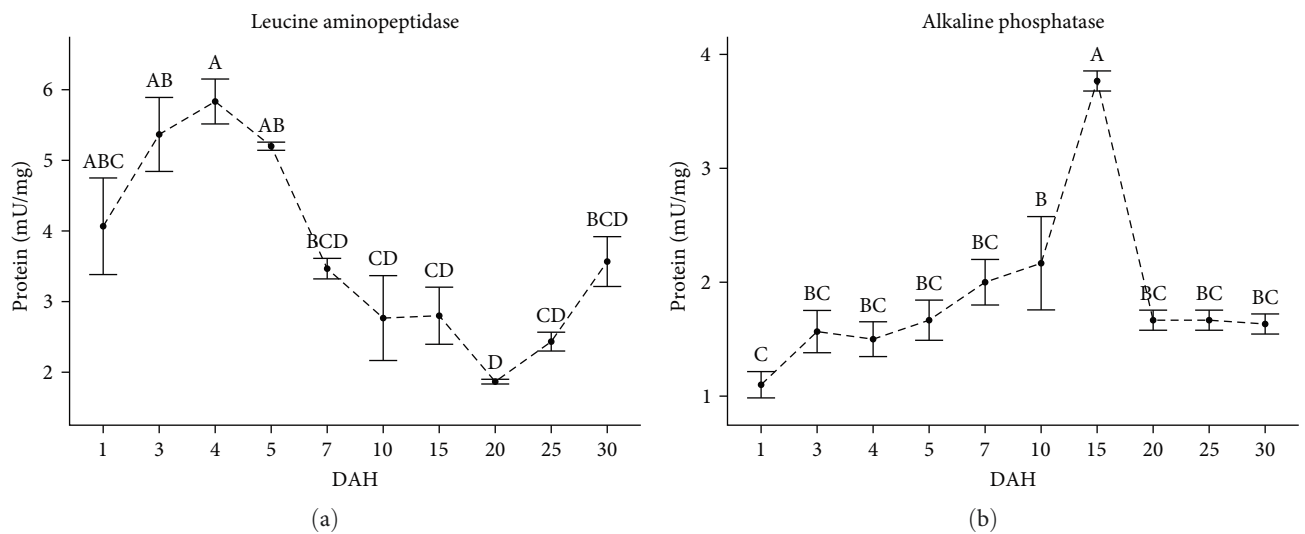


FIGURE 5: (a and b) Specific digestive enzyme activity (mU/mg protein) during larval development of the spotted seatrout (*C. nebulosus*); superscript letters indicate statistical differences among ages. The results are expressed as mean \pm standard error (SE) ($n = 3$ pooled samples).

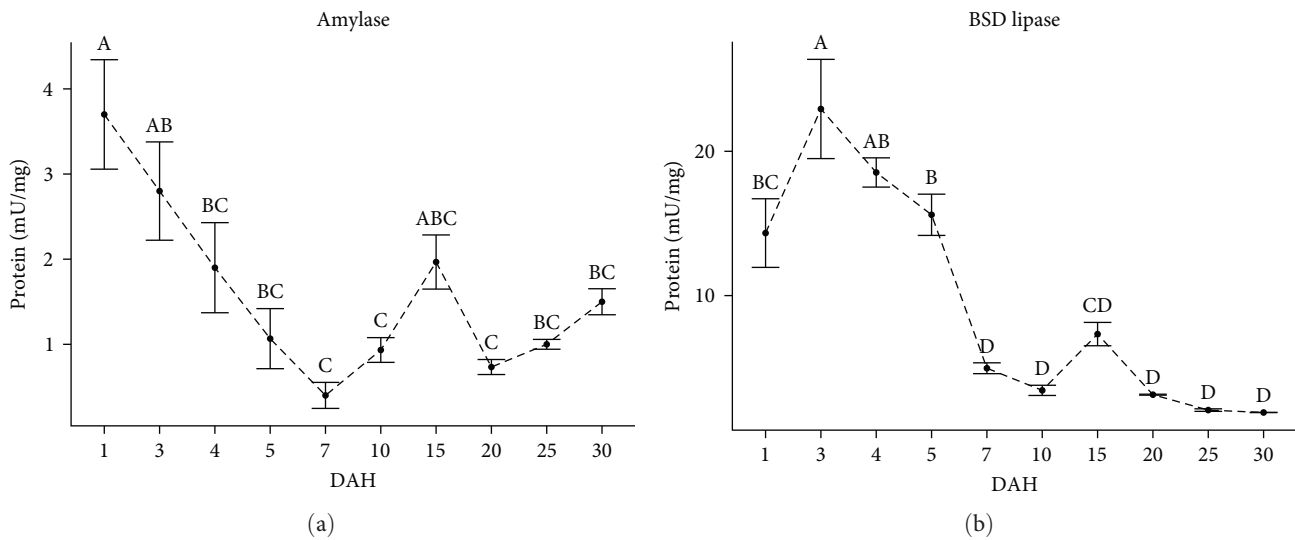


FIGURE 6: (a and b) Specific digestive enzyme activity (mU/mg protein) during larval development of the spotted seatrout (*C. nebulosus*); superscript letters indicate statistical differences among ages. The results are expressed as mean \pm standard error (SE); ($n = 3$ pooled samples).

7 DAH (231.2 UTC; 0.42 ± 0.48 mU/mg protein), and then two light increases were observed at 15 (462.4 UTC; 1.94 ± 0.98 mU/mg protein) and 30 DAH (895.9 UTC; 1.50 ± 0.41 mU/mg protein) ($p > 0.05$). BSD lipase-specific activity was detected at hatching (28.9 UTC; 14.29 ± 2.37 mU/mg protein) and then reached its maximum specific activity at 3 DAH (22.95 ± 3.44 mU/mg protein) ($p < 0.05$) but gradually decreased at 7 DAH (202.3 UTC; 4.95 ± 0.37 mU/mg protein) to remain relatively constant until the end of the study ($p > 0.05$).

Figure 7 shows the principal component analysis (PCA) graph of specific digestive enzyme activity. Dim 1 explained 43.8% of the variability, Dim 2 explained 22.8%, and both dimensions explained 69.6% of total variations. BSD lipase and leucine aminopeptidase enzymes contributed the most variation to Dim 1. In contrast, alkaline phosphatase and amylase enzymes contributed the most variation to Dim 2 (Figure 8). The PERMANOVA analysis found significant differences in the specific activity of digestive enzymes through time (DAH) in spotted seatrout larvae (PERMANOVA, $p = 0.0001$) (Table S1). In addition, a posteriori pairwise PERMANOVA analysis detected no differences between 1 and 3 DAH (PERMANOVA, $p = 0.0577$) and 25 (751.4 UTC) and 30 DAH (PERMANOVA, $p = 0.1075$); all other groups were different from each other (Table S2).

4. Discussion

This study measured the activity of major digestive enzymes from the stomach, pancreas, and intestine during larval development to provide a basic understanding of the digestive physiology (capabilities and limitations) of the early ontogeny of *C. nebulosus*.

4.1. Larval Growth and Survival Rate. The growth of marine tropical fish larvae is characterized by an exponential pattern that starts slowly, followed by a rapid increase [7, 42, 43]. Exponential growth in terms of weight gained (WG) and size (SL) was observed for the spotted seatrout. Still, interestingly, this species

showed faster growth compared with other tropical fish larvae, such as *Centropomus undecimalis* [10], *Lutjanus guttatus* [44], and *Totoaba macdonaldi* [45]. The survival in the present study (7.8%) was lower than that reported by [46] (30%), both studies at 30 DAH. However, the stocking density in this study was 93 larvae/L, while [46] used a stock of 1–2 larvae/L. In this study, the high mortality of spotted seatrout larvae could be attributed to cannibalism and aggression during rearing. High stocking densities promote cannibalism and aggressive behavior in the larvae of this species [47].

4.2. Ontogeny of Digestive Enzymes. Pepsin is a determinant enzyme in the digestion and assimilation of proteins in fish (altricial and precocial). Pepsin activity in fish larvae is associated with a functional stomach (gastric glands), which may take days or months [3]. In the spotted seatrout, the presence of pepsin activity was detected at 5 DAH (144.5 UTC). This aspect could suggest a functional stomach in the spotted seatrout larvae at 5 DAH. An early functional stomach was observed in *Atractosteus tropicus* (145 UTC) by Frías-Quintana et al. [9]. On the other hand, the levels of pepsin activity recorded at 5 DAH were similar to those recorded at the end of the larval period (Figure 3(a)). However, this is not consistent with the low degree of maturation of the stomach. Elevated levels of pepsin enzyme activity have also been observed in other fish species during the early stages of larval development [10, 48]. Elevated pepsin activity values during the early stages of larval development in fish may be overestimated due to the hydrolysis of cathepsins (intracellular acid hydrolases) [48]. Cathepsins are important enzymes in protein degradation during embryonic and early larval stages (eleutheroembryo) [49, 50], but in this study, acid hydrolysis was not detected before 5 DAH. Further research (molecular and histological tools) is needed to elucidate the mechanisms of stomach development in the spotted seatrout larvae.

During the first days of life of fish larvae (altricial and precocial), protein digestion in the digestive tract occurs in

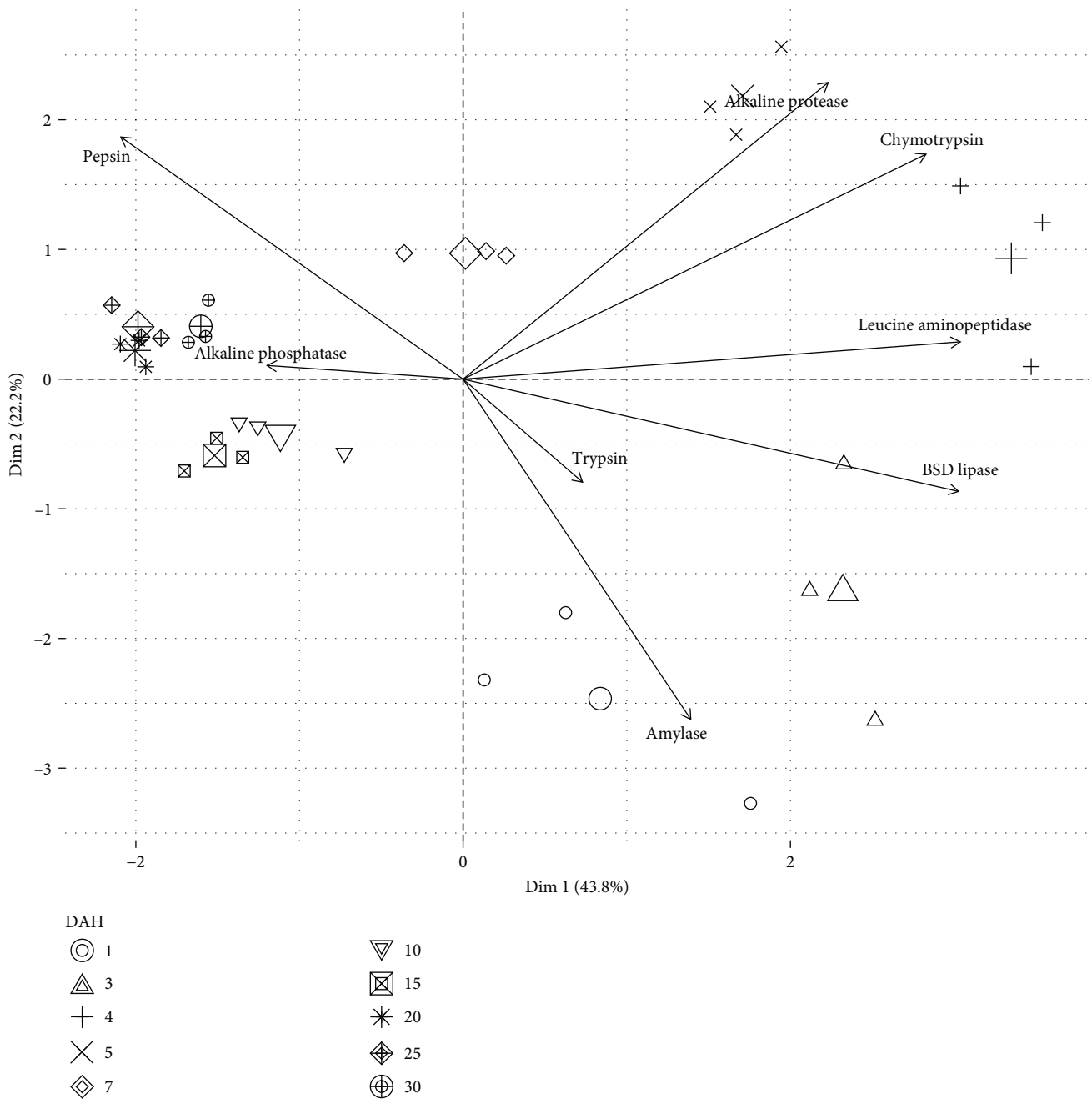


FIGURE 7: Principal component analysis (PCA) of specific digestive enzyme activity (mU/mg protein) during larval development of the spotted seatrout (*C. nebulosus*).

an alkaline environment by proteases of the pancreas and intestine (alkaline protease) until the development of a functional stomach (an acidic environment) [3]. In the spotted seatrout larvae, alkaline protease activity was detected at the onset of feeding (3 DAH), increased abruptly at 4 DAH, and gradually decreased until 7 DAH. The increase and decrease in alkaline protease activity during larvae transformation are considered physiological responses that are genetically pre-programmed [51]. The increase in alkaline protease after the onset of feeding is associated with a compensatory mechanism to maximize protein digestion to compensate for the lack of acid digestion, while the decrease is associated with a

functional stomach [7, 9, 48]. In the present study, the decline in alkaline protease activity coincides with the first pepsin activity detection, supporting the hypothesis of an early functional stomach in the spotted seatrout larvae.

Trypsin and chymotrypsin are the major or unique alkaline digestive proteases in the intestine of many fish, particularly in the larval stage due to the lack of a functional stomach [3]. In this sense, trypsin and chymotrypsin activities were detected in the spotted seatrout larvae at hatching (28.9 UTC) before opening the mouth; this finding is consistent with the report for *S. ocellata* larvae (~27.3 UTC) [52, 53]. The presence of trypsin and chymotrypsin activity

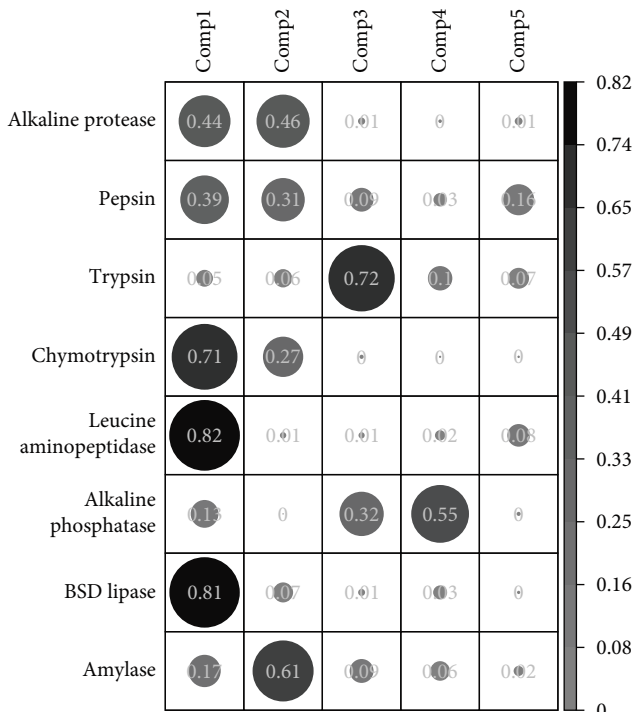


FIGURE 8: Contribution of each variable to the component.

in fish embryonic and eleutheroembryonic stages is associated with the cleavage of yolk proteins [54, 55]. The profile observed for chymotrypsin activity coincides with that obtained for alkaline proteases; in contrast, trypsin activity decreased sharply at 4–5 DAH, indicating that chymotrypsin was responsible for most of the total alkaline protease activity during this period. The diet has been shown to modulate the plateau levels of some pancreatic and intestinal proteases but not the genetically programmed timing of their rise or fall [51]. The decrease of trypsin activity a few days after hatching is a typical pattern in marine fish larvae [6, 10, 12, 54], which occurs regardless of feeding or starving conditions [56], live or inert food [52], and prey density [57, 58]. This decrease in trypsin levels may be associated with a programmed physiological event in the development of marine fish larvae. Due to their essential role in protein digestion, trypsin and chymotrypsin are considered good indicators for assessing the nutritional status of fish larvae [59]. Trypsin has been reported to be sensitive under conditions that favor growth, while chymotrypsin plays an important role when growth is restricted or repressed [60, 61]. When trypsin activity decreased at 4–5 DAH, chymotrypsin showed the highest activity values, indicating a critical physiological phase for the spotted seatrout larvae.

In fish, developing a fully functional intestine involves the complete differentiation of enterocytes, achieved by establishing the brush border membrane [3]. During larval to juvenile transformation, digestion moves from an intracellular to a luminal location in the intestine [51]. Leucine aminopeptidase and alkaline phosphatase are enzymes in the cytosol and brush border membrane, respectively, commonly used as enterocyte cytodifferentiation markers [29]. In the present study, higher leucine aminopeptidase activity

was observed in the early days of spotted seatrout larvae, indicating high intracellular digestion. This digestion is an expected response given the poor digestive tract development of the larvae a few days after hatching [9, 11, 62] (e.g., absorption and transport of nutrients). The increase in alkaline phosphatase activity coincided with a decrease in leucine aminopeptidase activity at least up to 15 DAH, indicating a change in digestion mode from intracellular to luminal location in the intestine.

In addition, it is interesting to note that alkaline phosphatase activity in the spotted seatrout declined sharply at 20 DAH. A decrease in the activity of this enzyme at the end of the larval stage has been reported in other fish species, such as *P. californicus* [6, 63], *P. maculatofasciatus* [7], *C. undecimalis* [10], and *C. nigrescens* [43]. In fish, intestinal alkaline phosphatase controls nutrient absorption and prevents the intestinal microbiota's inflammatory responses [64]. Alkaline phosphatase can reduce the toxicity of membrane-associated lipopolysaccharides of Gram-negative bacteria by removing their phosphate groups [65]. The resident microbiota also occurs during the development of the digestive tract in fish larvae [66]. In zebrafish, alkaline phosphatase has been suggested to be upregulated by the microbiota during gut colonization, preventing an inflammatory response [67]. The decrease in alkaline phosphatase activity at the end of the spotted seal larval period may be related to a physiological mechanism in response to the establishment of the resident gut microbiota.

Amylase is a pancreatic enzyme that catalyzes the endohydrolysis of α -1,4 glycosidic linkages of polysaccharides, such as starch and glycogen. In the spotted seatrout, amylase activity was higher at hatching and decreased steadily until 7 DAH (202.3 UTC). This pattern of amylase activity has been reported for other fish larvae [28, 44, 68]. High amylase levels during the early development of fish larvae have been suggested to be a genetically programmed event associated with a natural predisposition to digest carbohydrates [69]. This limitation should not be surprising; although carbohydrates are cataloged as non-essential to fish nutrition, they can be metabolized and utilized nutritionally [70]. In the present study, amylase was found to be significantly around 1 and 3 DAH, highlighting the importance of carbohydrates in the diet of the spotted seatrout at the onset of feeding (Figure 6). In *S. ocellata*, a second increase in amylase activity was observed at 14 DAH (386.4 UCT) [52]. A modulatory effect of dietary carbohydrates on amylase activity has been observed in this species between 11 and 24 DAH [71]. Differences in the pattern of amylase activity between spotted and *S. ocellata* could be explained by changes in diet composition; however, interspecific variation in the developmental process of this enzyme cannot be excluded.

Bile salt-dependent lipase, a pancreatic enzyme with broad substrate specificity (e.g., cholesteryl esters, fat-soluble vitamin esters, tri- and monoglycerides) [72], is considered the major lipolytic enzyme in teleost fishes [73]. The present study detected BSD lipase activity at hatching when the spotted seatrout larvae depended exclusively on yolk reserve. Lazo et al. [52] found similar results for *S. ocellata* larvae. Lipolytic activity during the yolk sac larvae stage in fish is

associated with lipid catabolism for energetic purposes [74]. This approach aligns with the reported changes in lipid composition found in the yolk reserve during this stage of the fish [75, 76]. The spotted seatrout showed the highest levels of BSD lipase activity at the onset of feeding and then decreased continuously until 7 DAH. The development of BSD lipase along the fish larvae transformation is highly variable among species [43, 52, 55]. The lipid content of the diet or live food has been observed not to have a modulatory effect on the development of BSD lipase during fish larval transformation [71, 77]. Therefore, the development of BSD lipase may follow an ontogenetic programming pattern [74]. Lipids are a source of energy, but they also provide essential fatty acids for cell structure and essential compounds [73]. In fish larvae, the onset of feeding is often followed by an exponential growth phase (intensification in metabolic processes), as observed for this species. The high lipolytic activity found in the spotted seatrout larvae at the beginning of feeding compared to later days suggests a high demand for lipids at this stage of development.

During the transformation of the spotted seatrout from larvae to juvenile, three development stages of digestive enzymes have been identified. The first stage occurred between 1 and 3 DAH, with amylase and BSD lipase being the main digestive enzymes. This period of digestive stability suggests that carbohydrates and lipids are significant components of the early diet of the spotted seatrout. The second stage occurred from 4 to 20 DAH; leucine aminopeptidase, chymotrypsin, and alkaline proteases were identified as the predominant enzymes at 4–5 DAH. At the beginning of this phase, an accelerated shift was observed towards protein digestion, indicating a high demand, which is consistent with the high growth rate during this stage. In addition, the lack of similarity between treatments means constant and accelerated physiological changes in the digestive tract of the spotted seatrout larvae. The third stage occurred between 25 and 30 DAH, where pepsin was the main enzyme. This aspect is a period of digestive stability that can be interpreted as the digestive tract maturation and transformation process completion. In addition, the fact that pepsin was the enzyme of higher activity during this period suggests that the spotted seatrout is highly protein-dependent, a typical characteristic of carnivorous fish species.

5. Conclusion

In the present study, spotted seatrout larvae showed an enzymatic profile typical of carnivorous species. Furthermore, pepsin activity at 5 DAH (144.5 UTC) suggests an early functional stomach; however, the biochemical activity of this enzyme needs to be complemented by molecular and histological techniques (presence of gastric cells). The period of digestive stability recorded between 25 (751.4 UTC) and 30 (895.9 UTC) DAH indicates that the maturation of the digestive system and the completion of the transformation process in spotted seatrout occur at 25 DAH. A holistic understanding of their digestive physiology is essential for determining an optimal weaning period for spotted seatrout larvae. Therefore, it is recommended to conduct studies on the RNAm expression of digestive enzymes, the gut

microbiota, and the histological development of the digestive tract. Furthermore, the digestion pattern obtained here should be considered when designing compound diets for weaning the spotted seatrout.

Data Availability

The data that support the findings of this study are available from the lead author.

Ethical Approval

Animals were handled in compliance with the Norma Oficial Mexicana NOM-062-ZOO-1999 from Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación, the Mexican standards, for good welfare practices of laboratory animals.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Martín Alberto Arenas Pardo, Martha Gabriela Gaxiola Cortés, and Carlos Alfonso Álvarez González contributed to conceptualization of this study. Martín Alberto Arenas Pardo, Alvaro Fabricio Barreto Altamirano, Adriana del Carmen Paredes Medina, Iveth Gabriela Palomino Albarrán, Patricia Margarita Balam Uc, and Juan Carlos Maldonado Flores contributed to methodology. Martín Alberto Arenas Pardo, Alvaro Fabricio Barreto Altamirano, Martha Gabriela Gaxiola Cortés, and Carlos Alfonso Álvarez González contributed to the formal analysis. Martín Alberto Arenas Pardo contributed to writing—original draft preparation. All authors of the manuscript contributed to writing—review and editing.

Acknowledgments

The authors gratefully acknowledge A. Rosales-Arce, F. Y. Cob-Pacheco, and J. Suárez-Bautista for their excellent technical assistance. The Consejo Nacional de Humanidades, Ciencia y Tecnologías (CONAHCYT) of Mexico, awarded a postdoctoral fellowship to the first author, Martín Alberto Arenas Pardo, and to D. Fischer for the English edition. This study was funded by the Programa de Apoyo a Proyectos para Innovar y Mejorar la Educación (PAPIME)-UNAM, PE207323, and the Consejo Nacional de Humanidades, Ciencia y Tecnologías (CONAHCYT).

Supplementary Materials

Permutational multivariate analysis of variance (PERMANOVA) information. (*Supplementary Materials*)

References

- [1] K. Hamre, M. Yúfera, I. Rønnestad, C. Boglione, L. E. C. Conceição, and M. Izquierdo, "Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing," *Reviews in Aquaculture*, vol. 5, no. s1, pp. S26–S58, 2013.

- [2] M. Planas and I. Cunha, "Larviculture of marine fish: problems and perspectives," *Aquaculture*, vol. 177, no. 1–4, pp. 171–190, 1999.
- [3] I. Rønnestad, M. Yúfera, B. Ueberschär, L. Ribeiro, Ø. Sæle, and C. Boglione, "Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research," *Reviews in Aquaculture*, vol. 5, no. s1, pp. S59–S98, 2013.
- [4] G. J. Holt, *Larval Fish Nutrition*, John Wiley and Sons, Chichester, UK, 2011.
- [5] J. L. Zambonino-Infante and C. L. Cahu, "Dietary modulation of some digestive enzymes and metabolic processes in developing marine fish: applications to diet formulation," *Aquaculture*, vol. 268, no. 1–4, pp. 98–105, 2007.
- [6] C. A. Alvarez-González, M. Cervantes-Trujano, D. Tovar-Ramírez et al., "Development of digestive enzymes in California halibut *Paralichthys californicus* larvae," *Fish Physiology and Biochemistry*, vol. 31, pp. 83–93, 2006.
- [7] C. A. Alvarez-González, F. J. Moyano-López, R. Civera-Cerecedo, V. Carrasco-Chávez, J. L. Ortiz-Galindo, and S. Dumas, "Development of digestive enzyme activity in larvae of spotted sand bass *Paralabrax maculatofasciatus*. 1," *Fish Physiology and Biochemistry*, vol. 34, no. 4, pp. 373–384, 2008.
- [8] R. Ahumada-Hernández, C. Alvarez-González, R. Guerrero Zarate et al., "Changes of digestive enzymatic activity on yellowtail snapper (*Ocyurus chrysurus*) during initial ontogeny," *International Journal of Biology*, vol. 6, no. 4, pp. 110–118, 2014.
- [9] C. A. Frías-Quintana, G. Márquez-Couturier, C. A. Alvarez-González et al., "Development of digestive tract and enzyme activities during the early ontogeny of the tropical gar *Atractosteus tropicus*," *Fish Physiology and Biochemistry*, vol. 41, no. 5, pp. 1075–1091, 2015.
- [10] L. D. Jimenez-Martinez, C. A. Alvarez-González, D. Tovar-Ramírez et al., "Digestive enzyme activities during early ontogeny in common snook (*Centropomus undecimalis*)," *Fish Physiology and Biochemistry*, vol. 38, no. 2, pp. 441–454, 2012.
- [11] A. Teles, J. Salas-Leiva, C. A. Alvarez-González et al., "Histological study of the gastrointestinal tract in longfin yellowtail (*Seriola rivoliana*) larvae," *Fish Physiology and Biochemistry*, vol. 43, no. 6, pp. 1613–1628, 2017.
- [12] A. Teles, J. Salas-Leiva, C. A. Alvarez-González, and D. Tovar-Ramírez, "Changes in digestive enzyme activities during early ontogeny of *Seriola rivoliana*," *Fish Physiology and Biochemistry*, vol. 45, no. 2, pp. 733–742, 2019.
- [13] C. L. Cahu, E. Gisbert, L. A. N. Villeneuve et al., "Influence of dietary phospholipids on early ontogenesis of fish," *Aquaculture Research*, vol. 40, no. 9, pp. 989–999, 2009.
- [14] S. A. Bortone, *Biology of spotted seatrout*, CRC Press, New York, 2002.
- [15] J. A. Bohnsack, "Impacts of Florida coastal protected areas on recreational world records for spotted seatrout, red drum, black drum, and common snook," *Bulletin of Marine Science*, vol. 87, no. 4, pp. 939–970, 2011.
- [16] DOF, "Carta Nacional Pesquera. Secretaria de Agricultura y Desarrollo Rural, México," 2022, <https://www.gob.mx/inapesca/documentos/carta-nacional-pesquera-2022>.
- [17] M. A. Banks, G. J. Holton, and J. M. Wakeman, "Age-linked changes in salinity tolerance of larvae spotted seatrout (*Cynoscion nebulosus*, Cuvier)," *Journal of Fish Biology*, vol. 39, no. 4, pp. 505–514, 1991.
- [18] J. Song, R. W. Brill, and J. R. McDowell, "Plasticity in standard and maximum aerobic metabolic rates in two populations of an estuarine dependent teleost, spotted seatrout (*Cynoscion nebulosus*)," *Biology*, vol. 8, no. 2, Article ID 46, 2019.
- [19] J. W. Tucker, "Growth of juvenile spotted seatrout on dry feeds," *The Progressive fish-culturist*, vol. 50, pp. 39–41, 1988.
- [20] K. M. Peters, "Early life history of spotted seatrout, *Cynoscion nebulosus* (Pisces: Sciaenidae), in Tampa, Florida," *Estuaries*, vol. 12, no. 2, pp. 98–110, 1989.
- [21] J. W. Tucker, *Marine Fish Culture*, Kluwer Academic Publishers, Boston, 1998.
- [22] R. Blaylock, E. Saillant, A. Apeitos, D. Abrego, P. Cason, and R. Veja, "The status of spotted seatrout (*Cynoscion nebulosus*) as a technologically feasible species for U.S. marine aquaculture," *Journal of the World Aquaculture Society*, vol. 52, no. 3, pp. 526–540, 2021.
- [23] R. L. Colura, "Induced spawning of the spotted Seatrout, *Cynoscion nebulosus*, (Cuvier)," *Proceedings of the annual meeting—World Mariculture Society*, vol. 5, no. 1–4, pp. 319–330, 1974.
- [24] J. W. Tucker and B. E. Faulkner, "Voluntary spawning patterns of captive spotted seatrout," *Northeast Gulf Science*, vol. 9, no. 1, pp. 59–63, 1987.
- [25] P. Thomas and N. W. Boyd, "Dietary administration of a LHRH analogue induces spawning of spotted seatrout (*Cynoscion nebulosus*)," *Aquaculture*, vol. 80, no. 3–4, pp. 363–370, 1989.
- [26] S. K. Lowerre-Barbieri, N. Henderson, J. Llopiz, S. Walters, J. Bickford, and R. Muller, "Defining a spawning population (spotted seatrout *Cynoscion nebulosus*) over temporal, spatial, and demographic scales," *Marine Ecology Progress Series*, vol. 394, pp. 231–245, 2009.
- [27] M. J. Wuenschel and R. G. Werner, "Consumption and gut evacuation rate of laboratory-reared spotted seatrout (Sciaenidae) larvae and juveniles," *Journal of Fish Biology*, vol. 65, no. 3, pp. 723–743, 2004.
- [28] D. Castro-Ruiz, M. T. Mozanzadeh, C. Fernández-Méndez et al., "Ontogeny of the digestive enzyme activity of the Amazonia pimelodid catfish *Pseudoplatystoma punctifer* (Castelnaud, 1855)," *Aquaculture*, vol. 504, pp. 210–218, 2019.
- [29] E. Gisbert, E. Nolasco, and M. Solovyev, "Towards the standardization of brush border purification and intestinal alkaline phosphatase quantification in fish with notes on other digestive enzymes," *Aquaculture*, vol. 487, pp. 102–108, 2018.
- [30] H. Nolasco-Soria, H.-R. Nolasco-Alzaga, and E. Gisbert, "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," *Reviews in Aquaculture*, vol. 12, no. 3, pp. 1928–1943, 2020.
- [31] H. Nolasco-Soria, "Improving and standardizing protocols for alkaline protease quantification in fish," *Reviews in Aquaculture*, vol. 13, no. 1, pp. 1–65, 2021.
- [32] B. Erlanger, N. Kokowsky, and W. Cohen, "The preparation and properties of two new chromogenic substrates of trypsin," *Archives of Biochemistry and Biophysics*, vol. 95, no. 2, pp. 271–278, 1961.
- [33] S. Maroux, D. Louvard, and J. Baratti, "The aminopeptidase from hog-intestinal brush border," *Biochimica et Biophysica Acta (BBA)—Enzymology*, vol. 321, no. 1, pp. 282–295, 1973.
- [34] E. G. Del-Mar, C. Largman, J. Brodrick, and M. Geokas, "A sensitive new substrate for chymotrypsin," *Analytical Biochemistry*, vol. 99, no. 2, pp. 316–320, 1979.
- [35] D. R. Gjellesvik, D. Lombardo, and B. T. Walther, "Pancreatic bile salt dependent lipase from cod (*Cadus morhua*)," *Biochimica et Biophysica Acta (BBA)—Lipids and Lipid Metabolism*, vol. 1124, no. 2, pp. 123–134, 1992.

- [36] H. Nolasco-Soria, "Amylase quantification in aquaculture fish studies: a revision of most used procedures and presentation of a new practical protocol for its assessment," *Aquaculture*, vol. 538, Article ID 736536, 2021.
- [37] M. M. Bradford, "A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248-254, 1976.
- [38] R Core Team, "R: a language and environment for statistical computing. R foundation for statistical computing, Vienna," Vienna, Austria, 2022, R version 4.2.2, <https://www.R-project.org>.
- [39] R. V. Lenth, "Emmeans: estimated marginal means, aka least-squares means," R package version 1.4. 2, 2021.
- [40] M. J. Anderson, "Permutational Multivariate Analysis of Variance (PERMANOVA)," in *Wiley StatsRef: Statistics Reference Online*, T. Colton Balakrishnan, B. Everitt, W. Piegorisch, F. Ruggeri, and J. L. Teugels, Eds., 2017.
- [41] K. R. Clarke, R. N. Gorley, P. J. Somerfield, and R. M. Warwick, *Change in Marine Communities: an Approach to Statistical analysis and Interpretation*, PRIMER-E, Ltd., Plymouth, 2014.
- [42] I. A. Hernández-López, L. Ibarra-Castro, C. A. Álvarez-González, J. M. Martínez-Brown, C. I. Maytorena-Verdugo, and E. S. Peña-Marín, "Characterization of digestive enzymes during early ontogeny of white Snook (*Centropomus viridis*)," *Aquaculture*, vol. 535, pp. 736-399, 2021.
- [43] C. Yanes-Roca, M. E. Toledo-Cuevas, L. J. Sánchez, A. Born-Torrijos, N. Rhody, and K. L. Main, "Digestive enzyme activity during larval development of black snook, *Centropomus nigrescens*," *Journal of The World Aquaculture Society*, vol. 49, no. 3, pp. 612-624, 2018.
- [44] I. Moguel-Hernández, R. Peña, H. Nolasco-Soria, S. Dumas, and I. Zavala-Leal, "Development of digestive enzyme activity in spotted rose snapper, *Lutjanus guttatus* (Steindachner, 1869) larvae," *Fish Physiology and Biochemistry*, vol. 40, no. 3, pp. 839-848, 2014.
- [45] M. Córdova-Montejo, C. A. Álvarez-González, L. M. T. López, D. Conal, C. A. Frías-Quintana, and M. A. Galaviz, "Changes of digestive enzymes in totoaba (*Totoaba macdonaldi* Gilbert, 1890) during early ontogeny," *Latin American Journal of Aquatic Research*, vol. 47, no. 1, pp. 102-113, 2019.
- [46] C. R. Arnold, J. L. Lasswell, W. H. Bailey, T. D. Williams, and F. JrWA, "Methods and techniques for spawning and rearing spotted seatrout in the laboratory," *Proceedings of the Annual Conference, Southeast Association Fish Wildlife Agency*, vol. 30, pp. 167-178, 1976.
- [47] C. B. Manley, C. F. Rakocinski, P. G. Lee, and R. B. Blaylock, "Stocking density effects on aggressive and cannibalistic behaviors in larval hatchery-reared spotted seatrout, *Cynoscion nebulosus*," *Aquaculture*, vol. 420-421, pp. 89-94, 2014.
- [48] F. J. Moyano, M. Diaz, F. J. Alarcon, and M. C. Sarasquete, "Characterization of digestive enzyme activity during larval development of gilthead sea bream (*Sparus aurata*)," *Fish Physiology and Biochemistry*, vol. 15, no. 2, pp. 121-130, 1996.
- [49] M.-F. Sire, P. J. Babin, and J.-M. Vernier, "Involvement of the lysosomal system in yolk protein deposit and degradation during vitellogenesis and embryonic development in trout," *Journal of Experimental Zoology*, vol. 269, no. 1, pp. 69-83, 1994.
- [50] P. Kestemont, J. Cooremans, A. Abi-Ayad, and C. Mélard, "Cathepsin L in eggs and larvae of perch *Perca fluviatilis*: variations with developmental stage and spawning period," *Fish Physiology and Biochemistry*, vol. 21, no. 1, pp. 59-64, 1999.
- [51] J. L. Zambonino Infante and C. L. Cahu, "Ontogeny of the gastrointestinal tract of marine fish larvae," *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, vol. 130, no. 4, pp. 477-487, 2001.
- [52] J. P. Lazo, J. G. Holt, and C. R. Arnold, "Ontogeny of pancreatic enzymes in larval red drum *Sciaenops ocellatus*," *Aquaculture Nutrition*, vol. 6, no. 3, pp. 183-192, 2000.
- [53] S. Applebaum, R. Perez, J. Lazo, and G. J. Holt, "Characterization of chymotrypsin activity during early ontogeny of larval red drum (*Sciaenops ocellatus*)," *Fish Physiology and Biochemistry*, vol. 25, no. 4, pp. 291-300, 2001.
- [54] M. A. Galaviz, A. García-Gasca, M. Drawbridge, C. A. Álvarez-González, and L. M. López, "Ontogeny of the digestive track and enzymatic in white seabass, *Atractoscion nobilis*, larvae," *Aquaculture*, vol. 318, no. 1-2, pp. 162-168, 2011.
- [55] R. Martínez-Lagos, D. Tovar-Ramírez, V. Gracia-López, and J. P. Lazo, "Changes in digestive enzyme activities during larval development of leopard grouper (*Mycteroperca rosacea*)," *Fish Physiology and Biochemistry*, vol. 40, no. 3, pp. 773-785, 2014.
- [56] K. Hjelmeland, I. Huse, T. Jorgensen, G. Molvik, and J. Raa, "Trypsin and trypsinogen as indices of growth and survival potential of cod (*Gadus morhua* L.) larvae. In: the propagation of cod *Gadus morhua* L an international symposium, Arendal," pp. 14-17, 1983, <http://hdl.handle.net/11250/115106>.
- [57] B. H. Pedersen, E. M. Nilssen, and K. Hjelmeland, "Variations in the content of trypsin and trypsinogen in larval herring (*Clupea harengus*) digesting copepod nauplii," *Marine Biology*, vol. 94, no. 2, pp. 171-181, 1987.
- [58] J. P. Lazo, M. T. Dinis, G. J. Holt, C. Faulk, and C. R. Arnold, "Co-feeding microparticulate diets with algae: toward eliminating the need of zooplankton at first feeding in larval red drum (*Sciaenops ocellatus*)," *Aquaculture*, vol. 188, no. 3-4, pp. 339-351, 2000.
- [59] S. L. Applebaum and G. J. Holt, "The digestive protease, chymotrypsin, as an indicator of nutritional condition in larval red drum (*Sciaenops ocellatus*)," *Marine Biology*, vol. 142, no. 6, pp. 1159-1167, 2003.
- [60] K. Rungruangsak-Torrissen, R. Moss, L. H. Andresen, A. Berg, and R. Waagbø, "Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar* L.)," *Fish Physiology and Biochemistry*, vol. 32, no. 1, pp. 7-23, 2006.
- [61] B. Ueberschär, "The use of tryptic enzyme activity measurement as a nutritional condition index: laboratory calibration data and field application," *ICES Journal of Marine Science*, vol. 201, pp. 119-129, 1995.
- [62] A. Teles, W. M. Costa, D. Ammar, Y. M. R. Müller, E. M. Nazari, and V. R. Cerqueira, "Ontogeny of the digestive tract of *Centropomus parallelus* larvae," *Fish Physiology and Biochemistry*, vol. 41, no. 2, pp. 549-559, 2015.
- [63] M. Zacarias-Soto, B. Barón-Sevilla, and J. P. Lazo, "Ontogeny and distribution of alkaline and acid phosphatases in the digestive system of California halibut larvae (*Paralichthys californicus*)," *Fish Physiology and Biochemistry*, vol. 39, no. 5, pp. 1331-1339, 2013.
- [64] J.-P. Lallès, "Intestinal alkaline phosphatase in the gastrointestinal tract of fish: biology, ontogeny, and environmental and nutritional modulation," *Reviews in Aquaculture*, vol. 12, no. 2, pp. 555-581, 2020.

- [65] C. Beumer, M. Wulferink, W. Raaben, D. Fiechter, R. Brands, and W. Seinen, "Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets," *Journal of Pharmacology and Experimental Therapeutics*, vol. 307, no. 2, pp. 737–744, 2003.
- [66] S. Egerton, S. Culloty, J. Wooley, C. Stanton, and R. P. Ross, "The gut microbiota of marine fish," *Frontiers in Microbiology*, vol. 9, Article ID 873, 2018.
- [67] J. M. Bates, J. Akerlund, E. Mittge, and K. Guillemin, "Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota," *Cell Host & Microbe*, vol. 2, no. 6, pp. 371–382, 2007.
- [68] T. M. Kortner, I. Overrein, G. Øie et al., "Molecular ontogenesis of digestive capability and associated endocrine control in Atlantic cod (*Gadus morhua*) larvae," *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, vol. 160, no. 2, pp. 190–199, 2011.
- [69] P. Ma, B. Sivaloganathan, P. K. Reddy, W. K. Chan, and T. J. Lam, "Ontogeny of alpha-amylase gene expression in sea bass larvae (*Lates calcarifer*)," *Marine Biotechnology*, vol. 3, no. 5, pp. 463–469, 2001.
- [70] B. S. Kamalam, F. Medale, and S. Panserat, "Utilisation of dietary carbohydrates in farmed fishes: New insights on influencing factors, biological limitations and future strategies," *Aquaculture*, vol. 467, pp. 3–27, 2017.
- [71] V. Buchet, J. L. Zambonino-Infante, and C. L. Cahu, "Effect of lipid level in a compound diet on the development of red drum *Sciaenops ocellatus* larvae," *Aquaculture*, vol. 184, no. 3-4, pp. 339–347, 2000.
- [72] D. R. Gjellesvik, "Fatty acid specificity of bile salt-dependent lipase: enzyme recognition and super-substrate effects," *Biochimica et Biophysica Acta (BBA)—Lipids and Lipid Metabolism*, vol. 1086, no. 2, pp. 167–172, 1991.
- [73] D. R. Tocher, "Metabolism and functions of lipids and fatty acids in teleost fish," *Reviews in Fisheries Science*, vol. 11, no. 2, pp. 107–184, 2010.
- [74] K. Hoehne-Reitan, E. Kjørsvik, and D. R. Gjellesvik, "Development of bile salt-dependent lipase in larval turbot," *Journal of Fish Biology*, vol. 58, no. 3, pp. 737–745, 2001.
- [75] A. J. Fraser, J. C. Gamble, and J. R. Sargent, "Changes in lipid content, lipid class composition and fatty acid composition of developing eggs and unfed larvae of cod (*Gadus morhua*)," *Marine Biology*, vol. 99, no. 3, pp. 307–313, 1988.
- [76] V. P. Voronin, S. A. Murzina, Z. A. Nefedova et al., "A comparative study of lipids and its dynamic during embryogenesis and early post-embryonic development of Atlantic salmon (*Salmo salar* L.) and brown trout (*Salmo trutta* L.)," *Russian Journal of Developmental Biology*, vol. 52, no. 2, pp. 87–96, 2021.
- [77] K. Hoehne-Reitan, E. Kjørsvik, and K. I. Reitan, "Bile salt-dependent lipase in larval turbot, as influenced by density and lipid content of fed prey," *Journal of Fish Biology*, vol. 58, no. 3, pp. 746–754, 2001.