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Gar biology and culture: status and prospects

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Abstract

Many lepisosteid populations in North America have declined and many are now threatened as a consequence of habitat loss and alteration and commercial and sport overfishing. Over the last two decades, morphological, histological and molecular studies allowed distinguishing between different phases of development and the nutritional condition of larvae. Ontogeny of the digestive enzymes of gar larvae indicated the possibility to feed them artificial feeds since early developmental stages. An *in vitro* digestibility system to test different feed ingredients has been used. Important characteristics of artificial diets were identified through different feeding experiments. Endocrinological studies showed the feasibility of altering larval development and the digestive capacity of larvae. Cloning of gar growth hormone opened new avenues to enhance growth in the gars. Plasmatic vitellogenin was isolated and purified, to develop a competitive enzyme-linked immunosorbent assay, which allowed the straightforward separation of males from females to establish appropriate proportions for reproduction and also was used to evaluate hormonal protocols to induce gonad recrudescence and spawning. This review analyzes the biology, ecology and physiology of different gar species as a basis for their domestication, mass production of larvae for repopulation experiments and for the culture of commercial-size gar.

Keywords: gar, biology, physiology, aquaculture, nutrition, reproduction

Introduction

The family Lepisosteidae is at present represented by two genera and seven extant species of non-teleost (Fig. 1), bony fishes that, together with the bowfin *Amia calva*, belong to the order Semionotiformes (Nelson 1994) and are sometimes grouped as holosteans. This is an ancient group of fish that is older than most teleosts, dating back to the Cretaceous period, approximately 180 million years ago (Wiley 1976). The genus *Lepisosteus* is composed of four species (*L. osseous, L. platostomus, L. oculatus* and *L. platyrhincus*), while the genus *Atractosteus* contains three extant species (*A. spatula, A. tristoechus, A. tropicus*).

At present, lepisosteids are distributed in North America, Central America and Cuba, including The Isle of Youth (Comabella, Mendoza, Aguilera, Carrillo, Hurtado & García-Galano 2006). The northernmost limit is reached by *L. osseus* in southern Quebec, while the southernmost limit is reached by *A. tropicus* in Costa Rica (Mora-Jamett, Cabrera & Galeano 1996; Orlando 2001). This is also the only species that ranges to Pacific slope drainages (from southern Mexico to Honduras).

In addition to their close phylogenetic relationships, the gars share similar ecological roles as top predators. Because of the high trophic position and relatively long life span of these fish, many lepisosteid populations have declined due to habitat loss and alteration (dams, channel straightening, road construction, flow alteration, pollution, etc.) and due to overfishing. The decline of many gar populations has resulted in research into the feasibility of using captive culture techniques to reduce fisheries pressure and to restore natural populations. Of the seven gar species, those belonging to the genus *Atractosteus* have recently motivated much interest in their culture. This importance is principally due to their rapid growth rate and large adult size, as is the case of alligator and Cuban gars (Aguilera, Mendoza, Rodríguez & Marquez 2002). Moreover, culturing gars offers

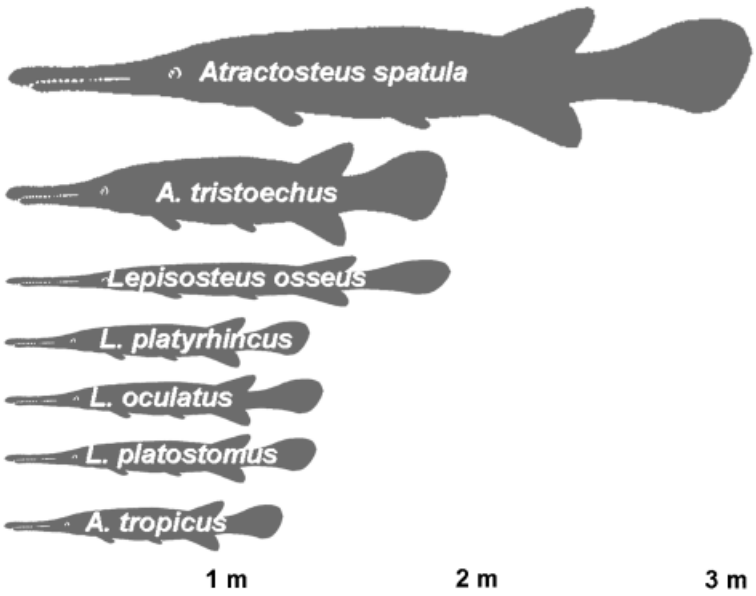

Figure 1 Relative total lengths of the seven extant gar species (based on Suttkus 1963; Page & Burr 1991; Mora-Jamett *et al.* 1997).

several additional advantages such as their ability to grow in waters of variable quality due to their capability to breathe atmospheric air (Hill, Renfro & Reynolds 1972; Smatresk & Cameron 1982) and tolerance to high ammonia and nitrite levels (Boudreaux, Ferrara & Fontenot 2007a, b), their resistance to several diseases (León, Aguiar & Hernández 1978; Kulakkattolickal & Kramer 1988) and excellent feed conversion rate (FCR) (Aguilera, Mendoza, Comabella & Marquez 2006; Márquez-Couturier, Alvarez-Gonzaléz, García-Galano, Contreras-Sánchez, Hernández-Franyitti, Mendoza-Alfaro, Aguilera-González, García-Galano, Civera. Cerecedo & Goytortua-Bores 2006).

Within this context, the use of new species for aquaculture production necessarily implies their domestication. This process requires, at least, the study of the capacity of those organisms of interest to live most of their life cycle under artificial conditions (FAO/PNUMA 1984). Among the main characteristics desired in a species to be domesticated are the ability to reproduce in captivity, the likelihood of spawning in captivity, the possibility of mass larval rearing, adaptability to the consumption of artificial diets and the capability to grow and be maintained at high densities (AQUACOP & Calvas 1990). However, most currently cultured species do not possess all of these characteristics (Mendoza 2005). In fact, the reasons to initiate the domestication of a species have been economical (commercial value of the species), sociocultural (traditional fisheries) and ecological (over-exploited or endangered species) (Mendoza, Aguilera, Rodríguez & Márquez 2000; Rojas & Mendoza 2000). This review summarizes recent research on the culture of *Atractosteus* species.

Reproductive biology

Gars spawn seasonally in the floodplain of large rivers, shallow lacustrine habitats, and tributaries that provide protection for their young from predators. Lepisosteids seldom display gregarious behaviours, with the exception of the spawning season, when groups of several individuals (some times 20 or more) may be observed together. Sex ratios of spawning aggregations are typically skewed towards males (Dean 1895; Holloway 1954; Suttkus 1963; Reséndez & Salvadores 1983; Aleman & Contreras 1987; Chavez-Lomelí, Matthews & Pérez-Vega 1989; Gómez-Gómez 1989; Pérez-Sánchez 1995; Bejerano, Marquez & Páramo 1997). The large spawning aggregations will divide into smaller parties containing a female that is attended by several males (from two to eight). The smaller groups will then enter very shallow waters where spawning takes place (Dean 1895). However, sex ratios vary among gar species. Differing sex ratios have been reported for tropical gar from 1:1, 3:1 to 5:1 males per female (Reséndez & Salvadores 1983; Chavez-Lomelí *et al.* 1989; Pérez-Sánchez 1995; Bejerano *et al.* 1997). Sex ratios of 1:1 male per female for *L. platyrhincus* and 3:1 males per female for *L. osseus* have been reported (Holloway 1954). Finally, ratios of 2:1 and 1:1 males per female have been reported for alligator gar (Morales 1987; Rodríguez, Banda, González,

Herrera & García 1998; Ferrara 2001). Because the sex of individuals in most populations of lepisosteids cannot be determined externally, or that techniques for external sex identification may need to be developed on a population by population basis, and sex ratios vary among species and may vary seasonally for a given area, techniques to accurately identify sex of broodstock are needed to establish proper sex ratios for captive spawning and to optimize fertilization rates.

Until recently, accurate sex identification of broodstock was a major obstacle in the culture of gars even though a few studies have documented sexually dimorphic external characteristics. Suttkus (1963) reported that males of *L. platostomus, L. oculatus* and *L. platyrinchus* have a smaller maximum size and reach maturation at a smaller size than females. Female spotted gar *L. oculatus* are reported to have larger bodies (Redmond 1964) and longer snouts (Hubbs & Lagler 1949; Suttkus 1963; Love 2001). Dean (1895) observed that the snouts of male gar (*Lepisosteous* spp.) had a lighter colour than female gar. León *et al.* (1978) noticed that *A. tristoechus* adult females showed a swollen abdomen and a prominent and coloured genital papilla before spawning. However, these differences are only apparent after several years or only during advanced reproductive stages (Netch & Witt 1962; Bejerano *et al.* 1997), thus hindering gender selection. The same problem has been observed for alligator gar (Morales 1987), in tropical gar that reaches sexual maturation more precociously (Chavez-Lomelí *et al.* 1989).

Vitellogenin (VTG)

Sex of mature gar was typically identified by gross examination of the gonads (Netch & Witt 1962; Reséndez & Salvadores 1983; Mora-Jamett *et al.* 1996; Ferrara & Irwin 2001; García de León, González-García, Herrera-Castillo, Winemiller & Banda-Valdés 2001), but this practice requires the sacrifice of animals. In addition to a lack of external sexually dimorphic features, other problems impeded the successful reproduction of captive gars. For example, the oviducts of female lepisoteids join in a common chamber (the urogenital sinus) before opening to the external environment (Pfeiffer 1933; Netch & Witt 1962; Suttkus 1963), thus preventing cannulation, which is commonly used in other fish to assess the degree of oocyte maturation. Development of an accurate, non-invasive and sensitive method for sex identification became a priority. Research efforts were directed to the purification and quantification of VTG as a sex-specific biochemical marker (Hernández 2002; Cortes 2003; Vela 2003; Santillán, Mendoza, Revol, Aguilera & Montemayor 2005; Santillán 2006; Orlando, Binczik, Denslow & Gillette 2007). Vitellogenin was selected for sex identification because it is the precursor of yolk and is thus specific to reproductive females. Moreover, the blood, muscle and mucus concentrations of VGT increase concurrently with ovarian development.

Because of the scarcity of wild alligator gar adults in northern and central Mexico, plasmatic VTG and ovarian lipovitellin (VTL) were isolated and purified from female and estradiol-injected male cultured Alligator gar. Purification of both molecules was carried out by selective precipitation ethylenediamine tetra acetic acid (EDTA–$MgCl_2$), followed by molecular weight filtration in Sepharose-6B and ion exchange chromatography using DEAE-Sephacel (Sigma Chemical, Sanint Louis, MO, USA). Purified molecules were characterized by their prosthetic groups and molecular weight was determined by regression analysis using the Stokes radius of the molecules according to Mendoza (1992). With the purified molecules, polyclonal antibodies were raised in rabbits following the method of Vaitukaitis (1981). Immunoglobulins (IgGs) were obtained by protein 'A' chromatography. With both purified antibodies and antigens, a competitive enzyme-linked immunosorbent assay (ELISA) was developed and submitted to a set of quality control tests, including sensitivity, recuperation, parallelism, reproducibility and specificity. The immunoassay was validated by measuring VTG concentrations in the plasma and mucus of female gar. Furthermore, the ELISA allowed for the straightforward separation of males from females to establish the appropriate sex ratios for reproduction and was also used to evaluate hormonal protocols for the induction of gonad recrudescence and spawning. VTG has also been used to follow the fate of key molecules such as amino acids during gar embryonic development (Finn, Marel, Mendoza, Aguilera, Evjen & Fyhn 2002). In conclusion, quantification of VTG by ELISA is a practical, reliable and quick method to identify the gender of alligator gar adults without sacrifice and can be used as a definitive marker for the onset and progress of maturation in female alligator gar. VTG assay in gar females has become a popular technique using various methods including qualitative determinations by immunoprecipitation and PAGE (Cortes 2003; Arias, Hernández & Contreras 2007) to quantitative determinations such as single radial immunodifussion (SRID) (Hernández,

Contreras, Martínez & Arias 2005) and ELISA (Santillán *et al.* 2005; Mendoza, Aguilera, Santillán *et al.* 2006;
Orlando *et al.* 2007). In some cases, due to a lack of specificity of antibodies, quantitative VTG determination was essential to prevent misidentifying females and males (Hernández *et al.* 2005).

Broodstock development

Broodstock development is a key phase for the domestication of a new species particularly for threatened species, in which the availability and reproductive condition of broodstock will determine the future availability of larvae and juveniles for the maintenance of the species in captivity. Within the last two decades, broodstocks have been established for each extant *Atractosteus* species.

Alligator gar. In northern Mexico, alligator gars have been spawned in captivity since 1982 to supplement the low numbers of wild larvae. Nevertheless, several obstacles hindered the successful captive reproduction of alligator gar including the species' short natural reproductive season, which is restricted to a few weeks per year. Additionally, the mean age of Mexican broodstock is estimated to be 15 years with some individuals as old as 35 years (G. Morales pers. comm.). The number of eggs spawned, fertilization rates and larval production have been erratic, probably due to the overall age of the broodstock. Owing to irregular annual fertilization rates (10–70%) and annual numbers of larvae produced (10 000–400 000), a protocol that uses the previously described VTG assay was developed to provide more consistent annual fertilization rates and larval production (Fig. 2).

Establishment of a new broodstock from wild and cultured juveniles coupled with the use of VTG assay would allow for gender identification and for the evaluation of different hormones to induce sexual maturation and spawning outside of the natural season to provide year-round availability of larvae and juveniles.

In the early 1980s, the Aquaculture Center 'Tancol', located in Tampico, Tamaulipas, Mexico, collected and maintained wild adult alligator gar. An unplanned spawning without the use of hormones occurred, thus prompting the maintenance of these individuals until the present. This broodstock is composed of 40 individuals, which have produced larvae annually and have been used to repopulate nearby water bodies (Morales 1987; Mendoza, Aguilera, Rodríguez *et al.* 2002; Mendoza, Aguilera, Cortés & Hernández 2003). Since 2000, a new broodstock was established using cultured and wild-caught juveniles. Wild individuals were caught in 'Vicente Guerrero' reservoir, and 'Soto la Marina' river in Tamaulipas. The new broodstock is maintained at the 'El Huasteco' aquaculture facility located in Gómez Farias, Tamaulipas. Each gar has been individually tagged with a microchip to follow their performance.

In the United States, two Fish and Wildlife Service National Fish Hatcheries and one Regional Fisheries Center have worked with the culture and genetics of alligator gar for re-stocking efforts. In 1999, the Private John Allen National Fish Hatchery in Tupelo, Mississippi, began spawning wild-caught adult alligator gar and producing alligator gar larvae and juveniles for re-stocking efforts in the southeastern US Tishomingo National Fish Hatchery, Tishomingo, Oklahoma, has also worked with the culture and production of juvenile alligator gar since 2000 (R. Campbell, US Fish and Wildlife Service, pers. comm.). The Conservation Genetics Lab at the Warm Springs Regional Fisheries Center in Warm Springs, Georgia, is currently developing microsatellite markers to assist in maintaining genetic diversity for the long-term viability of supplemented alligator gar populations (G. Moyer, US Fish and Wildlife Service, per. comm.)

Cuban gar. The first attempts to establish a Cuban gar broodstock were made in the early 1970s at the Center for Fluvial Repopulation in Loma de Tierra, Habana, Cuba. The observations and experiments conducted have produced the majority of the published information on the biology and culture of this species (León *et al.* 1978). Currently, a new Cuban gar broodstock has been established at the Center for Native Ichthyofauna Reproduction located in the Zapata Peninsula, Cuba (Comabella *et al.* 2006).

Tropical gar. Within their historical range, tropical gar are the most abundant *Atractosteus* species. The tropical gar is the smallest and earliest maturing species of *Atractosteus* (Fig. 1). The abundance of tropical gar and the species' relatively small size and early maturation as compared with other members of *Atractosteus* allowed for the establishment of several broodstocks. However, the erratic results of captive spawning and larval maintenance resulted in the dissolution of some of these broodstocks. The largest current broodstock assemblage is located at the Aquaculture facilities of the University Juárez

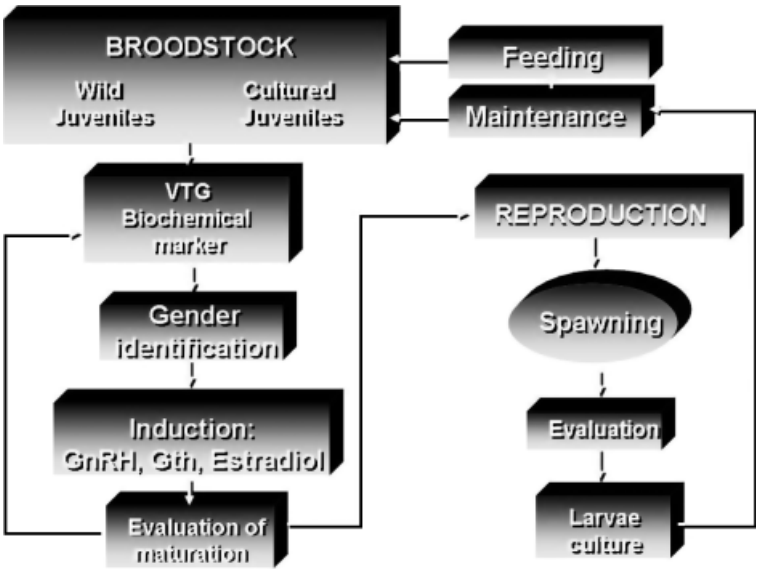

Figure 2 Protocol for the development and evaluation of broodstock reproductive quality in northern Mexico.

Autónoma de Tabasco (UJAT), Villahermosa, Mexico, which maintains up to 130 individuals (Hernández 2002). Ongoing efforts on wild-caught tropical gar induction to maturation and spawning are being carried out at the National University of Costa Rica (M. Protti, pers. comm.).

Sexual maturation and spawning induction

Most lepisosteids spawn naturally during spring and summer (Simon & Wallus 1989) when spawning occurs intermittently, resulting in six to seven effective spawning days during the spawning season (Dean 1895). An exception is the tropical gar that spawns from March to October (Chavez-Lomelí *et al.* 1989) with a peak in spawning activity in July and August (Pérez-Sánchez 1995). The prolonged spawning period of tropical gar may indicate that the length of the spawning season in lepisosteids is related to latitude and temperature. Captive *Atractosteus* were typically spawned annually during the natural spawning period for each species. In some cases, spawning of captive alligator, tropical and Cuban gar occurred without the use of hormones in earthen ponds containing vegetation or artificial spawning substrates, simulating the flooded grounds in which they normally spawn. Although spontaneous non-hormone-induced spawning of captive broodstock occurred, the numbers of larvae produced were not consistent from year to year.

The use of different hormones in the three *Atractosteus* species was evaluated for the induction of maturation and spawning in adults (Table 1). The best results were achieved for the three species using synthetic analogues of gonadotropin-releasing hormones (GnRH). Gonadotropin-releasing hormones are the major regulators of reproduction in vertebrates acting as a first signal from the hypothalamus to pituitary gonadotropes. Sherwood, Doroshov and Lance (1991) reported that alligator gar and two other ancient bony fishes, the reedfish *Calamoichthys calabaricus* and white sturgeon *Acipenser transmontanus*, have two forms of GnRH. Furthermore, GnRH analogs are effective in most fish and are more cost effective than are other hormones when used in large species such as alligator gar and Cuban gar. Implants induced spawning in tropical gar but negative results were obtained when used in alligator gar. This is presumably due to the weight of females in relation to the quantity of hormones released by the implants. For example, mean weights of female alligator gar broodstock range from 6.6 kg (González 2007) to 20 kg (Cortes 2003), whereas tropical gar females are reported to have a mean weight of 2.5 kg (Hernández 2002).

Larval culture

As in other fish species, the dependence of gar larvae and juveniles on live food has been one of the main impediments to the culture of these fish. Young gars grow rapidly and therefore require constant and adequate supplies of live prey items. Often, the cost and labour associated with the production of live food result in losses due to cannibalism and the production

Table 1 Homologous and heterologous hormones used to induce maturation and spawning in *Atractosteus* species

	Species		
Hormone	***Atractosteus spatula***	***Atractosteus tristoechus***	***Atractosteus tropicus***
Heterologous gonadotropins	Human chorionic gonadotropin (+) But low larval survival (Colunga 1996)	Human chorionic gonadotropin (−)	Human chorionic gonadotropin (−) (Bejerano *et al.* 1997)
	SG100 (−) (González 2007)*	Carp hypophisis (−) (León *et al.* 1978)†	(−) (Pérez-Sánchez 1995)‡ Promoted gonadal maturation but no spermiation was registered
Homologous gonadotropins		(+) After 35 days (León *et al.* 1978)†	(−) (Pérez-Sánchez 1995)
17b-Estradiol			(−) (Hernández 2002)
Ovaprim™	(+) Out of the regular spawning season – from April to August (González 2007)*		(−) (Pérez-Sánchez 1995) (+) (Hernández 2002)
Des-Gly^{10}-LHRHe	(+) (González 2007)*		(+) (Hernández 2002)
D-Ala^{6}-LHRHa	(+), Out of the regular spawning season (October) (González 2007)*	LHRHa: (+) (Comabella *et al.* 2006)	(+) (Hernández 2002; [43]Hernández & Contreras 2005)
Implants	Ovaprim: (−) (González 2007)*		GnRH-a: (+) (Hernández *et al.* 2007)

Plus sign (+) indicates successful induction of maturation and spawning, minus sign (−) indicates unsuccessful induction of maturation and spawning.

*Inductions reported by González (2007) were performed from 2003 to 2006.

†Inductions reported by León *et al.* (1978) were performed from 1971 to 1973.

‡Inductions reported by Pérez-Sánchez (1995) were performed from 1994 to 1995.

of larvae and juveniles of different size. Because growth rates vary among individuals, maintaining adequate live prey density to prevent cannibalism is difficult. Therefore, larvae and juveniles have been released at young ages to avoid cannibalism when maintained at high densities. Release of smaller, younger larvae rather than larger, older larvae or juveniles may result in reduced survival. In an attempt to prevent cannibalism among cultured alligator gar, larval growth and the development of the digestive tract were examined in an effort to formulate an effective feeding strategy using artificial feeds. A multidisciplinary approach aimed at describing the major morphophysiological changes that take place during the larval period was used and this helped to develop a feeding strategy using artificial diets for *A. spatula* and later for *A. tropicus*.

Morphology and growth of gar larvae

Morphological characteristics of early-life stages have been described for longnose gar *Lepisosteus osseous*, shortnose gar *L. platostomus* and spotted gar *L. oculatus* (Netch & Witt 1962; Pearson, Thomas & Clark 1979; Yeager & Bryant 1983; Simon & Wallus 1989; Simon & Tyberghein 1991) and for *A. tropicus* and *A. spatula* (Aguilera *et al.* 2002). Gar eggs are moderately large (egg diameter range 3–5 mm, León *et al.* 1978; Simon & Wallus 1989; Aguilera *et al.* 2002) and larvae are characterized by an extended yolk-sac period (10–30 days, Aguilera *et al.* 2002). As a consequence, larvae are large (23–30 mm, Pearson *et al.* 1979; Aguilera *et al.* 2002) and well developed when exogenous feeding begins. For alligator gar and tropical gar, morphometric studies indicated that the stage of larval development for both species could be differentiated by snout length, suggesting that this characteristic may be used to evaluate growth of gar larvae (Aguilera *et al.* 2002). Moreover, caudal peduncle depth, head width and preanal depth were found to be the morphometric characteristics that best differentiated between fed and starved gar larvae, and may be used to evaluate the nutritional condition of larvae of similar developmental stages. Alligator gar larvae approached adult snout-length proportion at an earlier size and age than did tropical gar larvae (Aguilera *et al.* 2002; Mendoza , Aguilera, Rodríguez *et al.* 2002). This suggests a faster metamorphosis from the larval to juvenile state in alligator gar.

The fastest larval growth rates (5.6 mm day^{-1}) were observed for 10 days post-hatching (dph) alligator gar larvae exceeding juvenile growth rates reported for juvenile spotted gar (1.3–1.7 mm day^{-1}) and longnose gar (2.33–4.5 mm day^{-1}) (Netch & Witt 1962; Echelle & Riggs 1972; Simon & Wallus 1989). Larval growth rates of tropical gar (1.0 mm day^{-1})

were similar to rates reported for larvae of longnose gar (0.8 mm day $^{-1}$) and spotted gar (0.83 mm day $^{-1}$) (Pearson *et al.* 1979; Simon & Tyberghein 1991). These data confirm that lepisosteids, and particularly alligator gar, are among fish with the fastest larval growth rates (Netch & Witt 1962).

Nutritional stages for alligator gar and tropical gar larvae were distinguished by Aguilera *et al.* (2002) according to the criteria established by Beccaria, Diaz, Connes and Chatain (1991). Utilizing these criteria, larvae between 6.8 and 13.5 mm TL (1–4 dph) were lecithotrophic. At this stage larvae remained attached to vegetation and received nutrition only from the yolk sac. Larvae between 12.5 and 22.5 mm TL (5–8 dph) were lecithoexotrophic, when exogenous feeding began, although fed and unfed larvae did not show differences in growth, indicating that yolk reserves are still present. Finally, the exotrophic stage began around 22 mm TL, when morphological differences were observed in unfed larvae, indicating a total dependence on exogenous food.

Despite the lack of detailed descriptions of morphology and larval development of Cuban gar, the observations reported by León *et al.* (1978) and Comabella *et al.* (2006) describe a development pattern similar to alligator gar.

Organogenesis

Embryonic development of lepisosteids shares characteristics with both telosteans and chondrosteans (e.g. egg segmentation) (Dean 1895). The appearance and development of different structures were histologically assessed to determine when the digestive tract of alligator gar was fully developed (Aguilera 1999; Álvarez, Sarmiento & Mendoza 1999; Mendoza & Aguilera 2001; Mendoza, Aguilera, Rodríguez *et al.* 2002). Upon hatching, the digestive tract develops from an undifferentiated epithelium surrounding the yolk sac. Differentiation takes place rapidly starting from the posterior end beginning with the formation of the spiral valve in 3 dph larvae. At the onset of exogenous feeding (5 dph) the stomach and pancreatic tissue can be observed, despite the presence of yolk reserves. These observations suggest that the digestive tract was completely formed. Digestive tract development at the start of exogenous feeding places gar among a small group of fish, including sturgeons and some salmonids, which possess a differentiated stomach at this stage of development (Buddington & Christofferson 1985; Buddington & Doroshov 1986; Dabrowski 1986; Gawlicka, Hung, Hinton & de la Nöue 1995; Gisbert, Rodriguez, Costelló-Orvary & Willot 1998). Once gar larvae started feeding, structural changes were observed, including the development of intestinal folds and the enlargement of enterocytes. According to Dabrowski (1986) these changes are indicative of a rapid maturation process during this period. In addition, the existence of yolk reserves in the body cavity until 8 dph was observed through histological analysis, confirming the proposal for the different nutritional phases in gar larvae (Aguilera *et al.* 2002). Moreover, the existence of yolk reserves help to explain the absence of qualitative and quantitative differences (enterocytes height) between starved and fed larvae until 8 dph. Once these reserves are exhausted (9–10 dph), conspicuous differences in the mid-gut cell height of fed and starved larvae were observed (Álvarez *et al.* 1999). Thus, mid-gut cell height served as a valuable tool for evaluating the suitability of artificial diets in gar larviculture, as in other studies. (Oozeki, Ishii & Hirano 1989; Theilacker & Watanabe 1989; McFadzen, Lowe & Coombs 1994; Theilacker & Porter 1995). At 15 dph, gastric glands of starved larvae were still underdeveloped or degenerated 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. From these results, it was possible to assume that the structural degree of development of the digestive tract of gar larvae would not hamper their weaning onto artificial diets.

Growth indicators

The need for sensitive and reliable indicators of growth and nutritional condition has led to extensive research on molecular indicators, particularly nucleic acids that play a major role in growth and development. The relationship between RNA and DNA is an index of the cell's metabolic activity and has been used to measure short-term growth in fish (Clemmesen 1996). The RNA/DNA ratio relies on the theory that transcription-dependent protein synthesis is positively correlated with ribosomal activity and an increase in cellular RNA levels, while DNA concentration does not increase during transcription-dependent protein synthesis, thus providing a point of reference (Mommsen 1998). The quantification of total nucleic acids concentration allowed for comparison of the parallel increase in weight and size, and DNA and RNA concentrations in gar larvae that were fed live prey or artificial diets. The RNA/DNA ratio

increased until 13 dph, remaining constant thereafter, which may imply that growth, as a product of protein accretion, stabilizes at this stage, while larvae continue growing with the same metabolic intensity. These data support morphological observations indicating the end of metamorphosis, the start of exponential growth and the stabilization of the cell height of enterocytes. In contrast, in starved larvae, RNA and DNA concentrations as well as the RNA/DNA ratio remained low throughout larval development. These observations allowed for the differentiation between starved and fed larvae, and among larvae that were fed different diets. Other indices including protein/dry weight, protein/DNA, RNA/protein, DNA/dry weight and RNA/total length were used to confirm the results of RNA/DNA ratio analysis to identify starved versus fed larvae and to identify larvae that were fed different diets (Mendoza, Aguilera, Carreón *et al.* 2002; Mendoza, Aguilera & Carreón 2002; Mendoza, Aguilera, Carreón, Montemayor & González 2007).

Enzymatic ontogenesis

As mentioned above, several digestive tract structures were found to be functional at the onset of exogenous feeding, thus supporting the ability of gar larvae to utilize artificial diets at the onset of exogenous feeding. Using substrate – PAGE – electrophoresis the quantity, type and appearance of digestive proteolytic enzymes were studied for alligator (Aguilera, Mendoza, García & Nolasco 1998; Aguilera 1999; Mendoza , Aguilera, Rodríguez *et al.* 2002), Cuban (Comabella *et al.* 2006) and tropical (Iracheta 2006) gar larvae. Seven different bands with proteolytic activity have been reported for each species (Table 2).

Alkaline proteolytic activity corresponded to six bands of different molecular weights. Only one band was related to acidic activity in alligator gar and tropical gar, whereas five bands had alkaline proteolytic activity and two had acidic activity in Cuban gar. Ontogenetically, alkaline proteases of lower molecular weight appeared before proteases of higher molecular weight for the three species. The expression of proteolytic activity was the most rapid in alligator gar, where at 5 dph (beginning of exogenous feeding) four alkaline proteases and one acidic protease were detected, while the two remaining alkaline proteases were distinguished at 15 dph. In Cuban gar, the appearance of proteases was slower. At 5 dph only one acidic protease was detected, at 7 and 8 dph a second acidic protease and one alkaline protease were detected respectively, and between 13 and 14 dph the remaining four alkaline proteases were distinguished in Cuban gar. Finally, the appearance of proteases in tropical gar was the most delayed. The acidic and first alkaline proteases were detected at 5 and 6 dph respectively, two alkaline proteases were detected at 13 dph, two additional alkaline proteases were detected at 19 and at 34 dph the last alkaline protease was detected (Fig. 3). Acidic activity was inhibited by pepstatin 'A' for the three species, indicating their similarity to aspartic proteases, particularly pepsin. All of the bands exhibiting alkaline proteolytic activity were inhibited by soybean trypsin inhibitor, known to inhibit serine proteases. Phenylmethylsulphonyl fluoride, also a serine proteases inhibitor, inhibited the activity of three to four bands. Three bands were inhibited for each species by the specific trypsin inhibitor *N*-tosyl-L-lysine-chloromethyl ketone, while only two were inhibited by L-1-tosylamide-2-phenylethyl chloromethyl ketone, a specific chymotrypsin inhibitor. Only one or two bands were inhibited for each species by EDTA, which is specific to metaloproteases (aminopeptidase or carboxipeptidase). Based on these results, alkaline proteolytic activity in lepisosteids is due to the activity of serine–proteases-like enzymes.

Despite the close phylogenetic relationships among *A. spatula, A. tristoechus* and *A. tropicus*, several differences in the maturation of the digestive tract, important to the feeding strategies of cultured larvae, were observed. The presence of pepsin-like activity, in all three species, from the onset of feeding (5 dph) reveals an unexpectedly rapid development of the digestive tract, and is reflected in the carnivorous food habits of these species. Notwithstanding, this precocious development is indicative of their ability to be fed artificial diets at the onset of exogenous feeding. Diets should be formulated with ingredients susceptible to acidic digestion. The gradual increase in alkaline digestion implies that the final maturation of the digestive tract takes place in the intestine and associated tissues (e.g. pancreas), unlike most marine fish larvae in which the stomach is the last functional region of the digestive tract to mature.

The fastest digestive tract maturation rate was observed in alligator gar, followed by Cuban gar and tropical gar and was related to growth rate and final size (Aguilera *et al.* 2006). However, in the final stage of digestive tract development, differences in the number and types of proteolytic enzymes are negligible, suggesting that juveniles of the three species share similar digestive abilities. This would allow for

Table 2 Digestive proteases detected by substrate-PAGE (zymograms) in lepisosteids larvae

	Gar species		
Protease enzyme no.	***Atractosteus spatula***	***Atractosteus tristoechus***	***Atractosteus tropicus***
1	Alkaline: 18.8 kDa	Alkaline: 21.8 kDa	Alkaline: 25.2 kDa
2	Alkaline: 23.3 kDa	Alkaline: 28.4 kDa	Alkaline: 28.5 kDa
3	Alkaline: 26.8 kDa	Alkaline: 34.9 kDa	Alkaline: 37.2 kDa
4	Alkaline: 33.7 kDa	Alkaline: 41.4 kDa	Alkaline: 44.1 kDa
5	Alkaline: 36.4 kDa	Alkaline: 46.4 kDa	Alkaline: 53.1 kDa
6	Alkaline: 43.7 kDa	Acid: 0.71 Rf	Alkaline: 54.8 kDa
7	Acid: 0.85 Rf	Acid: 0.88 Rf	Acid: 0.95 Rf

The quantity (no.), type (alkaline or acid), molecular weight (kDa) or mobility (Rf) are listed for each digestive protease.

the formulation of a single diet that could be used for the culture of all three species. In order to formulate the most effective diet, ongoing research is devoted to the purification of enzymes from alligator gar by chromatography for use in *in vitro* digestibility tests to select local ingredients for inclusion in artificial diets.

Hormones in larval development

Fish growth hormone (GH) is a pituitary hormone responsible for somatic growth; however, it can also be expressed in extrapituitary tissues of adults and in early embryos. Growth hormone is known to stimulate appetite (Pickford & Atz 1957; Higgs, Donaldson, Dye & McBride 1975), food conversion efficiency (Markert, Higgs, Dye & MacQuarrie 1977), lipid mobilization (Sheridan 1986), nitrogen retention (Matty 1962), amino acid incorporation into tissues (Cheema & Maty 1978; Fauconneau, Mady & LeBail 1996) and stimulation of energy and protein metabolism at the whole body level (Medale, Fauconneau & Kaushick 1988) and at the tissue level (Foster, Houlihan, Gray, Medale, Fauconneau, Kaushik & LeBail 1991). Within this context, the coding sequence of GH from alligator gar was obtained and its expression throughout larval development was studied (Mendoza, Garza *et al.* 2002; Revol, Garza, Hernández, Aguilera, Barrera & Mendoza 2005). The analysis of the fragment sequence confirmed that it corresponded to a GH gene, sharing 98% of nucleotide similarity with the GH previously reported for *L. osseus* (Rubin, Youson, Marra & Dores 1996). A single amino acid change (Val/Ala) in the fifth exon was observed between the two lepisosteids. The few residue changes observed between the GHs of these two ancient fish that diverged 180 million years ago support the theory of very slow evolution of the hormone within the ancient fish, as compared with the burst of changes observed in the euteleosts (Gayet, Meunier & Werner 2002). The relative expression of GH along larval stages indicates substantial expression of this hormone in unfertilized eggs, declining thereafter until 3 dph, then increasing around 5–7 dph larvae, corresponding to organogenesis. In 8 dph larvae the expression of GH decreases again, and is consistent with the depletion of yolk reserves and the onset of

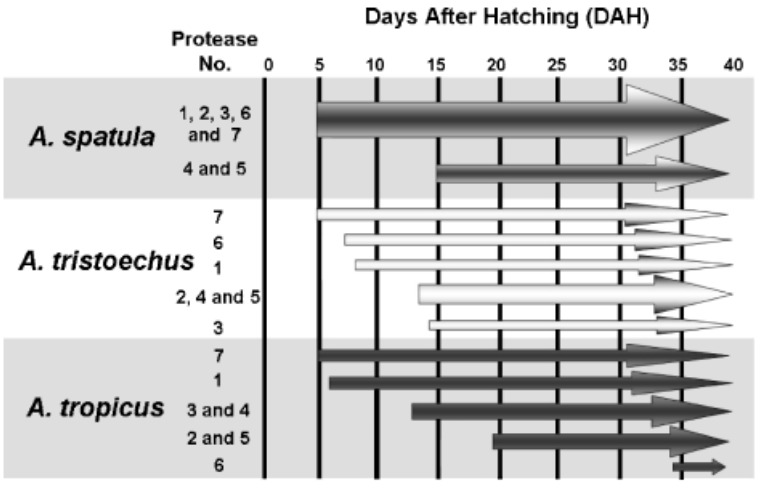

Figure 3 Ontogenetic appearance of proteases in *Atractosteus* species.

exogenous feeding. Lastly, the expression of GH increases in 9–10 dph larvae, probably corresponding to the formation of pituitary gland (Fig. 4).

Other key hormones implicated in larval development are thyroid hormones (TH). The nature of the growth promoting effect of TH in larval fish metamorphosis may involve induction of morphological changes in the digestive tract and accessory organs, stimulation of renewal processes of the intestinal epithelium, increasing nutrient absorption capacity, and growth enhancement in general (Dabrowski & Culver 1991). Thyroid hormones may have a great practical value in aquaculture because they not only act as growth promoters but also trigger and accelerate larval development (Lam 1980). In the case of alligator gar, 3, 3′, 5-triiodo-l-thyronine (T3) levels during embryonic development are low, as shown by the concentrations in recently spawned eggs and eggs just before hatching (0.0117 and 0.00995 ng egg^{-1} respectively). However, after hatching these levels increase, reaching 2.22 ng larvae^{-1} at 10 dph, decreasing thereafter to 0.3 ng larvae^{-1} at 13 dph (Aguilera 1999). These data, coupled with morphological observations, indicate that the metamorphic climax takes place around 10 dph. This contention is supported by the occurrence of two main events before and after 10 dph, the exhaustion of yolk reserves by 8 dph and the beginning of exponential growth at 11 dph. These observations together with the rising levels of GH explain the rapid growth rate of alligator gar at this stage. Considering the use and importance of exogenous TH and glucorticosteroids as potential regulators of larval gar development, the effects of the exposure of larvae to T3, hydrocortisone (HC) or thiourea (TU); (Mendoza, Aguilera & Montemayor 2001; Mendoza, Aguilera, Rodríguez *et al.* 2002) were determined. Concentrations of T3 were three times higher in the T3 treatment, when compared with the other treatments (Fig. 5). 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 in this group. Both hormones may have contributed to the lower survival and deformities observed in larvae treated with T3. Higher values of weight gain and total length were observed in the TU and control (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. These results reflect the feasibility of altering snout development and present novel alternatives for the control of cannibalism under culture conditions, through the retardation of snout growth by exposure to antithyroid compounds (TU) without affecting growth and survival.

Weaning

Based on the results obtained through the multidisciplinary research described above, it could be assumed that gar larvae should readily accept and ulitize artificial diets. However, there were no reports confirming this presumption for tropical gar or Cuban gar. On the contrary, most attempts to culture larvae of these species have failed, due to high mortality rates (León *et al.* 1978; García, Marquez & Paramo 1997; G. Morales 2000, pers. comm.; G. Marquez 2001, pers. comm.). In addition, poor results were obtained when juveniles of these species were fed live bait fish. (Maldonado 1991). Therefore, live prey was considered the only alternative for the successful production of lepisosteid larvae, particularly for tropical gar (Hernández, Marquez, Páramo, Félix

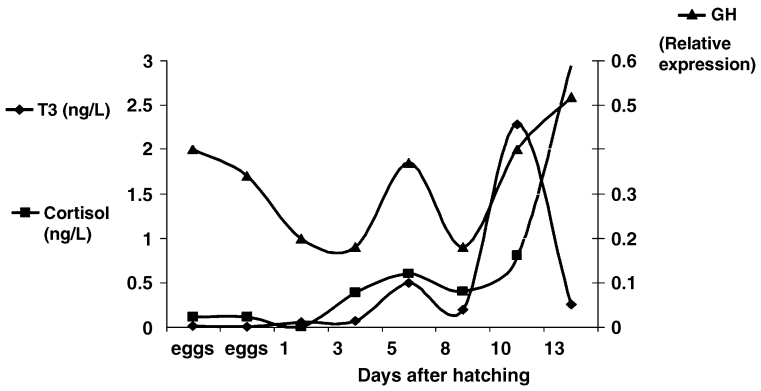

Figure 4 Concentration of T3, cortisol and relative GH expression throughout the larval development in alligator gar. T3, 3, 3′, 5-triiodo-l-thyronine; GH, growth hormone.

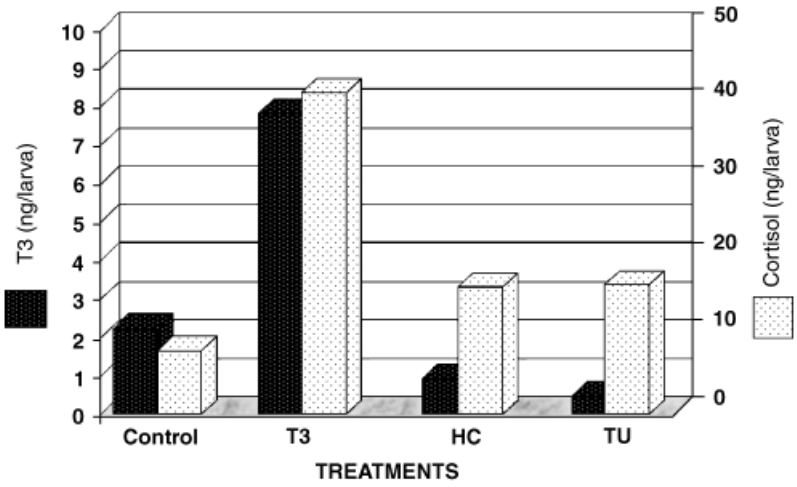

Figure 5 Concentration of T3 and cortisol from alligator gar larvae exposed to T3, hydrocortisone (HC) and thiourea (TU). T3, 3, 3′, 5-triiodo-l-thyronine.

& Hernández 1997; Rodríguez, Marquez & Paramo 1997; Hernández 1999). Feeding trials to produce alligator gar and Cuban gar larvae were of short duration, and were restricted to early developmental stages when food acceptance and cannibalism were not yet problematic (G. Morales 2000, pers. comm.). Between 1997 and 1999, the first attempts to culture gar larvae using artificial diets were also unsuccessful. For these trials gar larvae were fed nauplii and adults of *Artemia* sp., but the culture ended at 15 dph, when larvae reached a maximum size of 50 mm, before the type of food could affect survival and growth. Although good growth and survival rates were not attained, some important characteristics of artificial diets were identified, and the strategy for achieving the acceptance of artificial diets was defined (Aguilera 1999). The following year, the first successful results were obtained (Mendoza & Aguilera 2000; Mendoza, Aguilera, Montemayor *et al.* 2000). Observations of gar feeding behaviour indicated that larvae feed near the water surface and that chemical recognition of feed, indicated by the static position of larvae near the feed and the contact with the snout before ingestion, is important. This motivated the addition of feed attractants to diets for early weaning (Rodríguez, Mendoza, Aguilera & Montemayor 2000). Feeding must be initiated when larvae still carry yolk reserves and it is imperative to increase particle size as snout length increases. Gar larvae can be conditioned to the consumption of artificial diets from the first feeding provided that the feed floats. Because of the need for a floating feed, extruded diets and micro-spheres were chosen. In fact, these diets resulted in higher growth rates than did live prey (*Artemia* sp.). On the other hand, crumbled diets despite their higher floatability, tend to agglomerate, in contrast with micro-spheres that have lower floatability, but whose particles do not lose their shape and can be individually ingested (Mendoza *et al.* 2007). This determination of suitable characteristics of artificial diets was paramount for lepisosteids considering their strong predatory habits.

The feeding strategy for gar larvae based on that established for alligator gar can be summarized as follows: Free swimming larvae are transferred to fibre glass tanks to allow for even distribution of individuals at the water surface. Starter feed consists of a combination of floating particulate diet (0.5 mm diameter) and *Artemia* nauplii. As soon as yolk reserves are depleted a complete substitution of artificial feed for *Artemia* should occur. For alligator gar larvae, *Artemia* nauplii were eliminated from the diet without decreased growth or survival. When the highest growth rate is achieved, feed particle diameter should be increased. At this point it is necessary to adjust larval density according to the tank surface area to allow all individuals access to the water surface. Culture tanks with a large surface area and low turbulence, as opposed to tanks with a small surface area and high turbulence, allow for more effective distribution of feed and larvae. To reduce cannibalism, feed particle size should be increased when larval densities are adjusted and individuals should be sorted and separated according to size to prevent cannibalism. Unfortunately, cannibalism remains the first cause of mortality in alligator gar larvae ranging from 50 to 150 mm. However, it is seldom observed in individuals larger than 200 mm, even under starvation. Survival may be higher than 90% by 15 dph. Great care is required during the first month of culture due to continuous handling to adjust larval densities. This feeding strategy has been

successfully used to wean tropical gar larvae onto artificial feed (Iracheta 2006; Márquez-Couturier *et al.* 2006), and will be used to wean Cuban gar larvae onto artificial feed.

Juvenile production and growth to commercial size

The adoption of the above-mentioned feeding strategy using commercial trout feeds (45% protein) has allowed for the production of alligator gar juveniles of 30 cm and 250 g in 4 months (Mendoza , Aguilera, Rodríguez *et al.* 2002). Recent results in tropical gar culture have confirmed the utility of this diet and feeding strategy (Álvarez, Contreras, Castillo, Santana & Gallego 2007), achieving weights of 130–155 g in 5 months. This is due in part to the feeding efficiency and rapid growth of alligator gar. Juvenile alligator gar had a specific growth rate (SGR) between 5 and 8, a FCR lower than 1 and protein efficiency ratios (PER) between 2 and 5 (Aguilera, Mendoza, Marquez & Iracheta 2005). In contrast, values of FCR of 1.6, PER of 2 and a daily growth of 7% are reported for juvenile tropical gar (Márquez-Couturier *et al.* 2006).

Conclusion

At present, the basic technology for the commercial culture of different species of gar has been developed and aquaculture of gar species is expected to increase in Mexico and Cuba because gar is a staple food in some regions. Currently, further analysis of nutritional requirements for lepisosteids is being carried out (Márquez-Couturier *et al.* 2006). Polyculture trials of alligator gar and catfish in cages and earthen ponds are also under way (Mendoza, Aguilera, Montemayor *et al.* 2006). Because of their position in the food web, gars are being studied as indicators of contaminants (Orlando *et al.* 2007). A microsatellite library is being developed to study the genetic relationships of alligator gar from different regions of United States and Mexico (W. Karel pers. comm.).

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