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Morphophysiological studies on alligator gar (*Atractosteus spatula*) larval development as a basis for their culture and repopulation of their natural habitats

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Abstract

Natural populations of alligator gar (*Atractosteus spatula*) have declined recently due to the effects of commercial and sport fisheries. Aquaculture represents a short-term alternative to restore natural populations, and a first step to accomplish culture of this species is the study of early life stages. Therefore, multidisciplinary research was used to describe the major morpho-physiological changes taking place during this period. The studies serve as a basis for the introduction of artificial diets for culture. A morphological study distinguished different nutritional stages, as well as external indicators of starvation. A histological approach showed the digestive tract to be completely formed 5 days after hatching (DAH), at the beginning of exogenous feeding. Throughout larval development, intestinal maturation was followed and a nutritional indicator based on the mid-gut cell height was validated. The occurrence of pepsin-like proteolytic activity was detected from five DAH, while trypsin, chimiotrypsin and aminopeptidase-like activities gradually increased from two to nine DAH. The incidence of cannibalism in culture conditions was controlled by exposure to anti-thyroid compounds (thiourea – TU) to retard snout growth. This treatment did not effect growth and allowed juveniles to feed on live prey but prevented the consumption of gar larvae of the same size. Larvae exposed to 3,3′,5-triiodo-1-thyronine (T3) had faster development, a potentially advantageous characteristic for the repopulation of their natural habitat. Finally, artificial feeds were well accepted and resulted in growth rates similar to those of gar larvae that were fed natural prey. This has allowed the development of a feeding strategy that effectively reduced cannibalism and led to the production of 30 cm juveniles in four months.

Introduction

The alligator gar (*Atractosteus spatula*) is the largest freshwater fish inhabiting rivers draining into the Gulf of Mexico. These fish have traditionally been harvested in the northeast region of Mexico, where they are highly appreciated for their size and the quality of their meat (SEPESCA-INP, 1994). Therefore, commercial and sport overfishing has occurred and the populations have drastically declined. For example, estimated mean annual alligator gar harvest in the state of Tamaulipas declined from 13.2 m^3 in 1988 to 5.7 m^3 in 1990. In 1997, only 1.1 m^3 of alligator gar were harvested. Additionally, harvest occurs during the reproductive season thus affecting the recruitment of juveniles. Spawning grounds have also been affected by urban and agricultural expansion as well as by the construction of dams.

Within this context, the need to develop culture techniques has emerged as a short-term alternative to preserve the species and to restore natural populations. To accomplish this, the Mexican Ministry of Fisheries has maintained 30 adults in captivity since 1982. Reproduction and spawning of alligator gar are confined to one week per year, producing up to 400,000 larvae per year. To date, few attempts have been made to culture alligator gar past 7–10 DAH. Due to differences in larval growth rates, maintaining adequate live prey density to prevent cannibalism is difficult. Therefore, larvae have been released at young ages to prevent cannibalism due to high culture densities. Release of smaller, younger larvae rather than larger, older larvae may result in reduced survival.

In an attempt to prevent cannibalism among cultured alligator gar we have examined larval growth and the development of the digestive tract in an effort to formulate an effective feeding strategy using artificial feeds. We have utilized a multidisciplinary approach aimed at describing the major morpho-physiological changes that take place during the larval period (Figure 1).

Material and methods

Morphological, histological, enzymatic and endocrinological approaches constitute the different phases of this research. Eggs and larvae of *A. spatula* were obtained, between 1997 and 2000, from the reproduction of 30 adults maintained in captivity at the aquaculture facilities of the Environment, Natural Resources and Fisheries Ministry of Mexico, located in Tancol, Tamaulipas. Breeding adults were placed in two earth ponds measuring 30×20 m, with a water depth of 95 cm. In order to accommodate the spawning behavior of lepisosteids (Dean, 1985; Morales, 1987; Simon and Wallus, 1989), *Casuarina* spp. branches were spread throughout the ponds to provide spawning substrate. The following night, courtship and spawning took place. Eggs and larvae adhering to the *Casuarina* spp. branches were collected and transferred to the facilities of the Ecophysiology Laboratory of Universidad Autonoma de Nuevo Leon.

To determine the morphological and histological characteristics of larval development from the moment of hatching, larvae were placed in fiberglass tanks measuring 230×70×30 cm, with a water volume of 480 L. An initial density of 500 larvae/tank (approximately 1 larvae/L) was established. Water temperature was kept constant (27 °C ± 1) and oxygen was provided by a compressor to maintain oxygen levels above 6 ppm. A sample of 10 larvae/tank was taken every 12 hours from the beginning of hatching until the fourth DAH. Thereafter, samples were taken every 24 hrs until 15 DAH. Larvae were individually weighed with an Ohaus balance (±0.1 mg) and fixed in Bouin's solution. Morphometric characteristics were evaluated using an ocular micrometer and vernier calipers (±0.1 mm). Twenty morphometric and five meristic variables were determined for each specimen according to Simon and Wallus (1989) and Simon and Tyberghein (1991) for lepisosteid larvae. Descriptions of different stages were based on the presence of specific structures. Morphometric characters were analyzed using discriminant analysis using the stepwise method (Ferran, 1996), to identify characteristics describing each developmental stage. Larvae deprived of food were compared with larvae of the same age that were fed *Artemia nauplii*. Data were analyzed using one-way analysis of variance (ANOVA) to determine significant differences among morphometric traits. Discriminant analysis was used to identify characteristics that described the nutritional condition of larvae.

Because digestive tract development limits the utilization of artificial diets in most fish larvae (Lauf and Hoffer, 1984; Person-Le Ruyet, 1990), it was necessary to follow the appearance and development of different structures to determine when the digestive tract of alligator gar was completely formed. Digestive tract development was assessed histologically. Larvae fixed in Bouin's fluid were embedded in paraffin and

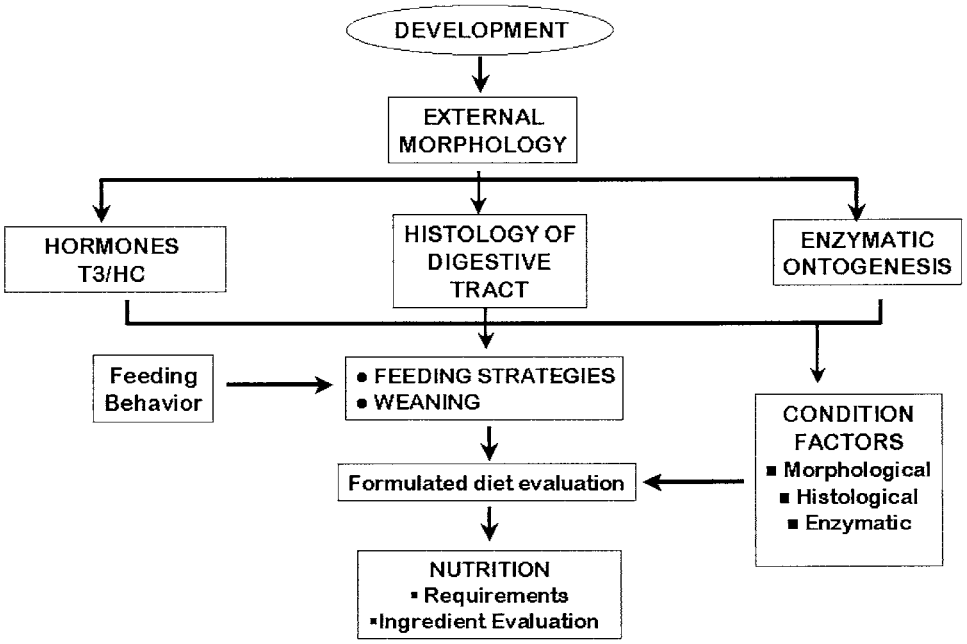

Figure 1. Research strategy for the study of alligator gar larval development.

7 μm serial sections were stained with hematoxylin-eosin for light microscopy. Mid-gut cell height was evaluated according to the methodology described by Theilacker and Porter (1995). Cell height of larvae at different stages was compared with a one-way ANOVA, while differences between starved and fed larvae were determined with Student's t-test.

Digestive tract function of different developmental stages was determined by spectrophotometric analysis of larval digestive tract extracts. Samples of 100 larvae were collected and stored in liquid nitrogen on a daily basis starting from 3 DAH. Digestive organs of individual larvae were dissected by placing larvae on a glass slide maintained at 0 °C. A cut was made at the boundary of the esophagus and intestine, and the whole intestinal segment, including the diffuse pancreas embedded in its outer surface, was removed. Following dissections, the guts were pooled and homogenized with distilled water in a 1:5 proportion (w/v) at 4 °C. Assays were conducted at 25 °C and the reaction mixtures consisted of 0.1 ml of enzymatic extract and 1 ml of substrate dissolved in buffers specific to each assay. Hemoglobin, dissolved in universal buffer (pH = 3), was used as substrate for measuring pepsin activity (Anson, 1938). Azocasein was used for total alkaline proteases (Galgani and Nagayama, 1986), while BAPNA, GPNA and LNA were used for trypsin, chymiotrypsin and aminopeptidase, respectively (Erlanger et al., 1961). Tris-HCl 50 mM, $CaCl_2$ 20 mM (pH = 8.2) buffer was used to dissolve substrates of all alkaline proteases. Starch in Na_2HPO_4 20 mM-NaCl 6 mM (pH = 6.9) buffer, p-nitrophenyl acetate in Tris-HCl 50 mM (pH = 7.1), and p-nitrophenyl phosphate in acetate (pH = 4.8) or diethalonamine (pH = 9.8) buffers were used as substrates for determining α-amylase, lipase, and acid and alkaline phosphatase activities, respectively (Moyano et al., 1996). These activities were confirmed by means of substrate-gel electrophoresis (Garcia-Carreño et al., 1993).

A crucial factor in fish larvae development is the participation of thyroid hormones and corticosteroids, particularly in digestive tract maturation. Immersion studies were used to determine the use and importance of exogenous thyroid hormones and glucorticosteroids as potential regulators of larval gar development. Four groups of 3 DAH larvae (30 larvae/group with four replicates) were exposed to 0.1 ppm of 3,3′,5-triiodo-l-thyronine (T3), 0.1 ppm hydrocortisone (HC) or 30 ppm TU. The fourth group, with no added material, served as a control (C). Survival, weight, total length, and snout length were compared (3 DAH and 15 DAH). Tissue levels of T3 and cortisol were determined by RIA according to the methods of Kobuke et

al. (1987) and De Jesús et al. (1991), respectively. The mid-gut cell height was used as a diagnostic index to assess differentiation and maturation of the digestive tract. Digestive acid and alkaline proteolytic activities were also measured, as previously described.

To determine the acceptance of artificial diets by gar larvae, two feeding experiments were conducted at different times, each with their own control. Both feeding trials were performed with 3 DAH larvae and were evaluated at 15 DAH. In the first experiment, three different diets were tested: a) *Artemia nauplii*, as a positive control; b) a sinking pelleted diet of 0.5, 0.75 and 1.2 mm diameter; c) a combination of *Artemia nauplii* and the pelleted diet in equal proportions. In the second experiment the test diets were: a) *Artemia nauplii*, as a positive control; b) an extruded 50% floating/50% slow-sinking diet of 0.5, 0.75 and 1.2 mm diameter; c) a combination of *Artemia nauplii* and the extruded diet in equal proportions. Diet particle size was increased through time (i.e., 0.5 mm pellets 5 to 10 DAH; 0.75 mm pellets 9 to 13 DAH; 1.2 mm pellets 12 to 15 DAH), as snout length increased. Both artificial diets were donated by Purina S.A de C.V. (Table 1). Initial weight and total length of larvae were determined. The animals were blotted dry before being weighed to the nearest 0.01g. At the start of the feeding trials, larvae were selected according to the uniformity of their weight and size as determined by a one-way ANOVA. Larvae with a mean initial weight of 16.2 $\pm$ 1 mg and a mean initial length of 11.7 $\pm$ 0.5 mm were randomly assigned to twenty 45 L fiberglass tanks provided with freshwater in a recirculating system. The tanks were continuously aerated throughout the experiment by an air compressor. Each test diet was fed to 4-replicate tanks with 30 alligator gar larvae per tank. *Artemia nauplii* and artificial diets were fed *ad libitum* three times a day. At the end of the study, individual weight and total length were measured. Survival and proteolytic activity were also evaluated.

Table 1. Proximate analysis of experimental diets

Proximate analysis	%
Moisture	12
Crude protein	50
Lipids	15
Fiber	4
Ash	12
Calcium	2
Phosphorus	1.2
Nitrogen free extract	7

Results

Morphological studies

Larval development is summarized in Figure 2. Hatching occurred after a 50 h incubation period (at 28 °C). Mean total length (TL) of recently hatched larvae was 7.2 mm. Larvae remained adhered to vegetation, by means of an adhesive suctorial disc at the tip of the snout, until 5 DAH when mean total length was 18 mm. Free-swimming larvae began to feed on zooplankton before complete absorption of the yolk sac occurred. With adequate prey (i.e., *Artemia nauplii*), larvae reached 23 mm TL by 10 DAH and had acquired the elongate body shape typical of juveniles. Until 10 DAH the mean growth rate was 1.5 mm/day and increased thereafter to 5 mm/day until they reached nearly 50 mm TL (15 DAH). Starved larvae grew more slowly, reaching 20 mm TL by 15 DAH, than did larvae that received sufficient food ($t = 14.4$; d.f. 18; $P < 0.001$). Small larvae were typically preyed upon by large larvae. A comparison between fed and starved larvae indicated that yolk reserves were exhausted by 8 DAH, which suggests the existence of a mixed nutritional phase (lecito-exotrophic) between 5–8 DAH. Discriminant analysis showed that pre-anal depth was the best indicator of nutritional condition of larvae. Pigmentation pattern was also indicative of nutritional condition of larvae: starved larvae displayed darker pigmentation patterns than fed larvae. The general pigmentation pattern observed for *A. spatula* larvae in this study was similar to descriptions from other studies of larvae of similar size (Suttkus, 1963; May and Echelle, 1968; Echelle and Riggs, 1972; Moore et al., 1973; Simon and Wallus, 1989). A conspicuous, lightly pigmented line along the dorsal region, distinctive of this species, was clearly visible in individuals less than 139 mm TL. Thus, the absence of this line was considered an indicator of transformation to the juvenile stage at approximately 30 DAH. Snout length was related to total length but was not affected by nutritional condition because the metamorphosis of the suctorial disk into the characteristic lepisostid snout occurred before yolk reserves were depleted. Finally, these

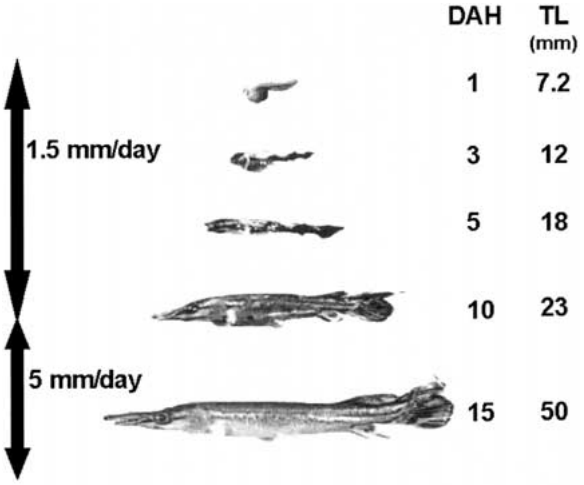

Figure 2. Daily growth rate of alligator gar during 15 days culture at 28 °C. The figure shows the total length (TL) along larval development at different days after hatching (DAH).

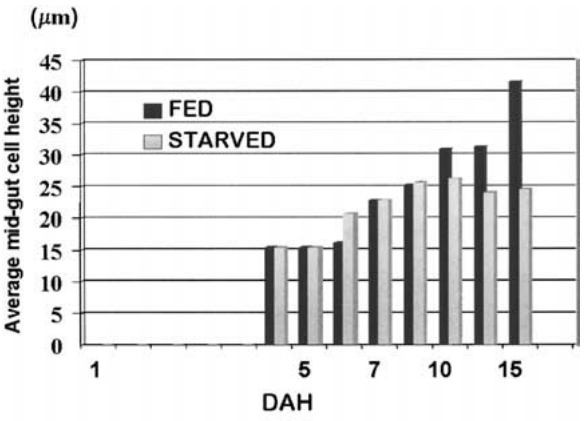

Figure 3. Average mid-gut cell height (μm) of fed and starved larvae of alligator gar at different days after hatching (DAH).

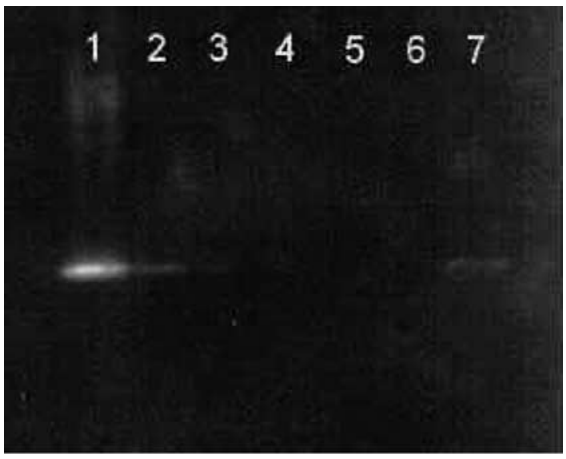

Figure 4a. Native-PAGE, 11% PAA, incubated in Hemoglobin 0.25%, Buffer Gly-HCl, pH 2.0, 90 min, 25 °C.

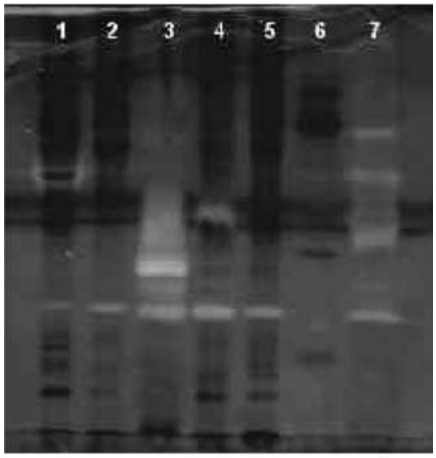

Figure 4b. SDS-PAGE, 12% PAA, incubated in Casein 1%, Buffer Tris-HCl, pH 9.0, 90 min, 25 °C. Enzymatic extracts of samples 1 to 5 correspond to different sections of the digestive tract of gar juveniles (1: stomach, 2: fore-gut, 3:mid-gut, 4:hind-gut, 5:pyloric caeca). Samples 6 and 7 are extracts of 1 DAH and 5 DAH larvae, respectively.

results indicate that the release of larvae between 7–10 DAH, which has been the common practice until now (G. Morales, 2000, personal communication), reduces cannibalism rates of cultured larvae. Although this research only examined larvae, the release of older and larger individuals (beyond the larval stage) may result in higher survival rates. Unfortunately, care and maintenance of juveniles may be too time intensive, too expensive, and require additional facilities.

Histological studies

We observed a straight intestine formed by simple epithelia in 1 DAH larvae. By 5 DAH, the stomach, spiral valve and pancreatic tissues were formed despite the presence of yolk reserves. From 10 DAH significant differences ($t = 25.95$; $P = 0.00001$; d.f. = 34) in the mid-gut cell height of fed and starved larvae were observed (Figure 3). By 15 DAH, gastric glands of starved larvae were underdeveloped or degenerating when compared with those of fed larvae. Hepatic cells of starved larvae contained condensed cytoplasm with no intracellular spaces (areas of glycogen and lipid storage) and muscle fibers were atrophied.

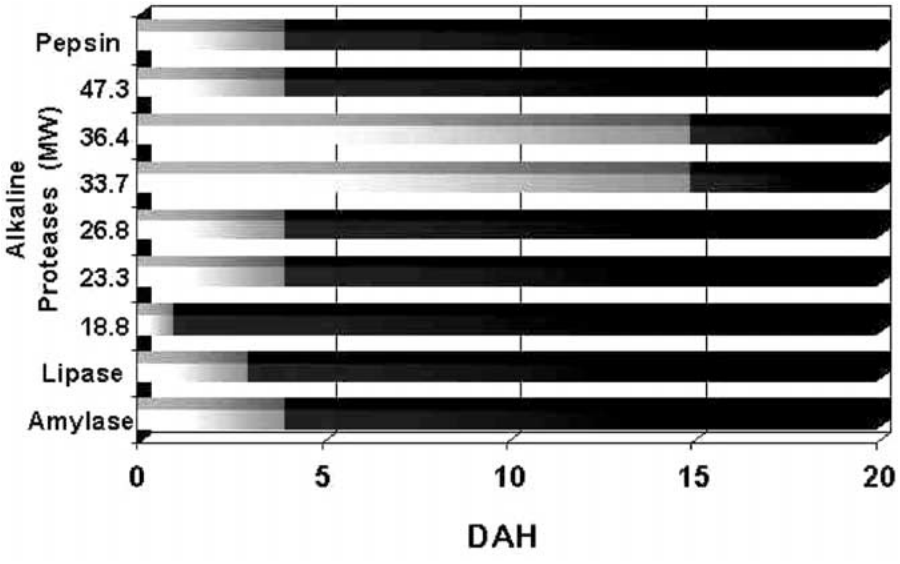

Figure 5. Main digestive enzymes spectrum of alligator gar larvae during early developmental stages.

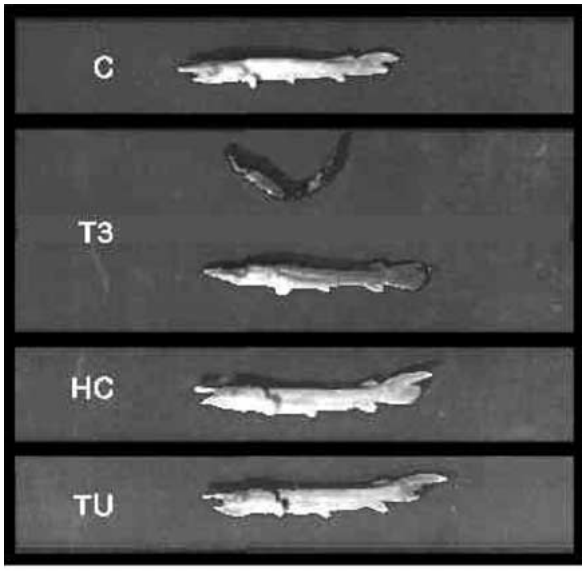

Figure 6. Morphological modifications of larvae treated with thyroid hormones (T3: triiodo-1-thyronine), glucocorticoids (HC: hydrocortisone) and goitrogens (TU: Thiourea). C: control.

Enzymatic studies

Pepsin-like acid proteolytic activity was detected from the beginning of exogenous feeding (5 DAH); before complete absorption of the yolk sac. Trypsin, chymiotrypsin and aminopeptidase-like alkaline proteolytic activity gradually increased from 2–9 DAH. Enzymatic activity differed by digestive tract region. For example, trypsin and chymiotrypsin-like activity was greatest in the fore-gut, whereas, aminopeptidase-like activity was greatest in the hind-gut. Digestive enzyme activity, molecular weight, and number of enzymes within the two digestive tract regions, were confirmed using substrate-gel electrophoresis (Figures 4a and 4b). Important lipase and acid phosphatase activities in 3–8 DAH larvae were related to yolk utilization. In contrast, increased activity of alkaline phosphatase at 8 DAH coincided with the maturation of enterocytes. Amylase activity was low throughout larval development and may be due to the carnivorous food habits of gar larvae. In contrast, fish with herbivorous or omnivorous diets have high levels of amylase activity (Vonk and Western, 1984; Munilla-Moran and Saborido, 1996). Similar to salmonids and sturgeons (Buddington, 1985; Buddington and Dorshov, 1986; Gawlicka et al., 1995), alligator gar posses a well developed intestinal tract, characterized by a functional stomach and the secretion of pepsin-like enzymes, from the beginning of exogenous feeding (Figure 5). These characteristics are indicative of precocious digestive tract maturation.

Endocrinological studies

Survival rates were near 100% with the exception of the T3 group that had relatively higher mortality (80%). Concentrations of T3 were three times higher in the T3 group, when compared to the other groups.

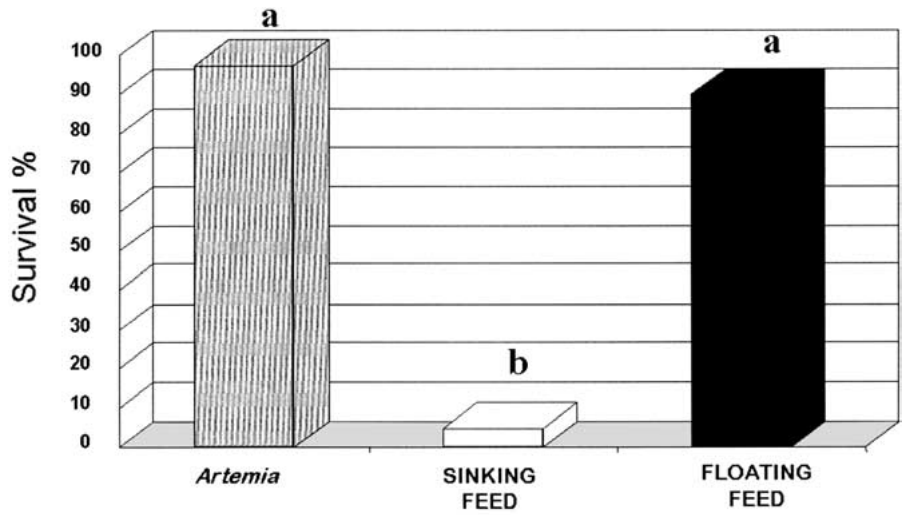

Figure 7. Survival of larvae fed natural preys and sinking or floating artificial diets. Different letters refer to significantly different means.

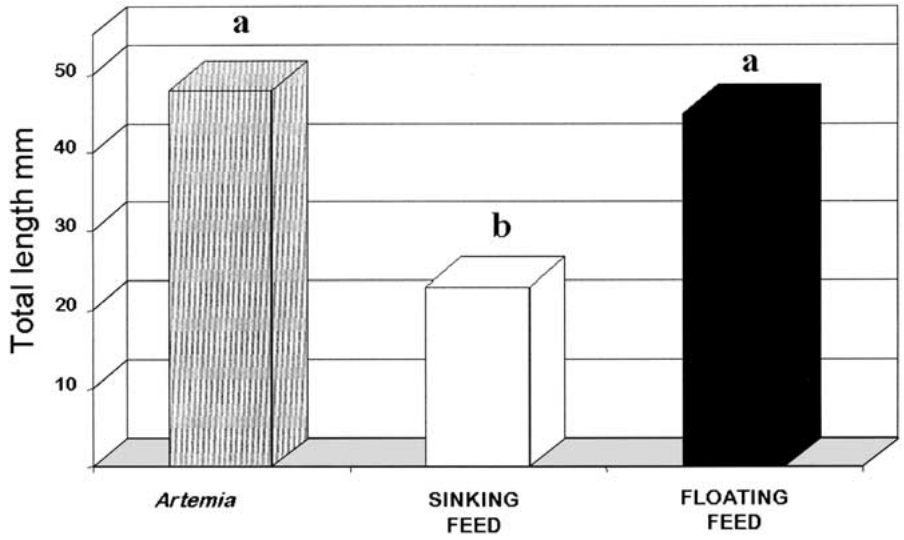

Figure 8. Total length of larvae fed natural prey and sinking or floating artificial diets. Different letters refer to significantly different means.

Figure 9. Alligator gar juvenile after 4 months of culture on artificial diets.

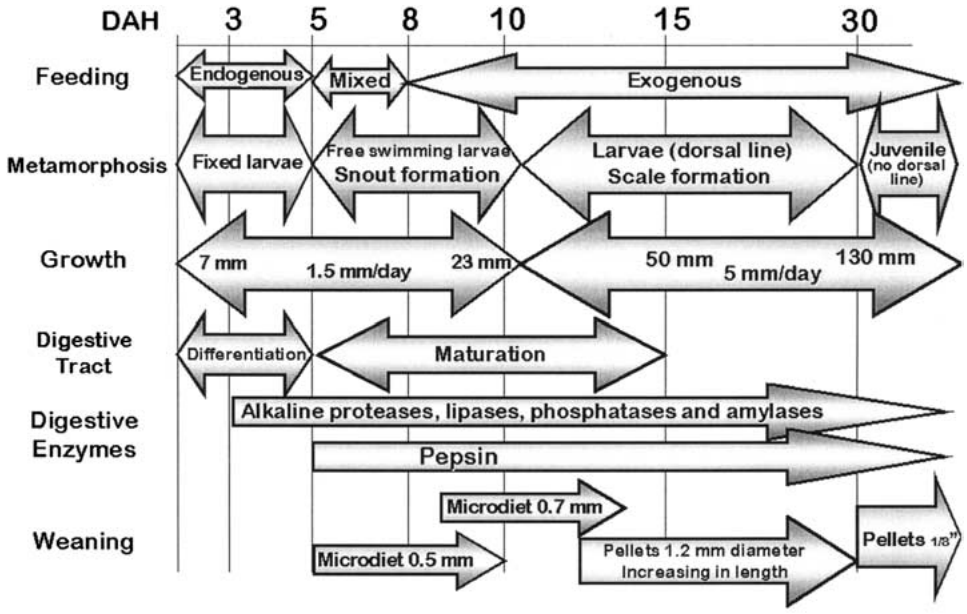

Figure 10. Summary of alligator gar larval development.

This may have resulted from exposure of larvae to exogenous T3 added to the naturally high endogenous levels of T3 found in individuals of all groups. Abnormally high levels of T3 may be related to the high concentrations of cortisol determined in this group. Both hormones may have contributed to the lower survival and deformities, such as a curvature of the spine similar to lordosis, observed in larvae treated with T3. Higher values of weight gain and total length were observed in the TU and C groups. The lower weight attained by larvae exposed to T3 could be explained by energy invested in premature metamorphosis. Snout length was significantly reduced in the TU group, whereas in the T3 group snout development was accelerated (Figure 6). Mid-gut cell height was highest in the HC group followed by T3 and TU. Acid and alkaline proteolytic activities were highest in the T3 and TU groups.

Feeding trials (weaning)

In the first experiment, larvae generally rejected the sinking pellet diet, despite the addition of a commercial betain-based feeding attractant (*Langobuds*^{TM}, Quali-Tech, Chaska, Minnesota, USA, use of trade or manufacturer names does not imply endorsement). This resulted in 20% survival compared to 95% survival of larvae fed *Artemia nauplii*. This could be explained by the feeding behavior of gar larvae, which feed mostly at the water surface. It was not possible to maintain artificial feed in the water column even with air-lifts. Moreover, air-lifts produced turbulence within the aquaria and gar larvae, which are not active swimmers, avoided such areas even though they may or may not have contained suspended food pellets. These observations were confirmed in the second experiment where gar larvae consumed extruded microparticulated feed to which the same commercial attractant was added. In this case, in a similar experimental period, survival increased to 80% (Figure 7) and no significant differences were detected regarding growth and length when compared to the *Artemia nauplii* treatment group (Figure 8).

Conclusion

Morphological studies allowed us to distinguish between different phases of development and nutritional condition of larvae as well as select external indicators of starvation. Histological studies indicated that the digestive tract was completely formed at the beginning of exogenous feeding. The maturation process of different structures, including the digestive glands, spiral valve, liver, and pancreas were also observed. Mid-gut cell height was shown to be an indicator of larval nutritional condition. Because the

digestive system of alligator gar developed rapidly, we were able to use artificial feeds starting 5 DAH. Based on our results, growth rates of larval gar that were fed artificial diets were similar to those of larval gar that were fed live prey. Information gained from the isolation and purification of the main digestive enzymes may be used to develop an *in vitro* digestibility system to test different feed ingredients, resulting in the formulation of a cost-effective diet for cultured gar. The endocrinological approach showed the feasibility of altering snout development and the digestive capacity of larvae. These results present novel alternatives for the control of cannibalism under culture conditions, through the retardation of snout growth by exposure to anti-thyroid compounds (TU) without effecting growth and survival. Further investigations are needed to determine the optimal dose of T3. Finally, the results of the feeding trials made it possible to culture larvae and juveniles using artificial diets. This feeding strategy produced 30 cm juveniles in four months (Figure 9). Our current research focuses on the determination of nutritional requirements of larval and juvenile alligator gar. Through our multidisciplinary approach, we determined that artificial diets could be used to replace diets of live prey and, when used in conjunction with anti-thyroid treatments, reduce cannibalism among cultured alligator gar larvae. This research provides a basis for mass production of larvae for repopulation experiments and for the culture of commercial-size alligator gar (Figure 10).

Acknowledgements

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