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Digestive enzymatic activity on Tropical gar (*Atractosteus tropicus*) larvae fed different diets

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Abstract Digestive enzymatic activity and growth performance on tropical gar (*Atractosteus tropicus*) larvae fed Artemia nauplii (LF), frozen adult Artemia (AB), an artificial diet (AF) with 46% protein and 16% lipids and a starvation group (SG) from first feeding (5 days after hatching—5 DAH) to 34 DAH were studied. All larvae under starvation (SG) died at 15 DAH. By the end of the experimental period, morphological variables (total length, wet weight and specific growth rate) were significant in larvae fed AF compared to LF and AB. All enzymes studied in the experiment were present since the start of exogenous feeding (including pepsin) and the enzymatic activity varied with the diets. Low levels of enzymatic activity were observed until the 29 DAH; however, after this moment, there was a significant increase (eightfold), particularly for the AF treatment. In vitro protein digestibility tests performed with enzymatic extracts showed that artificial diets with 52% protein and 14% lipids were better digested by larvae before 30 DAH, while diets with 45% protein and 11% lipids were better digested after this age. Taking into account the better growth performance, higher enzymatic activity and better protein digestibility obtained, artificial diets can be used since the start of exogenous feeding on tropical gar larvae, as in other lepisosteids.

Keywords Tropical gar · Atractosteus tropicus · Artificial diets · Digestive enzymes · Protein digestibility

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Introduction

The family Lepisosteidae is represented by seven extant species of non-teleost bony fishes that are distributed in North America, Central America and Cuba. The southernmost limit is reached by tropical gar (*Atractosteus tropicus*). Many of the lepisosteid populations have declined due to factors such as high trophic position and relatively long life span of these fish, habitat loss, alteration and also overfishing (Mendoza et al. 2008a). In the state of Tabasco, Mexico, fisheries constitute one of the main economic activities, and 30% of the total catch corresponds to freshwater species. Among these species, the tropical gar outstands as one of the most popular, being captured in 32 official registered fisheries in the state (CONAPESCA 2008). Tropical gar capture peaked in 1996 attaining more than 500 tonnes; however, capture volumes, as well as the size of

captured fish, have been markedly declining in the last years, similar to other lepisosteids like alligator gar (*A. spatula*) and Cuban gar (*A. tristoechus*) (Mendoza et al. 2008a).

The decline of tropical gar populations has resulted in research into the feasibility of using captive culture techniques to reduce fisheries pressure and to restore natural populations (Maldonado and Ponce 1991; Mendoza et al. 2008a; Castro-Mejia et al. 2009). Larval feeding stands as one of the key aspects for the commercial aquaculture and one of the greatest challenges to accomplish fish larvae domestication and production in controlled culture systems (Zambonino and Cahu 2007; Nguyen et al. 2010). Formulation and use of artificial diets is paramount for fish larval culture, constituting the best alternative compared to live feed which is labor intensive and expensive (Lazo et al. 2007; Conceicao et al. 2010). However, after two decades of research on the formulation of microdiets, only limited success has been achieved due to problems related to the ingestion and digestion of these diets, among other factors (Zambonino and Cahu 2007; Conceicao et al. 2010). These problems have been attributed to the incomplete functioning of the digestive system in larvae. A clear relationship has been established between the increase in digestive enzymatic activity and the ability of larvae to use artificial diets; thus, the replacement of live feed must coincide with the maturation of the digestive system (Moyano et al. 1996; Zambonino and Cahu 2007).

Previous studies indicate the feasibility to successfully wean lepisosteid larvae into artificial diets without affecting growth or survival (Mendoza et al. 2008b). Digestive processes were elucidated using substrate-PAGE–electrophoresis, which allowed describing the quantity, type and ontogeny of digestive proteolytic enzymes for alligator gar (Mendoza et al. 2002), Cuban gar (Comabella et al. 2006) and tropical gar (Mendoza et al. 2008a). Despite the close phylogenetic relationships among these species, several differences in the maturation of the digestive tract, important to the feeding strategies of cultured larvae, were observed. The fastest digestive tract maturation rate was observed in alligator gar, followed by Cuban gar and tropical gar, which is in relation to their growth rate and final size (Mendoza et al. 2008a).

Within this context, in order to develop a successful feeding strategy for tropical gar larvae, the present study was aimed to evaluate the growth of larvae fed different feeds and to gain understanding of the digestive processes of larvae in response to these feeds along their development.

Materials and methods

Animals

Tropical gar larvae were obtained from the controlled spawn of breeding adults at the Aquaculture laboratory of the Juarez Autonomous University of Tabasco. The spawning was made in a circular tank (60 cm height, 2 m in diameter), in which one adult female (2,495 g) previously injected with Ovaprim (0.5 mL/kg) and two males (1,072.2 ± 3.87 g) were placed. In order to accommodate the spawning behavior of lepisosteids (Mendoza et al. 2008a), polyethylene strips of raffia were spread throughout the tank to provide spawning substrate, simulating the flooded grounds in which they normally spawn. Broodstocks were removed from the pond after spawning. Larvae remained in this tank until the third day after hatching (3 DAH), when they were transported to the experimental tanks where the feeding bioassay was carried out.

Experimental conditions

Twelve circular plastic tanks (90 × 70 × 30 cm) were used as experimental units. Water flow was provided with a 1/8-hp pump, and water level was adjusted by means of valves. During the first 15 days, water volume was maintained at 40 L, from this time onwards the volume was increased at 80 L, until the end of the experiment. A water volume of 30% was exchanged every third day to maintain water quality. Water temperature was maintained at 28°C with the use of thermostats, and oxygen was provided throughout the experiment by a compressor to maintain dissolved oxygen levels above 6 mg/L. At this time, 12,000 larvae with a mean initial weight of 16 ± 2 mg and a mean initial length of 12.06 ± 1.2 mm were randomly distributed into the 4 experimental groups.

Diets

Starting from 5 DAH, when free-swimming larvae initiate exogenous feeding, each test diet was fed in

four daily rations to three replicate tanks with 1,000 tropical gar larvae per tank. At the start of the feeding trials, initial weight and total length of larvae were determined. Larvae were selected according to the uniformity of their weight and size as determined by a one-way ANOVA. Four treatments were tested: (i) LF consisting of recently hatched *Artemia* nauplii (Salt Creek Select, Great Salt Lake, Utah), as a positive control. (ii) Frozen adult *Artemia* biomass (AB) supplied by H_2O LIFE Aquarium Foods (San Diego, CA). (iii) Commercial artificial feed (AF) for trout (Silver Cup), with 46% protein and 16% lipids. The AF was crumbled and sieved to particles of 0.5, 1 and 1.5 mm diameter and was fed to larvae at 5–14 DAH, 14–29 DAH and 29–34 DAH, respectively. (iv) A starvation group (SG) to which no feed was offered.

Artemia cysts were prepared 24 h prior to each feeding. Just before being fed to the larvae, the nauplii were washed with freshwater and equally distributed between the tanks. *Artemia* treatments (LF and AB) and artificial diets were fed ad libitum.

Experimental design

Samples were collected starting from 3 DAH and finished at 34 DAH. During the first 12 days, 100 larvae per treatment were sampled daily. During the following 4 samplings, at 5-day interval, 50 larvae per treatment were collected. Samples were taken every morning before first feeding. Larvae were euthanized with tricaine methanesulfonate (MS222), blotted dry and then weighed to the nearest 0.1 mg for wet weight (WW) determination. Total length (TL) was recorded for each larva using a caliper rule with 0.05-mm precision. Larvae were dissected on a cold plate (4°C). The head was separated at the operculum, and the digestive tracts were removed and pooled by sampling day and treatment and were freeze-dried. Enzymatic analyses were performed at the Ecophysiology laboratory of the Autonomous University of Nuevo León.

Digestive enzymes

Digestive tracts were homogenized in distilled water 1:5 (w/v) using a Wheaton-glass homogenizer at 4°C. The resulting homogenates were centrifuged at 15,300×*g* during 15 min at 4°C. Supernatants were stored in 0.5-mL aliquots at −20°C to later be used as enzymatic extracts. The reaction mixtures consisted of 0.02 mL of enzymatic extract and 0.3 mL of substrate dissolved in specific buffers for each assay. Hemoglobin, dissolved in universal buffer (pH = 3), was used as substrate for measuring total acid proteolytic (pepsin) activity (Anson 1938). Azocasein was used to measure total alkaline protease activity (Galgani and Nagayama 1986), while N-benzoil-arginine (BAPNA), N-gutaril-phenilalanine-p-nitroaniline (GPNA) and L-leucine-p-nitroaniline (LNA) were used to determine trypsin, chymotrypsin and aminopeptidase activities (Erlanger et al. 1961; Lauff and Hofer 1984). Alkaline and acid phosphatase activities were measured in a similar way using p-nitrophenyl phosphate with 1.0 M diethanolamine/50 mM $MgCl_2$ (pH 9.8)/0.1 M sodium acetate-HCl (pH 4.8) as buffers, respectively (Moyano et al. 1996). Lipase (non-specific b-esterase) activity was estimated following the method of Munilla and Stark (1989) using 50 mM Tris–HCl (pH 7.2) as the buffer and p-nitrophenyl acetate as the substrate. α-Amylase was assayed in 0.1 M sodium acetate-HCl/6 mM NaCl (pH 4.8) with a 1% starch solution as the substrate (Bernfeld 1951). In a glass tube, 0.5 mL of the starch solution was added to 25 µL of homogenate and 0.5 mL of buffer. The tube was incubated at 25°C for 30 min and the reaction was stopped by the addition of 1.5 mL of dinitrosalicylic acid (DNS), followed by boiling for 15 min. The resulting solution was diluted with 2.5 mL of distilled water, and α-amylase activity was determined spectrophotometrically at 540 nm.

All assays were carried out in triplicate at room temperature (25°C) using a Tecan Sunrise micro-plate spectrophotometer and Nalgen Nunc flat-bottom 96-well microplates (Fisher Scientific, Hampton, N.H.), except for the assays of amylase and total protease activities, where a Spectronic spectrophotometer (Thermo Electronic, Waltham, Mass.) with quartz cells was used. All reactions were run at saturating substrate concentrations and compared with an appropriate blank. Digestive enzymatic activities were presented as total activity (U/larva) or specific activity (U/mg protein). For all the assays, one unit of activity (U) was defined as the amount of enzyme that catalyzes the substrate causing a 0.001 change in absorbance per minute (Walter 1984; Comabella et al. 2006). The total soluble protein concentrations in the enzyme extracts were determined using the method of

Bradford (1976) with bovine serum albumin (BSA) as a standard.

Protein digestibility

Enzymatic extracts of 10, 29 and 34 DAH larval digestive tracts were used to determine in vitro protein digestibility using the protocol described in Alarcon et al. (1999). Artificial feeds with different nutrient composition and obtained by different processes were used: (1) a micro-extruded-marumerized (Barrows 2000) micro-spheres diet with 45% protein and 11% lipids (MP45/11). (2) An extruded diet with 52% protein and 14% lipids (E52/14). (3) An extruded diet with 46% protein and 16% lipids (E46/16). Artificial feeds were homogenized in distilled water (100 mg/mL) and centrifuged at 21,000×*g* for 20 min, and the resulting supernatant was used as the substrate in the reaction mixture.

Hemoglobin and casein at 1% were used as reference substrates to test acid and alkaline activities, respectively. The reaction mixture consisted of 0.5 mL substrate, 0.5 mL sodium acetate-HCl buffer (0.1 M, pH 3.0) and 0.5 mL enzymatic extract. The mixture was then incubated at 37°C for 30 min, and the enzymatic reaction was stopped with cold 12% trichloroacetic acid (TCA). A control tube to which TCA was added before incubation was used as a reference. Samples were centrifuged at 9,520×*g*, at 4°C for 5 min and non-digested material was discarded. Tris–HCl (50 mM, pH 8.5) buffer was used for estimating alkaline activity under the previously described conditions. The difference in absorbance at 280 nm between test tubes and the control was recorded. Protein digestibility, as a result of released amino acids and peptides, was expressed as a direct function of absorbance at 280 nm.

Variables and statistical methods

The morphometric TL, WW and specific growth rate in TL or WW (SGR = [100 (ln TL_2 − ln TL_1)/#days)] or [100 (ln WW_2 − ln WW_1)/#days] were recorded. Enzymatic activities were determined by means of three independent assays on the extracts of each treatment. The analyses were conducted using the SPSS 9.0 software, Chicago, IL, USA. Data were submitted to Kolmogorov–Smirnov normality test. Significant differences were determined using one-way ANOVA analysis. The new Duncan multiple-range test (Steel and Torrie 1980) was used to identify the differences among mean values at the 0.05 level.

Results

Growth

At 10 DAH, survival was above 90% for larvae in all treatments, including the SG. At 15 DAH, treatments LF, AB and AF still showed a high survival rate (near 80%), whereas by this time, all larvae had already died in SG. From this moment on, cannibalism was the main cause of mortality in the rest of the treatments. Survival at the end of the experiment (34 DAH) was 50, 47 and 48% for LF, AB and AF, and no significant differences among them were registered.

Wet weight and total length results for the experimental treatments at different DAH are shown in Tables 1 and 2, respectively. A similar growth performance of larvae from the different treatments can be observed until 8 DAH. This could be explained by the existence of yolk reserves during this stage. However, once that these reserves are exhausted, and the larvae start depending exclusively on exogenous food, growth variations among treatments are evident, particularly because growth stops in the SG. Larvae fed *Artemia* nauplii (LF) showed a significant higher growth, expressed in terms of wet weight since 9 DAH, as well as in total length at 10 DAH. This better growth performance was maintained only until 14 DAH; thereafter, larvae fed AB and AF performed better. Larvae fed AB showed the best growth performance between 10 and 14 DAH, while larvae fed AF despite showing the lower growth performance during the first days gradually recover since 14 DAH and outperformed the rest of the treatments from 19 DAH, showing significant differences in WW and TL. From this moment onwards, and until the end of the feeding trial, the differences became more conspicuous and the AF treatment showed a statistically significant better growth performance, followed by the AB and LF treatments.

Similar results were observed regarding the SGR. While SGR declines through time, those fed AB and AF showed a gradual and constant increase until the

end of the experiment. Again, this increase was more noticeable for larvae fed AF, which reached a 2.19 mm/day SGR-TL, while larvae fed AB ended with a 1.25 mm/day SGR-TL (Fig. 1).

Digestive enzymes

In order to clearly show the variation in enzymatic activities along the experiment, results were separated from the beginning of the experiment until 29 DAH and from this time until the end of the experiment (34 DAH), considering the eightfold increase in the magnitude of enzymatic activities that in this last period.

Pepsin and total alkaline proteolytic activities were observed since the start of exogenous feeding at 5 DAH in all treatments. Both activities were similar in the LF and SG treatments, with the exception of activities at 8 and 14 DAH when a marked difference in pepsin was observed (Fig. 2). In contrast, in larvae fed AB and AF, the total proteolytic activity was lower during this period. In particular, the activity at 8 DAH observed in the LF and SG treatments was lower compared to the AB and AF treatments. Only two slight increases in activity were noticed at 6 and 10 DAH. A conspicuous decrease in activity between the 10 and 14 DAH followed by a rapid increase at 15 DAH was observed in all treatments. From this moment, a different enzymatic behavior was observed for the experimental treatments. In larvae fed LF, the total proteolytic activity started declining until 29 DAH. In larvae fed AB, activity levels were quite similar during this period, while in larvae fed AF, the total proteolytic activity tends to rise significantly, outperforming even the activity peak observed at 8 DAH in LF fed larvae. Overall, these differences explain the variations in total proteolytic activity observed at the final stage of the experiment (34 DAH). A higher activity was observed in larvae fed AF, followed by larvae fed AB and a considerably lesser activity in larvae fed LF (Table 3).

Trypsin, chymotrypsin and aminopeptidase activities in LF, AB and AF treatments were very similar to the total alkaline activity from 3 to 10 DAH, except that a slight activity peak was observed at 6 DAH, although this decreases rapidly Fig. 3. It is worth noting that the activity observed at 29 DAH in AF fed larvae was lower than that observed at 8 DAH. Despite this similarity, at 34 DAH, larvae fed AF exhibit the highest activities for these enzymes, followed by larvae fed AB and LF (Table 3). At this age, the increase in activity was eight times higher on average than the highest value recorded at earlier ages. The increase was 21 times higher for trypsin and the lowest increase was of only two times for chymotrypsin (Fig. 3).

The activity pattern of lipase, alkaline and acid phosphatases was very similar to that described for the other enzymes (Fig. 4; Table 3); however, an increase in enzymatic activity for these enzymes was

Table 1 Total length (mm) of tropical gar (*Atractosteus tropicus*) larvae fed a commercial artificial diet (*AF*), frozen adult *Artemia* biomass (*AB*) a live food diet of *Artemia* nauplii (*LF*) and a starved group (*SG*)

DAH	Starved group (SG)	Live food (LF)	Artemia biomass (AB)	Artificial feed (AF)
4	12.06 ± 0.016^{a}	12.06 ± 1.2^{a}	12.06 ± 1.2^{a}	12.06 ± 1.2^{a}
5	14.4 ± 0.024^{a}	14.4 ± 0.98^{a}	14.39 ± 0.98^{a}	14.39 ± 0.98^{a}
6	14.82 ± 0.026^{a}	15.48 ± 0.99^{a}	15.35 ± 1.06^{a}	15.19 ± 1.06^{a}
7	16.08 ± 0.02^{a}	16.12 ± 1.14^{a}	16.11 ± 1.25^{a}	15.91 ± 1.33^{a}
8	18.44 ± 0.03^{a}	18.16 ± 0.89^{a}	18.31 ± 0.67^{a}	18.21 ± 0.67^{a}
9	18.85 ± 0.029^{b}	18.93 ± 0.90^{a}	18.63 ± 0.58^{a}	18.54 ± 0.86^{a}
10	18.97 ± 0.029^{bc}	19.88 ± 1.36^{a}	19.42 ± 0.70^{a}	18.84 ± 0.60^{b}
11	19.04 ± 0.028^{cd}	21.39 ± 0.96^{a}	19.94 ± 1.60^{b}	18.87 ± 1.12^{c}
12	18.7 ± 0.025^{d}	21.52 ± 1.32^{a}	19.90 ± 1.47^{b}	18.32 ± 1.52^{c}
13	18.94 ± 0.032^{c}	22.77 ± 1.19^{a}	21.96 ± 1.90^{a}	19.91 ± 1.66^{b}
14	19.11 ± 0.023^{c}	24.09 ± 0.98^{a}	22.61 ± 1.70^{b}	22.32 ± 2.51^{b}
19		27.62 ± 1.68^{c}	29.17 ± 2.48^{b}	33.23 ± 2.92^{a}
24		29.15 ± 2.37^{c}	33.71 ± 3.98^{b}	39.07 ± 4.64a
29		33.51 ± 2.72^{c}	38.38 ± 4.50^{b}	51.87 ± 6.16^{a}
34		39.47 ± 3.30^{c}	47.79 ± 4.84^{b}	66.15 ± 4.69^{a}

Values are means ± standard deviation. Means within a row with the same superscript are not significantly different ($P > 0.05$)

Table 2 Wet weight (mg) of tropical gar (*Atractosteus tropicus*) larvae fed a commercial artificial diet (*AF*), frozen adult *Artemia* biomass (*AB*) a live food diet of *Artemia* nauplii (*LF*) and a starved group (*SG*)

DAH	Starved group (SG)	Live food (LF)	Artemia biomass (AB)	Artificial feed (AF)
4	16 ± 1.9[a]	16 ± 2[a]	16 ± 2[a]	16 ± 2[a]
5	24.5 ± 1.8[a]	25 ± 2[a]	25 ± 2[a]	25 ± 2[a]
6	26 ± 2.2[a]	26 ± 2[a]	26 ± 2[a]	27 ± 2[a]
7	26 ± 2.7[a]	25 ± 0.002[a]	26 ± 2[a]	25 ± 3[a]
8	31 ± 2[a]	30 ± 3[a]	30 ± 1[a]	30 ± 2[a]
9	29 ± 1.6[b]	32 ± 4[a]	30 ± 3[b]	29 ± 2[b]
10	29 ± 1.6[bc]	35 ± 6[a]	32 ± 4[b]	30 ± 4[b]
11	28 ± 1[cd]	42 ± 4[a]	35 ± 9[b]	29 ± 5[c]
12	25.5 ± 1.7[d]	43 ± 6[a]	34 ± 8[b]	28 ± 7[c]
13	24.8 ± 1.7[d]	48 ± 6[b]	49 ± 12[a]	34 ± 9[c]
14	23.8 ± 1.8[c]	58 ± 6[a]	54 ± 11[ab]	49 ± 17[b]
19		78 ± 11[c]	98 ± 27[b]	141 ± 35[a]
24		92 ± 16[c]	146 ± 49[b]	224 ± 69[a]
29		127 ± 28[c]	193 ± 62[b]	459 ± 143[a]
34		198 ± 44[c]	347 ± 85[b]	866 ± 183[a]

Values are means ± standard deviation. Means within a row with the same superscript are not significantly different ($P > 0.05$)

observed at 13 and 14 DAH in the SG larvae contrary to the rest of the treatments. Amylase activity could not be detected with the technique employed.

Protein digestibility

Protein digestibility of different diets increased with age of tropical gar larvae (Fig. 5). Differences in protein digestibility were observed in function of the different treatments. The highest protein digestibility was observed for E52/14 at 10 and 29 DAH, while protein digestibility was similar for MP45/11 and E46/16 for these ages. In the same way as in digestive enzymatic activity, at 34 DAH, protein digestibility increased distinctly for the artificial diets evaluated. At this time, the best protein digestibility was observed with the MP45/11 diet, outperforming both extruded feeds (E52/14 and E46/16).

Discussion

Observations of tropical gar larval development agree with those made by other authors (Aguilera et al. 2002; Hernández 1999; Mendoza et al. 2008a). The SGR-TL registered from 3 to 8 DAH (1.3 mm/day in TL and 11 mg/day in WW) was similar for all treatments, including the SG. This highlights the importance of yolk reserves during the first day of exogenous feeding. After this age, larvae from the SG group showed a slower growth than those of the rest of the treatments. This was very conspicuous at 14 DAH when the SGR-TL was close to zero for (0.05 mm/day) and became negative for SGR-WW (−4.4%). This indicates that larval length remains constant, while their weight began to decline. This also shows the complete exhaustion of yolk reserves at 8 DAH, concurring with observations in other lepisosteids (Aguilera et al. 2002; Comabella et al. 2006). The SGR-TL (1.3 mm/day) observed in LF larvae was slightly higher than that previously reported for tropical gar larvae also fed *Artemia* nauplii (Márquez 1998; Aguilera et al. 2002). These differences may be the result of diverse factors such as the quantity and quality of food, larval density and broodstock quality (Blaxter and Hempel 1963). However, this SGR-TL value is lower to that attained by Cuban and alligator gar larvae fed *Artemia* nauplii (Comabella et al. 2006; Mendoza et al. 2008b).

At 9 DAH, LF fed larvae exhibited a higher performance than larvae of other treatments, followed by AB and AF fed larvae, respectively. This tendency is also reflected in the SGR although declining in the three treatments is still higher in the LF treatment. However, it can be noticed that growth recovers at 13 DAH in AB fed larvae attaining a size similar to the LF treatment. A possible explanation is that food size in the AB treatment was not adequate during the first

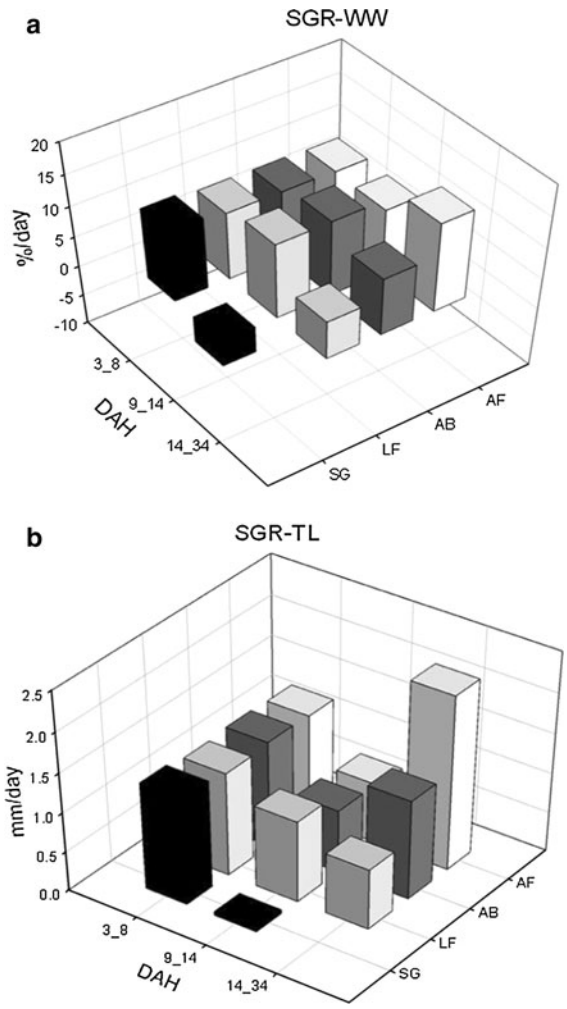

Fig. 1 Specific growth rate in wet weight (SGR-WW) = [100(ln WW_2 − ln WW_1)/#days] (**a**), and total length (SGR-TL) = [100(ln TL_2 − ln TL_1)/#days] (**b**), of tropical gar (*Atractosteus tropicus*) larvae fed a commercial artificial diet (*AF*), frozen adult *Artemia* biomass (*AB*) a live food diet of *Artemia* nauplii (*LF*) and a starved group (*SG*)

day of exogenous feeding, which was reflected in the SGR. However, when larvae were developed enough to properly ingest AB, their SGR increased. The relationship between feed size and snout length has been stressed as a key factor in lepisosteid larvae feeding (Mendoza et al. 2008b). This assumption is supported by the observed rapid adaptation in the enzymatic activity of AB fed larvae. Indeed, pepsin and intestinal enzymes (phosphatases and aminopeptidases) activities start increasing at 10 DAH. Furthermore, considering the similar composition of AB and LF, the growth response and enzymatic activity would be chiefly determined by the ingestion rate of these foods.

On the other hand, AF fed larvae only showed a better performance after 19 DAH, when they reached higher sizes than those attained by larvae of other treatments. Because tropical gar larvae present a well-developed digestive tract at the beginning of exogenous feeding (Mendoza et al. 2008a), it is possible that the lower performance of AF fed larvae during the first day of exogenous feeding could be the result of a low ingestion rate, as a part of the recognition and acceptance process of artificial feed (Mendoza et al. 2008b). At the same time, it could be assumed that a rapid adaptation of the digestive tract to the chemical composition of artificial feed takes place, as shown by the progressive increase in both acidic and alkaline proteolytic activities at 14 DAH, followed by an increase in intestinal enzymatic activity until 19 DAH. This physiological response has been observed in the larvae of other fish species, in which the use of artificial feed caused a variation in the activity of pancreatic enzymes (trypsin and chymotrypsin) that was inverse to the complexity and digestibility of their components, as well as reduced the activity of brush border enzymes (phosphatases and aminopeptidases) (Zambonino and Cahu 2007).

After 19 DAH, a net growth increase is observed in the AB treatment, but it is even more conspicuous in the AF treatment. This is confirmed by the SGR values of these treatments. In contrast, the SGR of LF fed larvae declines markedly. The lower performance of LF fed larvae may in part be explained by the small size of the *Artemia* nauplii, in relation to the large size of the snout of gar larvae. Larvae fed live food may have obtained a lower energetic reward compared with AF fed larvae, due to the high energy expenditure of larvae in the search and capture of *Artemia* nauplii, even if these were highly available (Aguilera et al. 2002; Mendoza et al. 2008a). In other treatments, larvae of a similar age may have spent less energy during the feeding process, considering the bigger size of AB and the variable size of AF, in addition to the lack of mobility of these foods. This feeding behavior seems to be a common feature in lepisosteids, as a similar SGR decline was reported in alligator gar larvae fed live prey, when compared with larvae fed artificial diets (Mendoza et al. 2008b; Clay 2009).

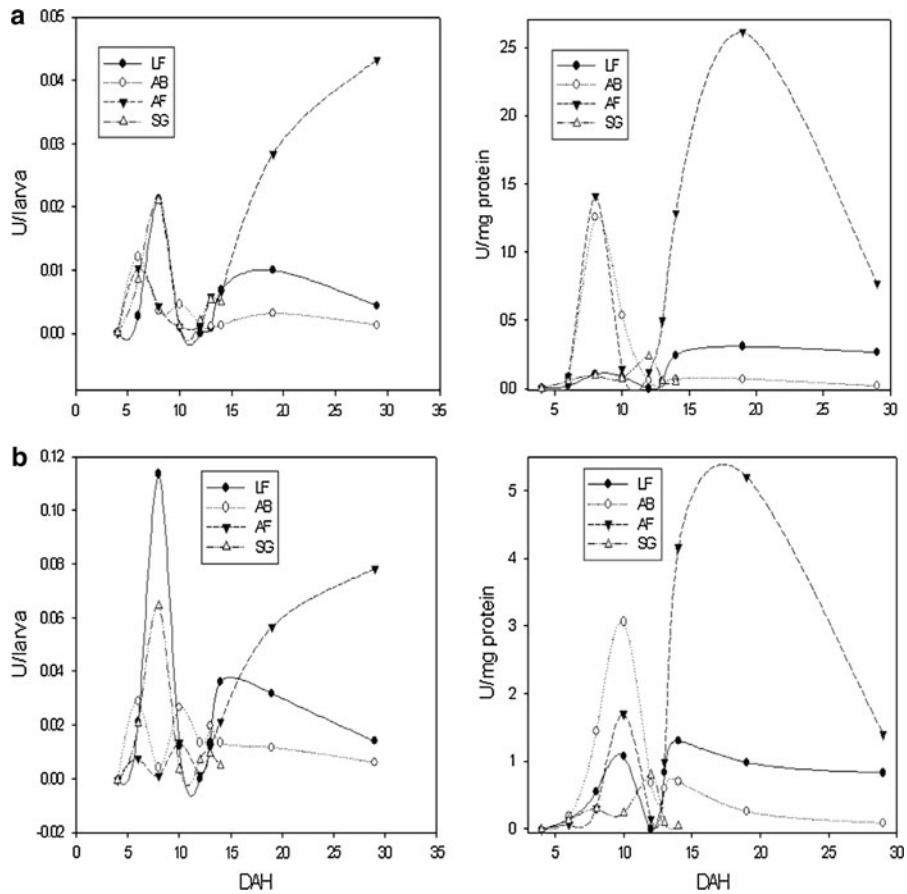

Fig. 2 Total alkaline proteolytic (**a**) and pepsin (**b**) activity (U/larva and U/mg protein) from 3 to 29 DAH of tropical gar (*Atractosteus tropicus*) larvae fed a commercial artificial diet (*AF*), frozen adult *Artemia* biomass (*AB*) a live food diet of *Artemia* nauplii (*LF*) and a starved group (*SG*)

Table 3 Digestive enzymes in total activity (U/larva) and specific activity (U/mg protein) at 34 DAH of tropical gar (*Atractosteus tropicus*) larvae fed a commercial artificial diet (*AF*), frozen adult *Artemia* biomass (*AB*) and a live food diet of *Artemia* nauplii (*LF*)

Digestive enzyme	Live food (LF)		Artemia biomass (AB)		Artificial feed (AF)	
	U/Larva	U/mg protein	U/Larva	U/mg protein	U/Larva	U/mg protein
Pepsin	0.056 ± 0.0012^{c}	1.353 ± 0.0304^{a}	0.231 ± 0.0254^{b}	0.857 ± 0.094^{b}	0.956 ± 0.0323^{a}	1.321 ± 0.0447^{a}
Total alkaline proteases	0.016 ± 0.0027^{b}	0.397 ± 0.0665^{a}	0.013 ± 0.0073^{b}	0.051 ± 0.0270^{b}	0.338 ± 0.0546^{a}	0.467 ± 0.0755^{a}
Trypsin	0.011 ± 0.0008^{c}	0.280 ± 0.0207^{b}	0.091 ± 0.0038^{b}	0.337 ± 0.0143^{b}	1.750 ± 0.0879^{a}	2.418 ± 0.1214^{a}
Chymotrypsin	0.002 ± 0.0007^{c}	0.048 ± 0.0029^{c}	0.022 ± 0.0039^{b}	0.081 ± 0.0051^{b}	0.083 ± 0.0076^{a}	0.115 ± 0.0009^{a}
Aminopeptidase	0.009 ± 0.0002^{c}	0.236 ± 0.0060^{c}	0.22 ± 0.0061^{b}	0.814 ± 0.0228^{b}	0.652 ± 0.038^{a}	0.901 ± 0.0525^{a}
Alkaline phosphatase	0.49 ± 0.0201^{c}	11.74 ± 0.4819^{a}	2.571 ± 0.4634^{b}	9.511 ± 1.7137^{b}	8.848 ± 0.358^{a}	12.22 ± 0.4947^{a}
Acid phosphatase	0.037 ± 0.005^{c}	0.886 ± 0.297^{c}	0.408 ± 0.009^{b}	1.509 ± 0.162^{b}	1.813 ± 0.137^{a}	2.504 ± 0.313^{a}
Lipase	0.057 ± 0.0038^{c}	1.364 ± 0.0987^{b}	1.811 ± 0.0915^{b}	6.697 ± 0.391^{a}	5.223 ± 0.3569^{a}	7.214 ± 0.364^{a}

Values are means ± standard deviation. Means within a row with the same superscript are not significantly different ($P > 0.05$)

The similar pattern in the enzymatic activity was observed between LF fed larvae and those from the SG during the initial developmental stage (3–14 DAH), as has been reported for other fish species (Baragi and Lovell 1986; Dabrowski et al. 1992). In these treatments, an important increase in both

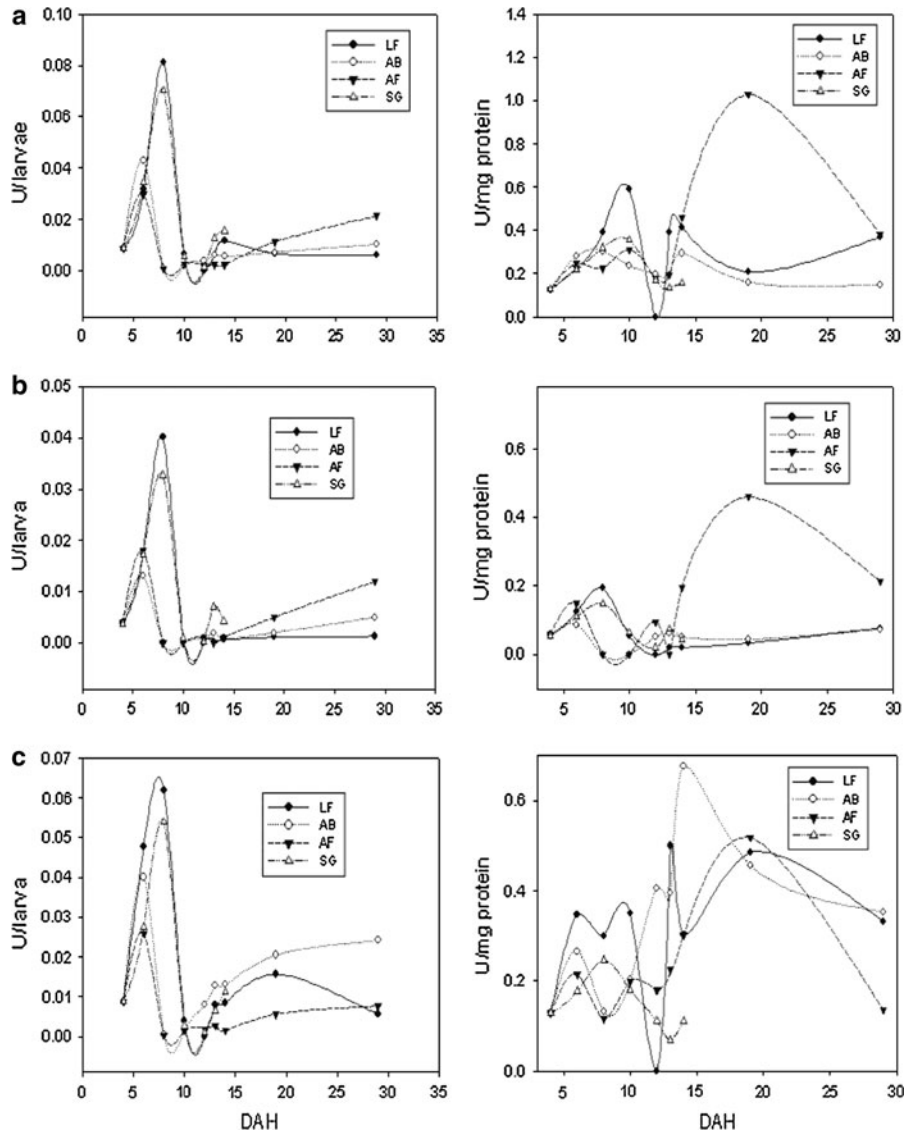

Fig. 3 Trypsin (**a**), chymotrypsin (**b**) and aminopeptidase (**c**) enzymatic activity (U/larva and U/mg protein) from 3 to 29 DAH of tropical gar (*Atractosteus tropicus*) larvae fed commercial artificial diet (*AF*), frozen adult *Artemia* biomass (*AB*) a live food diet of *Artemia* nauplii (*LF*) and a starved group (*SG*)

proteolytic alkaline and acidic enzymatic activities was observed at the beginning of exogenous feeding (5 DAH), followed by a sharp decline in these activities, at the moment when yolk reserves were exhausted (8 DAH), and a descent below basal levels at 10 DAH and thereafter remained until the 13 DAH. Some authors (Buddington 1985; Buddington and Doroshov 1986; Martínez et al. 1999) relate this change in enzymatic activity to the onset of metamorphosis, a process that varies between species and that depends specifically on digestive tract differentiation (e.g., gastric glands) (Tanaka 1971). In tropical gar, metamorphosis and digestive tract differentiation including functional gastric glands take place since the 5 DAH (Hernández 1999; Aguilera et al. 2002; Mendoza et al. 2008a), which could contribute to explain the sudden increase in enzymatic activity at the start of exogenous feeding. The observed sudden drop in enzymatic activity during the following days is probably a consequence of the adaptation period that the digestive tract has to pass through in preparation to the new exogenous feeding phase. The similar pattern of enzymatic activity between LF and SG until 10 DAH suggests that these enzymes are involved in yolk reserve utilization, since the activity starts decreasing at 8

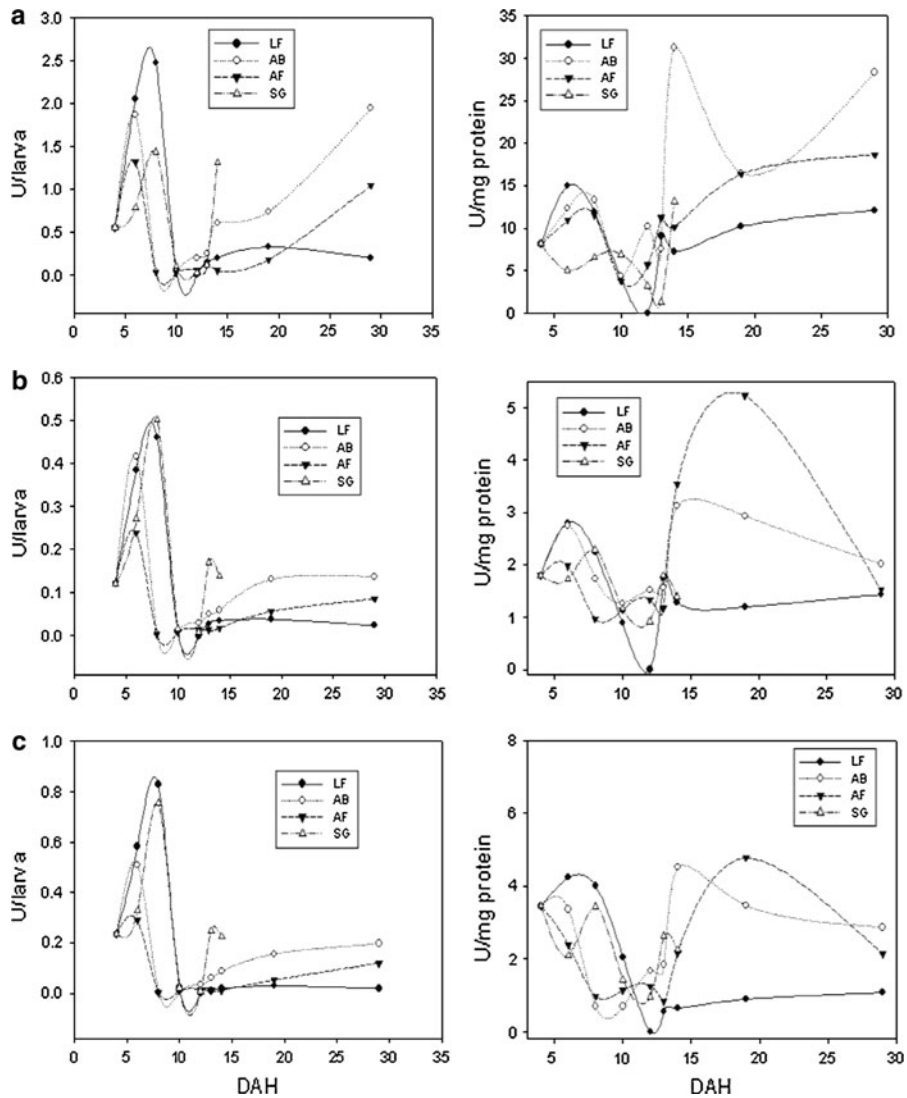

Fig. 4 Alkaline phosphatase (**a**), acid phosphatase (**b**) and lipase enzymatic activity (U/larva and U/mg protein) from 3 to 29 DAH of tropical gar (*Atractosteus tropicus*) larvae fed a commercial artificial diet (*AF*), frozen adult *Artemia* biomass (*AB*) a live food diet of *Artemia* nauplii (*LF*) and a starved group (*SG*)

DAH. However, the lower activity in pepsin, specific hydrolases, namely alkaline phosphatases, and lipases in SG larvae during this period shows the influence of first feeding (5 DAH) on the level of secretion of enzymes. In relation to this, Hjelmeland et al. (1988) found that the difference in response to trypsin secretion of starved larvae and larvae to which inert feed was offered was the result of the stimulation of the digestive tract caused by the ingestion of feed particles, which would explain the lower enzymatic activity in the SG treatment. During the last days before SG larvae die (13–14 DAH), enzymatic activity reached higher levels of alkaline and acidic phosphatases and lipase, than those registered in fed larvae. With regard to this, most of the scientific reports indicate a reduction in the digestive enzymatic activity in later stage of starvation, particularly at the *point of no return*; however, most of these studies have focused on the activity of proteases (Shan et al. 2008). Nevertheless, it has been demonstrated that food deprivation did not seem to significantly reduce the lipase activity in yellowtail kingfish (*Seriola lalandi*); similarly, it was noticed that alkaline phosphatase was not a sensitive enzyme reflecting food deprivation (Chen et al. 2007). Our observations on the activity of these enzymes concur with these findings. Acidic and alkaline phosphatases, as well as aminopeptidases, are typical intestinal

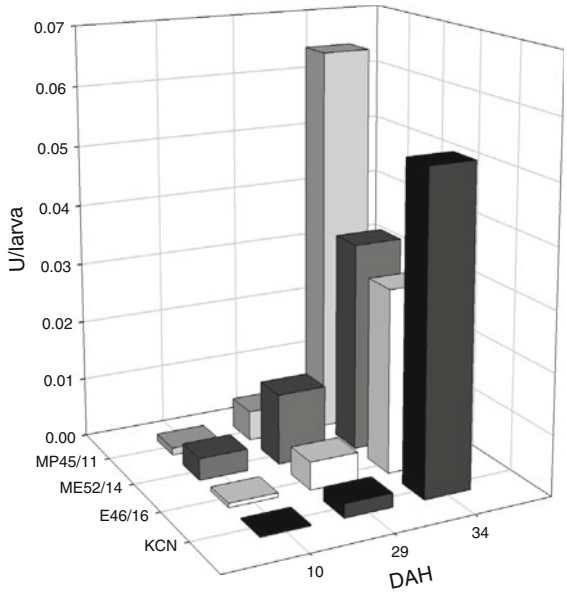

Fig. 5 In vitro protein digestibility of artificial diets (MP45/11, E52/14 and E46/16) using enzymatic extracts of 10, 29 and 34 *DAH* larval dissected digestive tracts. Hemoglobin was used as a control for acid digestion and casein for alkaline digestion (*KCN*). Protein digestibility is expressed as absorbance units at 280 nm

enzymes responsible for nutrition, mineralization and intestinal transport processes as well as the hydrolysis of phosphorlyated peptides and proteins. In fish larvae of certain species, high levels of acidic phosphatase activity have been detected before the maturation of the stomach, suggesting that this activity together with that of aminopeptidases represents a compensatory mechanism to palliate the low extracellular digestion by the lack of pepsin (Moyano et al. 1996; Lazo et al. 2007; Zambonino and Cahu 2007). However, this is not the case in tropical gar and other lepisosteids larvae, in which the extracellular digestion by pepsin is functional from the beginning of exogenous feeding at 5 DAH (Mendoza et al. 2008a). As acidic phosphatase activity levels remain low along the development, with the exception of a slight peak at 8 DAH, the observed activity could be more related to yolk assimilation, as vitellin has an important phosphorus content (Hoque et al. 1998). On the other hand, enzymatic activities of alkaline phosphatases and aminopeptidases follow the same pattern of progressive maturation. These enzymes have been suggested as reliable intestinal maturation indicators as well as important for the nutritional condition of fish larvae (Moyano et al. 1996; Lazo et al. 2007; Zambonino and Cahu 2007).

Larvae of AB and AF treatments showed a similar enzymatic activity pattern to those larvae of LF and SG treatments until 10 DAH; however, in the former treatments, the activity was lower presumably due to the complexity (e.g., protein digestibility) of these foods, compared to LF (Zambonino and Cahu 2007). The variation in enzymatic activity at this stage does not seem to have had an effect on larvae development since no significant differences in growth were observed until 8 DAH. Variations in enzymatic activity of larvae of all treatments were evident until 15 DAH, when the enzymatic activity level was close to that observed at the start of exogenous feeding (5 DAH), and after 15 DAH, enzymatic activity progressively increased until 29 DAH. The highest increase was observed in the total alkaline proteolytic activity of AF fed larvae. Observations on the characterization of digestive proteases during enzymatic ontogeny of lepisosteids may contribute to explain this variation (Mendoza et al. 2008a). Tropical, Cuban and alligator gars larvae have seven proteases; however, they appear at different times in different species. While in the Cuban and alligator gars by 15 DAH, all proteases have already been detected, in the tropical gar, just four of them are functional. It is only after 30 DAH when the rest of the proteases are detected in tropical gar. This indicates the slower maturation of the digestive tract of tropical gar and explains the low enzymatic activity levels until 29 DAH. However, just after this maturation period, the enzymatic activity increases rapidly. This is more evident in the total alkaline activity that exhibits an eightfold increase from 29 to 34 DAH in AF fed larvae (Table 3). In those fish larvae that have a functional stomach at the onset of exogenous feeding, lipases have too an important role in yolk digestion (Oozeki and Bailey 1995), which would explain the descent of activity levels well below the initial levels after the end of the transitional stage (5–9 DAH). Amylase could not be detected in the extracts of tropical gar digestive tract. This was expected as in the Cuban and alligator gars, this kind of activity was only detected at very low levels, presumably as a result of the carnivorous behavior of these species (Mendoza et al. 2002; Comabella et al. 2006). In support of this contention, it has been reported that fish with herbivorous or omnivorous behavior show higher amylase activity

levels than carnivorous fish (Munilla and Saborido 1996).

The in vitro protein digestibility tests showed the effectiveness of the enzymatic extracts of larvae of different age to digest test foods. Higher digestion levels were observed in the extracts of 34 DAH, whereas the levels remained at a low level in the extracts of larvae between 10 and 29 DAH. This confirms the slower maturation process of the digestive tract of tropical gar larvae compared to other lepisosteids and attests that this process is only reached after 29 DAH, when the digestive capacity increases markedly. This validates the utility of protein digestibility tests in the evaluation and selection of adequate nutrients and feeds for different phases of the culture of larvae and juveniles (Bassompierre et al. 1998; Alarcon et al. 1999). Within this context, from the test feeds, the E52/14 would be the better option for the nutrition of larvae until 29 DAH, while, after this age, the micro-extruded-marumerized feed MP45/11 would ensure a better performance than other feeds.

Overall, the results of this study show that all digestive enzymes tested (pepsin, trypsin, chymotrypsin, aminopeptidase, alkaline and acidic phosphatases and lipase) were present since the start of exogenous feeding in tropical gar larvae, in agreement with observations on digestive tract development of other lepisosteids (Mendoza et al. 2002; Comabella et al. 2006). Enzymatic activity showed variations during the early development related to the transitional phase of maturation and to the food. A relevant aspect is the low level of enzymatic activity until the 29 DAH and the important increase from this moment onwards. This confirms the slower maturation process of tropical gar larvae, compared to Cuban and alligator gar, that could be related to the growth rate and final size of the species as have been suggested (Mendoza et al. 2008a). Despite the slow digestive tract development, artificial diets can be used since the start of exogenous feeding, as in other lepisosteids, with even better results than live food (Mendoza et al. 2008b; Clay 2009). Taking into account the in vitro protein digestibility results, it could be advisable to feed tropical gar larva with a feed with a similar quality of E52/14 until 29 DAH and with similar quality of MP45/11 after 30 DAH. These results also open the possibility to explore the inclusion of ingredients with different protein quality to evaluate the growth of larvae and juveniles, as has been recently suggested by Castro-Mejia et al. (2009).

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