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Morphological changes, peptidase activity, and effects of exogenous enzymes in the early ontogeny of Nile tilapia, *Oreochromis niloticus*

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Abstract
During the early ontogeny, the transition from endogenous (yolk protein) to exogenous feeding (artificial diets) represents a critical period linked to the undifferentiated digestive system, with low digestibility of food protein. The objectives of this work were to characterize the morphology of the early Nile tilapia (*Oreochromis niloticus*) developmental stages and determine the activity of alkaline and acid peptidase enzymes during the ontogenesis from hatching to 20 days post-hatching (DPH). Also, the in vitro effect that exogenous enzymes from Argentine red shrimp (*Pleoticus muelleri*) waste have on the alkaline peptidases of larvae from 6 to 20 DPH (which correspond to the age at which fish eat exogenous food) was studied. Both acid and alkaline peptidase activities varied throughout early ontogeny development (from 0.1 to 1, and from 0.1 to 7.1 UE mg protein^{-1}, respectively). The patterns of both enzyme activity variation would be related with changes in endogenous, mixed and exogenous feeding. Our studies show that the additions of the enzyme extract of shrimp have a synergistic effect (from 3 to 6 times) on endogenous in vitro activity. Moreover, the zymogram analysis demonstrates that the bands corresponding to the activity of each species (tilapia and red shrimp) remain active when they are mixed. The increase in peptidase digestive capacity by addition of exogenous enzymes would maximize the assimilation of nutrients from artificial food during early development.

Keywords Digestion · Enzymes · Ontogeny · Peptidase · Tilapia

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Introduction

Nile tilapia (*Oreochromis niloticus)* (Linnaeus 1758) constitutes one of the most important commercial fish species for aquaculture and it global production is almost 6.3 million tons (FAO 2020). The high demand for tilapia in domestic and international markets has led to a greater intensification of production systems and the use of balanced diets specifically formulated to increase growth. In aquaculture, food takes about 40–50% of production costs (Prabu et al. 2019), thus knowledge about nutrition demand and digestive capacity is essential for food management (de Moura Pereira et al. 2019).

Eating habits of Nile tilapia depend on many factors such as age and size (El-Sayed 2019). For instance, early stages of development require a high protein diet ($35 \geq 50\%$) that ensures their high metabolism and muscle mass growth (El-Sayed 2019). Larvae preferably consume zooplankton (Ibrahim et al. 2015) which represents a source of protein and also provides some exogenous enzymes (Kurokawa et al. 1998). However, culture of zooplankton is costly even though their nutritional contribution to fish diet could be variable (Drossou et al. 2006). In that context, the use of commercial food with added enzymes could constitute an advantage, since it allows a balanced nutrition with a better digestibility.

During the ontogeny, the digestive tract of Nile tilapia undergoes through morphological and histological modifications (Morrison et al. 2001). However, changes in digestive physiology are not totally understood yet, especially those related with enzymes involved in protein hydrolysis. Since the assimilation of amino acids is essential for the synthesis of structural and functional proteins (El-Sayed 2019), knowing the timing in which these enzymes are activated during ontogeny is essential for the design of diets that promote larval growth. Previous studies performed in tilapia larvae until 10 days post hatching (DPH) showed that dietary protein level modifies acid peptidase activity (pepsin) (de Moura Pereira et al. 2019). Nevertheless, information about the peptidase enzyme profile in early and more advanced ontogeny stages (until 20 DPH) and the potential effects of the addition of exogenous enzyme on endogenous hydrolysis capacity is still unknown.

Studies have shown that in several species of fish, including tilapia, the incorporation of exogenous enzymes into the diet increases the ability to digest the nutrients present in the food (Zheng et al. 2019; Huang et al. 2020; Maas et al. 2020; Monier 2020). Nowadays, waste from various industries is being studied as a source of exogenous enzymes. These recovered enzymes could have a lower cost and at the same time can reduce the risk of contamination that such wastes produce (Rodriguez et al. 2017). Fisheries' residues represent a good source of exogenous enzymes, including those from processing of the Argentine red shrimp (*Pleoticus muelleri)* (Bate 1888). During fresh shrimp processing, the cephalothorax — which represents more than 60% of the body-weight — is discarded. Global shrimp production has reached about 4 million tones (FAO 2020). Therefore, a high amount of residual material is available for several potential uses.

The first step to prove the consequence of the exogenous enzymes (inhibition, addition or neutral effect) on digestive capacity is to perform in vitro studies. This information is essential before including shrimp processing waste in the tilapia diet to avoid adverse effects in their digestive process. Thus, the first objective of this work was to assess the morphological characteristics of the early Nile tilapia developmental stages, from hatching to 20 DPH, and determine the activity of acid and alkaline peptidase enzymes during its ontogenesis. Also, the in vitro effect that exogenous enzymes from red shrimp residues have on the endogenous alkaline peptidases of fish from 6 to 20 DPH (which correspond to the age at which larvae eat exogenous food) was studied.

Materials and methods

Tilapia sampling

The care and use of experimental animals complied with "Institutional Committee for the Care and Use of Experimental Animals" animal welfare laws, guidelines, and policies as approved by FCEyN-UNMdP (RD 395–19).

Larvae were obtained from spawning and natural fertilization of adult Nile tilapia on the Aquaculture Laboratory of the National Technological University (Mar del Plata, Argentina). For this, 2 females and 4 males of average weight 600 g were kept in circular tanks of 2000 L in a Recirculating Aquaculture System (RAS) with controlled conditions of photoperiod (10 h dark–14 h light cycle) and temperature (28 ± 1 °C). Fish were fed three times daily (3% of biomass) during 60 days with formulated feed according to the known requirements of broodstock of tilapia (Table 1).

Eggs were removed from the female oral cavity by flushing a stream of water into the mouth. Then, they were incubated in 30 L aquaria. Three days post-hatching (DPH), the larvae formulated food was started to offer ad libitum four times daily (Table 1). For sexual reversion to males, such feed contained 17 α metil-testosterone ALFAREVER® (Jiménez-Badillo and Arredondo-Figueroa 2000; Marjani et al. 2009) which was dissolved in ethanol and then sprayed on the diet (60 mg k^{-1} diet).

Fish were sampled at 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, 15, and 20 DPH. For this, they were cold-anesthetized and euthanized by rapid chilling (2–4 °C). Individuals were transferred from aquaria with acclimation temperature (28 °C) into a small recipient with ice water. A

Table 1 Ingredient composition and proximate composition of formulated feeds to broodstock (1) and for larvae (2) of tilapia (*Oreochromis niloticus)*

Ingredients (g 100 g $diet^{-1}$)	(1)	(2)
Fish meal[1]	25.0	39.0
Soybean meal[2]	35.0	38.0
Wheat meal[2]	20.0	9.0
Rice meal[3]	10.0	4.0
Corn starch[4]	6.0	6.0
Fish oil[1]	3.8	3.8
Vitamins and minerals[5]	0.2	0.2
Proximate composition[6] (% of dry weight)		
Dry mater	93.7	92.8
Ash	7.7	9.0
Total protein	31.0	41.0
Total lipid	8.3	8.5
Carbohydrate	46.7	34.3
Energetic value (Kcal 100 g $diet^{-1}$)	385.5	282.9

[1]Agustiner® (Argentina). [2]Molino Chabas (Argentina). [3]Dos Hermanos (Argentina). [4]Padoan (Argentina). [5]Supradyn® Vitamins (mg g − 1) 1.5 vitamin B1, 1.7 vitamin B2, 2.2 vitamin B6, 0,003 vitamin B12, 60 vitamin C, 15 vitamin E, 5.5 calcium pantothenate, 0.2 biotin, 0.1 folic acid, 19 nicotinamide. (IU g^{-1}) 3.33 vitamin A and 300 vitamin D3. Minerals (mg g^{-1}) 62.5 Ca, 62.5 P, 50 Mg, 4.5 Fe, 0.5 Cu, 0.5 Mn, 3.75 Zn. [6]The proximal analyses of food were performed by standard procedures according to AOAC (1995)

thermometer was used to ensure chilling water temperature (Wallace et al. 2018; Underwood and Anthony 2020). Then, ten individuals of each development stage were placed in 10% buffered formalin for two hours and then transferred to 70% ethanol. Also, 28 additional fishes of each stage were stored at − 20 °C for biochemical analysis. Morphological characteristics, standard and total lengths for each group of fixed fishes were observed by stereo microscopic OlympusSZX16 and photographed using a dark field (Olympus DP73). The relationship between the days after hatching and the appearance of different body structures, such as quantities of caudal fin elements and other morphological characteristics, was studied.

Biochemical analysis

Pools of tilapia were homogenized separately in Eppendorf tubes using plastic rods. Four pools of seven individuals were done for each development stage, due to the small size of individuals.

On the other hand, samples of local fishery red shrimp residuals used as source of exogenous enzymes were provided by manufacturing plants from Mar del Plata, Argentina (38°02′ S, 57°30′ W). Samples (cephalothorax of shrimp) were transported to the laboratory (UNMdP) and immediately the exoskeleton was removed and the tissues were homogenized on an ice bed in a borisilicate-teflon glass tube. Homogenates of tilapia and shrimp were centrifuged (Presvac EPF-12R, Argentina) for 30 min at 4 °C and 10,000 g. The supernatants were frozen (− 20 °C) for subsequent tests.

Acid peptidase activity of enzymatic extracts of tilapia samples was determined according to Anson (1938) with 0.5% (w/v) bovine hemoglobin (Sigma H2625) in 200 mM pH3 glycine–HCl buffer, whereas alkaline peptidase activity of enzymatic extracts of tilapia and shrimp was measured according to García-Carreño (1992), with 0.5% (w/v) azocasein (Sigma A2765) dissolved in 50 mM pH 8 Tris–HCl buffer was used as substrate. Enzymatic activities were performed by triplicate and the data were expressed as total enzymatic activity (UE mL^{-1}, where UE = Abs min^{-1} and as specific activity (UE mg $protein^{-1}$).

In all enzyme extracts, the soluble protein concentration was evaluated according to the method of Bradford (1976) and bovine albumin was used as the standard protein (Sigma A9647). This data was used to express peptidase specific activity.

To assess possible synergistic cooperation between alkaline peptidases of tilapia and exogenous enzymes (shrimp extract), the enzymatic activity of each extract and their mixtures (for example, 5 µL 6 DPH of Nile tilapia extract + 5 µL shrimp extract) were evaluated, according to the protocol explained previously. The possible synergistic cooperation was assayed from 6 to 20 DPH, which correspond to the age at which fish eats exogenous food.

The proteins of each enzyme extract were separated by 12% SDS-Page (Laemmli 1970). Aliquots of homogenates containing 20 µg of protein were diluted in 1:1 v/v sample buffer; and also 5 µL of molecular standard marker (Protein™ Standards BIO-RAD) was loaded in different lanes of the gel. The electrophoresis was run at 30 milliAmperes per gel in a recirculation bath cooled to 4 °C (Mini PROTEAN Tetra System BIO-RAD). The gels were removed and stained with aqueous solution containing 0.5% w/v Commassie Blue R-250 in 40% v/v methanol, 7% v/v acetic acid. Then it was washed with an aqueous solution with 40% v/v methanol and 7% acetic acid. The Rf of each band was calculated to determine the molecular weight of the bands observed (Image lab 6.0 BIO RAD software, CA, USA).

Zymograms were performed to show endogenous enzyme (alkaline peptidases of tilapia), exogenous enzymes (shrimp extract), and their mixtures. Zymograms corresponding to mixture enzymes (endogenous + exogenous) were done from 6 to 20 DPH when fish starts exogenous feeding. For this, enzyme preparations, containing 10 mU of each protein extract (diluted 1:1 with sample buffer), were loaded into individual gel wells. The mixtures (5 µL each tilapia stage extract + 5 µL shrimp extract) were incubated for 60 min, at room temperature before electrophoresis run.

Enzyme extract of each tilapia stage containing 10 mU of activity was incubated with an inhibitor of peptidases belonging to the serine class. The solution of soybean trypsin inhibitor (SBTI), 250 µM in distilled water, was added to each enzyme extracts at a ratio of 1:2 (inhibitor/extract) and incubated at room temperature for 60 min. Distilled water replaced inhibitors in zero inhibition controls. Then, the treated enzyme preparations were subjected to SDS-Page as described above. After electrophoresis, gels were treated according to Fernández-Gimenez et al. (2001).

Statistical analysis

Statistical analyses were performed using the Sigma-Stat 3.0 statistical package for Windows operating system, which automatically performs a previous test for equal variance and normality. A parametric one-way ANOVA analysis of variance was used. A posteriori ANOVA test using the Holm-Sidak method was used to identify in peptidase activity among different development stages. Differences between total alkaline peptidase activity of tilapia and mixture activity (enzymatic extract of tilapia plus enzymatic extract of shrimp) for each ontogeny stage (from 6 to 20 DPH) were evaluated using a *t*-test. A significant level of 0.05 was considered for all analyses.

Results

Morphological characterization of tilapia developmental stages

The morphological aspects as total length, standard length, caudal fin ray elements (CFRE), and other characteristics of different developmental stages of tilapia are shown in Fig. 1. The fish of the earliest stages (1–3 DPH) have a standard length between 6.5 and 7.7 mm and are characterized by a large amount of yolk surrounded by capillaries; while individuals from 4 to 6 DPH present a standard length between 7.9 and 8.7 mm. During these stages, yolk begins to decrease and the ossification of the dorsal fin rays takes place. The mouth opens and becomes functional around 6 DPH. Noticeably, an enhancement in melanophores density was observed in the head and dorsal area of the body.

Also, between 7 and 20 DPH, the standard lengths ranged between 7.9 and 14.8 mm. During this period, there is an increase in the number of CFRE and pigmentation. In stage 7 DPH, the yolk is completely included in the body cavity whereas in the 20 DPH stage, yolk is absent. Moreover, at 20 DPH, the melanophores spread to the ventral region of the body and individuals have a very similar appearance to that of the adults.

DPH	Total lenght (mm)	Standard lenght (mm)	Caudal fin ray elements	Characteristics
1	7.9±0.19	6.5±0.19	2	Dorsal and anal fin fold- large yolk covered by capillaries
2	8.2±0.24	6.7±0.19	3	Large yolk covered by capillaries
3	9.2±0.29	7.7±0.26	4	Gills covered by operculum
4	9.8±0.64	7.9±0.39	5-6	Yolk absorption initiated- pelvic fin bud over yolk- soft rays on dorsal and anal fins
5	10.4±0.27	8.3±0.18	6	Yolk partially absorbed and covered (75%) by white tissue- evidence of lateral line
6	10.8±0.54	8.7±0.38	7	Yolk partially absorbed and covered (90%) by white tissue- hard and soft rays on dorsal fin
7	10.1±0.24	7.9±0.20	8	Yolk completely absorbed into body cavity walls
9	10.3±0.64	8.2±0.49	8-9	Rays on pelvic fin
11	11.5±0.27	9.1±0.24	9-10	Pelvic fin more developed
13	13.6±1.00	11.0±0.83	11	Patch of pigmentation on lateral trunk-tail region
15	14.4±0.91	11.7±0.57	12	
20	18.9±0.18	14.8±0.15	14	Darkness of patch of pigmentation on lateral trunk-tail region

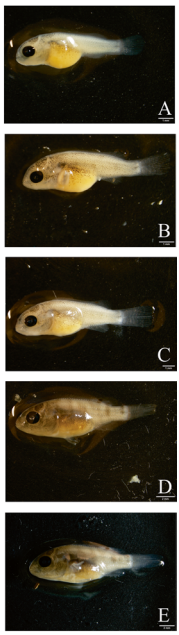

Fig. 1 Total length, standard length, caudal fin ray elements (CFRE), and morphological characteristics of developmental stages of Nile tilapia (*Oreochromis niloticus*). Photographs: **A** 2 DPH, 38.8×; **B** 4 DPH, 38.8×; **C** 6 DPH, 31×; **D** 13 DPH, 24.8×; **E** 20 DPH, 21.7×. *DPH* days post-hatching. ($n = 10$)

Peptidase activities of the different tilapia developmental stages

Acid specific peptidase activity was detected from hatching. The activity varied throughout the studied stages showing two "waves" of secretion. The first peak was detected at 7 DPH (0.8 ± 0.1 UE mg protein^{-1}) and the second at 13–15 DPH (1 ± 0.2 UE mg protein^{-1}). On day 20 after hatching, the activity decreased to minimal values (Fig. 2a).

Alkaline peptidase activity was detected from the first day post-hatching. However, the specific activity was low until 9 DPH (from 0.1 ± 0.05 to 1 ± 0.2 UE mg protein^{-1}). From day 11, an increase of approximately 70% of activity was observed compared to day 1 after hatching, reaching the highest value at 20 DPH (7.1 ± 0.6 UE mg protein^{-1}) ($P < 0.05$) (Fig. 2b).

Effects of exogenous enzymes addition

The addition effect of shrimp enzymes on the alkaline peptidase activity of Nile tilapia was tested from 6 DPH because tilapia began exogenous feeding at this time. In all

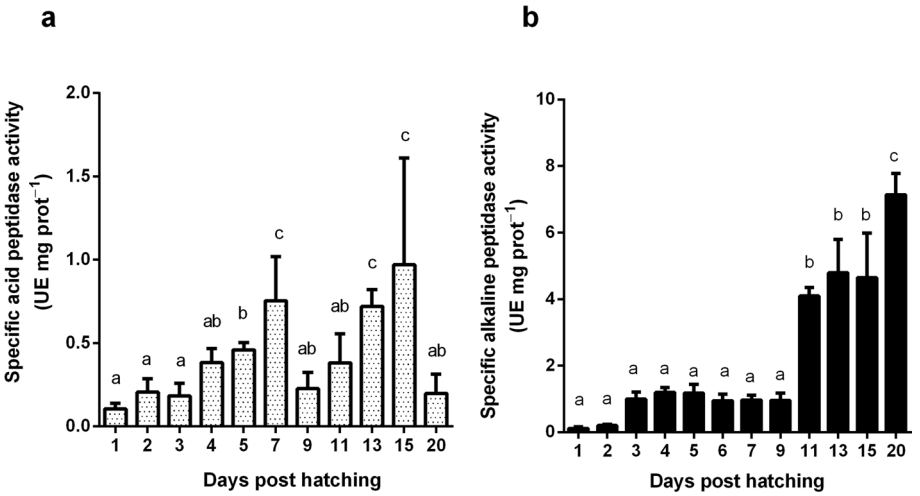

Fig. 2 Acid (**a**) and alkaline (**b**) specific peptidase activity of Nile tilapia (*Oreochromis niloticus*) in different development stages. Different letters indicate significant different ($P < 0.05$) ($n = 4$ of 7 pooled individuals)

Table 2 Effects of in vitro addition of exogenous enzymes from Argentine red shrimp (*Pleoticus muelleri)* on the total alkaline peptidase activity of Nile tilapia (*Orechromis niloticus*) of different days post hatching (DPH)

DPH	Nile tilapia	Nile tilapia + shrimp
6	13.3 ± 2.97	84.5 ± 4.42*
7	13.6 ± 2.04	95.5 ± 2.56*
9	13.5 ± 3.02	96.5 ± 5.04*
11	23.0 ± 1.49	104.8 ± 3.01*
13	26.1 ± 5.43	83.9 ± 3.07*
15	35.1 ± 10.16	97,3 ± 2.89*
20	47.1 ± 4.20	131.4 ± 5.86*
Shrimp	99.6 ± 2.20	

Total peptidase activity was expressed as UE ml^{-1}. Data are the mean ± SE. $n = 4$ of 7 pooled individuals, *indicate significant differences on row ($P < 0.005$)

the studied stages, total peptidase activity was raised approximately 3 to 6 times by the addition of exogenous enzymes from shrimp ($P < 0.05$) (Table 2).

The zymogram of the alkaline peptidase activity corresponding to the different studied stages of tilapia is shown in Fig. 3. Stages 1 and 2 DPH present three active bands with molecular weight of 33.1, 47.4, and 91.1 kDa. Three bands were observed for stage 3 DPH: the same two enzymes bands (33.1 and 47.4 kDa) that were observed in the previous stages and also, a new band of 44.8 kDa. From 4 to 20 DPH, four bands of 33.1, 44.8, 47.4, and 69 kDa were observed. All enzymes detected by SDS-Page were inhibited after incubation with SBTI inhibitor, which suggests that these enzymes are serine-type (data not shown). Zymogram of alkaline peptidase from shrimp extract exhibited 4 bands of 22.1, 29.4, 57, and 65.9 kDa. All performed zymograms show that both tilapia and shrimp kept their catalytic activity after incubation mixed (Fig. 3).

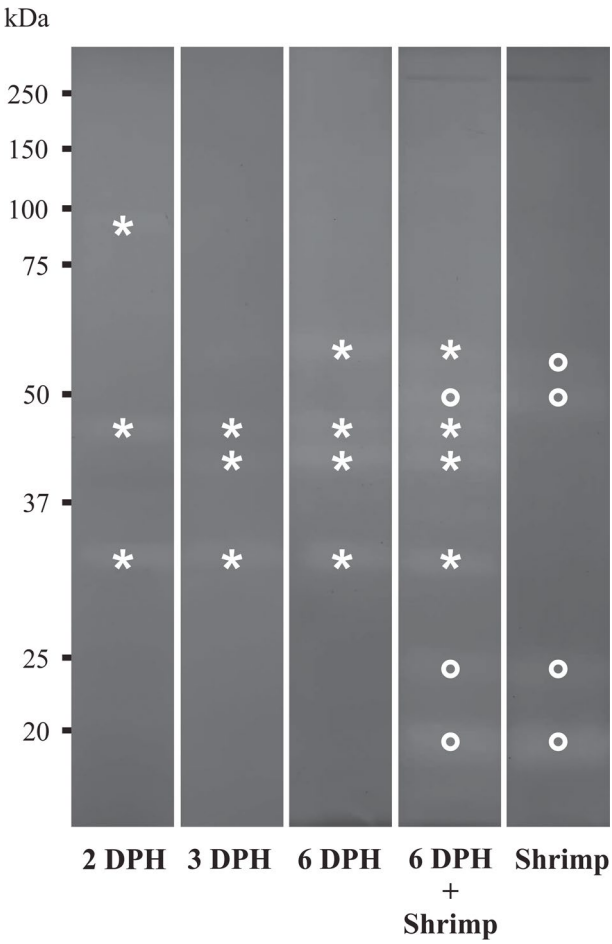

Fig. 3 Zymogram of alkaline enzyme extracts and their mixture. (*) Nile tilapia (*Oreochromis niloticus*) activity bands. (o) Exogenous enzymes activity bands; 2, 3, and 6: enzyme extract of tilapia of 2, 3, and 6 DPH respectively; shrimp (*Pleoticus muelleri*) cephalothorax enzyme extract. *DPH* days post-hatching. Some bands appear weak due to the low activity of the Nile tilapia extracts and the impossibility of adding more extract to the gel lane

Discussion

Morphological characterization of tilapia developmental stages

The growth of tilapia depends on several factors (i.e., temperature, oxygenation, feeding management, etc.) that can affect the time of onset and the degree of development of different morphological characters of larvae after hatching (Fujimura and Okada 2007; El-Sayed 2019). For teleost fish, the first step of larval development is a critical period with high energetic demands, mainly when yolk reserves become scarce and exogenous feeding begins. In this stage, the consumption and digestive efficiency determines both growth and survival (Robert et al. 2014). Throughout their ontogeny, the larvae exhibit three types of feeding. First, they metabolize endogenous yolk reserves, then go through a mixed endogenous and exogenous feeding, and finally rely only on exogenous consumption (Khalil et al. 2011). Once the mouth is open, the digestive system becomes functional and increases in length and mass, but the switch to different ways of feeding depends on the size of the mouth gape and the degree of development of their digestive tract (Yúfera and Darias

2007). Thus, feeding transition constitutes a bottleneck with high mortality rates characterized by low digestive enzyme activity and poor food digestibility because of the fast passage of digesta (Khalil et al. 2011).

Peptidase activities of the different tilapia developmental stages

The digestive enzyme activities during ontogeny stages must be known in order to determine the ability of fish to utilize a given diet. This study revealed the presence of acid and alkaline peptidase activities in all the stages studied — from 1 to 20 DPH — of tilapia and demonstrated their ability to hydrolyze protein dietary substrates. Both activities were detected from the first day of hatching. However, during development, the variation pattern of acid and alkaline peptidase activities was clearly different. Such modulation of peptidase activity could be related with the transition of energy acquisition, from endotrophic to exotrophic nutrition.

On the other hand, the eggs of teleost fish have a yolk protein called vitellogenin. These high molecular weight proteins (300–600 kDa) constitute the main source of amino acid to allow adequate embryonic and larval growth (Hiramatsu et al. 2015; Sullivan and Yilmaz 2018; Riddle and Hu 2021). In this study, it was demonstrated that from 1 to 6 DPH, fish get the energy from yolk reserves since they just open their mouth at 6 DPH. Moreover, the low level of acid and alkaline peptidases activity found in this period (1–6 DPH) could be enough to allow the degradation of egg yolk proteins. In teleost fish, yolk nutrients are hydrolyzed and transported to larvae embryonic cells by an extra-embryonic tissue, the yolk syncytial layer. This syncytial layer is located between two germinal layers surrounding all the yolk: ectoderm and mesoderm (Kondakova et al. 2016). Therefore, yolk absorption occurs without connection with endoderm cells of the primitive intestine (Kunz-Ramsay 2013).

Additionally, the compositions of required nutrients and their metabolism vary during early ontogeny in order to support energy requirements. Although this mobilization may differ among species, most teleosts show a sigmoid pattern, in which carbohydrates are used before hatching and proteins and lipids are hydrolyzed thereafter. Amino acids from protein degradation are the last nutrients to be used from endogenous reserves (Heming and Buddington 1988; Zavala 2011). Peptidases called cathepsins play an important role in the degradation of yolk reserves during early development. Among them, cathepsin B and D are cysteine and aspartic peptidases respectively, whose transcript levels increase after fertilization (Gwon et al. 2017; Palomino et al. 2017; Oh et al. 2018). In some teleost fish, it was demonstrated that both cathepsins and serine peptidases participate in egg yolk degradation and mobilization (Raldúa et al. 2006; Gupta et al. 2020; Riddle and Hu 2021). Indeed, this work demonstrated that those fish belonging from 1 to 6 DPH have activities of alkaline peptidases (serine-type) and acid peptidases. This evidence suggests that this level of peptidase activity is enough to hydrolyze yolk protein since a decrease in the bulk reserves was notorious at early developmental stages.

For teleost, the development of stomach coincides with the beginning of exogenous food consumption. Histological studies performed in tilapia found a fledgling stomach at 4 DPH with a few gastric glands. Simultaneously with the development of the stomach, the intestine appears as a structure with thin walls and few folds (Khalil et al. 2011). The first step for extracellular digestion occurs in the stomach when gastric glands start to develop and secretes HCl who activates pepsin. The digestion of dietary protein thus begins in the stomach and continues in the intestine by means of brush border aminopeptidase that are

present in early stages of tilapia (Tengjaroenkul et al. 2002; de Moura Pereira et al. 2019). Acid peptidases are aspartic endoproteases that digest proteins at a pH range from 3 to 4. The activity of these peptidases such as pepsin is required to initiate hydrolysis of dietary proteins (Khalil et al. 2011; Silva et al. 2019). Although the complete development of stomach digestive glands in Nile tilapia occurs about 1–6 DPH (Morrison et al. 2004), in the present work, it was found that the activity of acid peptidases are present from the first day after hatching. Moreover, low activity was also described at gastrula and blastula stages by de Moura Pereira et al. (2019). These results differ from those described for the euryhaline species of Mozambique tilapia (*Oreochromis mossambicus)* in which pepsin activity is detected from day 3 DPH (Lo and Weng 2006) and highlights the existence of interspecific differences in the activation of these enzymes during ontogeny. In the present work, tilapia larvae maintain the low acid peptidase activity detected at the first DPH until 7 DPH, when a first increase occurs in coincidence with the beginning of exogenous feeding; similar results were found by de Moura Pereira et al. (2019). A second increase in activity (day 13) appears in concomitance with the increase observed in the activity of alkaline peptidases. It is likely that this second enhancement in acid peptidase activity (corresponding to gastric pepsin activity) will provide more substrate availability for the serine peptidases (trypsin, chymotrypsin, and elastase) released by the pancreatic gland toward the intestine. It was suggested that the schedule of proteolytic enzymes activation involved in protein metabolism occurs in order to enhance protein utilization and assimilation (Tengjaroenkul et al. 2002; Con et al. 2019; Silva et al. 2019). Hormonal regulation of the physiology of gastrointestinal tract occurs in response to food intake. Several hormones secreted by digestive tract, accessory organs (pancreas, liver), and other organs signals (i.e., central nervous system, thyroid, adrenal cortex) are involved in the modulation of digestion and absorption processes to match energy demands during life metamorphosis (Buddington and Krogdahl 2004; Volkoff 2016).

The existence of a functional pancreas indicates a step further in the growing digestive function (Tengjaroenkul et al. 2002). Development of exocrine pancreas occurs at hatching, when few cells are surrounding the main endocrine islet (Morrison et al. 2001; 2004). Thus, small quantities of pancreatic enzymes could be released into the digestive tract at the early larval stage. Exogenous feeding in fish begins at 9–10 days post-fecundation (5–6 DPH) when the pharyngeal skeleton becomes functional and the oropharyngeal membrane that covers the mouth starts to open (Morrison et al. 2001; 2004; Fujimura and Okada 2007). We provided evidence that the addition of exogenous enzymes of shrimp waste enhanced the proteolytic activity. Noticeably, alkaline peptidase activity increased on 11 DPH suggesting that there is a concomitant increase in enzyme activity following the start of exogenous food intake. This enhancement in alkaline peptidases activity could be associated with a progressive development of exocrine pancreas. Histological studies performed in Nile tilapia found an expanding exogenous pancreas at 16 DPH that connects to the portal hepatic system, forming the hepatopancreas (Morrison et al. 2004).

During metamorphosis, the variations in amino acid profile are marked and therefore, the protein requirements also change during ontogeny (Conceição et al. 2011). Several studies in fishes have shown that the addition of exogenous enzymes to food increases protein digestive efficiency (Zheng et al. 2019; Huang et al. 2020; Monier 2020). Particularly, in advanced stage of development as juveniles (older than 30 DPH), Rodriguez et al. (2017) found that the dietary addition of red shrimp waste processing synergistically increased the alkaline peptidase activity of the fish intestine. However, no studies have been done about the possible effect of the use of exogenous enzymes in food during early ontogeny of tilapia (1 to 20 DPH). In fish culture, the use of solid food allows farmers to become

independent of the complexity and the high costs of using live food. It also enables them to do sex reversal by adding hormones but, generally, dry pellets show less digestibility that live food.

Effects of exogenous enzymes addition

The first step toward learning the consequence of the addition of exogenous enzymes on digestive capacity is to perform in vitro studies. These studies allow us to evaluate whether the exogenous enzyme increases, decreases, or has no effect on the activity of endogenous enzymes. The results of this study show that the addition of the enzyme extract of red shrimp has a synergistic effect on endogenous activity of the early developmental stages of Nile tilapia. Moreover, the zymogram analysis demonstrates that the bands corresponding to the activity of each species, Nile tilapia and red shrimp, remain active. This maintenance of bands is reflected in an increase of six times in the activity of alkaline peptidase. The increase in proteolytic capacity by the addition of exogenous enzymes would maximize the assimilation of nutrients from artificial food. Younger fish have higher requirements of protein attributable to high rate of specific growth and metabolism (Otieno et al. 2014). In particular, the larvae of Nile tilapia demands approximately 35–45% of dietary protein to reach their maximal growth (El-Sayed 2019). Thus, the addition of exogenous enzymes in food could lead to a better digestion and absorption of dietary protein. Therefore, its use in aquaculture could improve larvae growth to reach the required commercial size in less time. However, future in vivo bioassays using diet supplemented with exogenous enzyme should be performed to validate this hypothesis. In addition, the recycling of shrimp wastes as an exogenous enzyme for fish feed would significantly reduce the cost of food and would allow farmers to become independent from the expenditure and the time required for zooplankton production. This novelty knowledge complements our previous information about the addition of exogenous peptidases for other growing stages of Nile tilapia. Moreover, the recycling of shrimp waste inside a design economy circular would decrease environmental pollution and at the same time, could lead to a profit in tilapia farming.

Conclusion

Aquaculture is a growing source of protein for human consumption but at the same time, this activity could be economically and environmentally unsustainable, due to the high cost of balanced fish feed and the large amount of waste generated by its processing. Protein supplementation of fish diet is generally assumed as a solution to increase growth, but protein surplus cannot always be metabolized and assimilated by fish. One solution to these problems is to increase the efficiency of feed consumption by exogenous enzymes addition in diets. Due to the fact that teleost fishes exhibit different patterns of organ and physiological development, it is necessary to know the timing in which the activation of digestive enzymes occur to synchronize the addition of exogenous enzymes in the food. The larvae of Nile tilapia as a biological experimental model allow to verify that exogenous enzymes from fishing residues would lead to the optimization of protein digestion and concomitantly to the reduction of the negative environmental effects of aquaculture and fisheries.

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Data availability Not applicable.

Code availability Not applicable.

Declarations

Ethics approval The care and use of experimental animals complied with "Institutional Committee for the Care and Use of Experimental Animals" animal welfare laws, guidelines, and policies as approved by FCEyN-UNMdP (RD 395–19).

Conflict of interest The authors declare no competing interests.

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