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# Cloning of the growth hormone cDNA of alligator gar *Atractosteus spatula* and its expression through larval development

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**Abstract**

The alligator gar (*Atractosteus spatula*) is the largest freshwater fish inhabiting rivers draining into the Gulf of Mexico. This primitive fish shows a fast growth rate since its early larval stages. This is attributed to the action of growth hormone (GH), an anterior pituitary gland hormone responsible for linear growth in vertebrates that can also be expressed in extrapituitary adult tissues and in fish embryos. The present research was aimed at obtaining the GH coding sequence of the alligator gar and studying its expression through larval development. A cDNA was obtained by RT-PCR, cloned and sequenced. The alligator gar GH cDNA sequence shares 98% nucleotide similarity with that reported for *Lepisosteus osseus,* indicating a very slow evolution of the GH within the primitive fish, in contrast with the burst of changes observed in euteleosts. Using RT-PCR and RNA nuclease protection assays, GH transcripts were detected at very high levels in eggs, embryos and in several larval stages. These data suggest that the GH may play an important role during embryogenesis in fish. The better understanding of alligator gar larval physiology will facilitate the culture of larvae and juvenile gar and consequently may allow the restoration of their natural populations.


*Keywords:* Growth hormone; *Atractosteus*; Fish larvae; Development

## 1. Introduction

The alligator gar (*Atractosteus spatula*) is the largest freshwater fish inhabiting rivers draining into the Gulf of Mexico. Although this species presents several remarkable anatomic characteristics that distinguish it as a very primitive fish, its phylogenic relationship with other fish including other gars, is still vague. The alligator gar presents a significantly fast growth rate from early larval stages throughout its life cycle (Aguilera et al., 2002), allowing adults to reach up to 2 and even 3 m. Unfortunately, little is known regarding the physiology behind this process (Mendoza et al., 2002a).

Growth hormone (GH) is a well-conserved hormone expressed mainly in the anterior pituitary gland of all vertebrates and is responsible for linear growth. The hormone also participates in the regulation of nitrogen, lipid, carbohydrate and mineral metabolism. Its growth-promoting action is exerted indirectly through inducing hepatic or local IGF-I production, but also directly through binding to its own receptors that are expressed in several tissues (Perez Sanchez et al., 1991; Sakamoto and Hirano, 1991). Although the growth-promoting effect of GH is well documented, growth in fish is multifactorial and GH may

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act at different levels (see Mommsen, 2001, for review). In juvenile and adult euteleosts, GH is known to stimulate appetite (Pickford and Atz, 1957, Higgs et al., 1975), food conversion efficiency (Markert et al., 1977), lipid mobilization (Sheridan, 1986), nitrogen retention (Matty, 1962), amino acid incorporation into tissues (Cheema and Matty, 1978; Fauconneau et al., 1996), as well as energy and protein metabolism at tissue level (Foster et al., 1991) and at whole body level (Medale et al., 1988). Furthermore, the hormone promotes intestinal growth, digestive capacity and transport phenomena (Sun and Farmanfarmaian, 1992; Farmanfarmaian and Sun, 1999). Recent studies suggest that the anabolic effects of GH could be mediated through decreasing myostatin expression levels, a negative regulator of muscle growth (Roberts et al., 2004). Moreover, this hormone is involved in many physiological processes that are not overtly growth related, such as saltwater osmoregulation (Riley et al., 2003), antifreeze synthesis (Idler et al., 1989), the regulation of sexual maturation (Bjornsson, 1997; Li et al., 2002) and immune functions (Yada et al., 2001, 2002). Since the growth-promoting effect of exogenously administered GH has been well established, over the past two decades, advances in gene transfer technology have allowed creating GH transgenic fish that present a dramatically enhanced growth (Devlin et al., 1994; Nam et al., 2001). In this way, the use of GH has been proposed to improve modern commercial aquaculture practices (Farmanfarmaian and Sun, 1999). Although some fast-growing fish strains created after the transfer of GH transgenes will be soon commercially available, more knowledge is needed on the exact functions of GH.

Besides its expression in the somatotrophic cells of the pituitary *pars ventralis*, GH transcripts have also been reported in various extrapituitary tissues (brain, heart, gill, lymphoid tissues, testis, ovaries, head kidney, spleen, intestine and leucocytes) of rainbow trout (*Oncorhynchus mykiss*) adults (Yang et al., 1999). The physiological function of its local production is still uncertain but it may act in paracrine or autocrine ways on local processes that are strategically regulated by pituitary GH, such as immune and reproductive functions (Loir, 1999; Yada et al., 2002). Until recently, the dogma was that GH had no role in embryo growth, but GH transcripts have been detected in mouse embryos at morula stage (Pantaleon et al., 1997), in rainbow trout embryos before pituitary organogenesis and even in mature oocytes (Yang et al., 1999). Additionally, functional GH receptors have been found to be expressed very early in mouse embryos (Pantaleon et al., 1997), sheep blastocyst and fetal tissue (Lacroix et al., 1999). These data led to give another dimension of GH action in vertebrates in early development. Although GH expression has been extensively described in euteleosts, until now little is known of GH physiology in primitive fish.

Because GH is present and functionally constrained within a large number of taxa, comparison of GH sequences has been informative in a variety of phylogenetic analyses. This has been possible because the GH gene meets several criteria to be used in this kind of analysis including enough conservation of the sequence avoiding saturation; sufficient length; and minimal amount of homoplasy (Marins et al., 2003). Given the phylogenetic position of the gars relative to the chondrostean fish, the bowfin (*Amia calva*) and the teleosts, the characterization of GH from alligator gar, as in the case of *Lepisosteus osseus* (Rubin et al., 1996), should be useful for interpreting evolutionary trends in GH structure within the Neopterygii and among the Actinopterygii.

Within this context, we aimed our research at obtaining the coding sequence of GH and studying its expression through larval development of the alligator gar, a primitive fish native to North America.

## 2. Materials and methods

### 2.1. Biological material

Unfertilized and fertilized eggs, as well as larvae of alligator gar (*A. spatula*) collected at different days after hatching (DAH) were obtained from the Ecophysiology Group, Biological Sciences Faculty, UANL, Monterrey, Mexico. Collected specimens were stored in liquid nitrogen.

### 2.2. RNA extraction and RT-PCR

Total RNA was extracted by the Chomczynski and Sacchi method (1987) from a pool of three sample units (eggs or larvae). Extractions were carried out directly from the whole samples and after dissection from the larvae heads and headless bodies.

After verification of acceptable quality of RNA extractions, reverse transcription reactions were performed using 300 ng of RNA, random primers and Superscript-II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA first strands of rRNA and GH were specifically amplified by PCR using primer sets designed on conserved regions of both genes (Table 1). Selection of the oligonucleotides was based on alignment of the GH

Table 1
Primers used in this study

| Sense oligonucleotide | Antisense oligonucleotide | Amplified sequence |
|---|---|---|
| CT5: cac caa tgc act gat aag agc | CT3 aag tct cca ctt tga gca tgt c | GH cDNA from position 115 to 605 in *L. oseus* sequence (S82528) |
| 5r18: tcg aat gtc tgc cct atc aac | 3r18 gct gat ggc acc aga ctt g | 18S rRNA from position 286 to 562 in *L. oseus* sequence (AF188369) |

sequences reported for ancestral fish (*L. osseus*: GenBank:S82528 and bowfin, *Amia calva*: S73969) and of European eel *Anguilla anguilla* (AY148493). The same approach was followed to design the primers for 18S rRNA using sequences of longnose gar (*L. osseus*: AF188369), white sturgeon (*Acipenser transmontanus* AF188380), bowfin (*A. calva*: AF188368) and king snake eel (*Ophichthus rex*, X98843). Primers were positioned within conserved stretches of the genes (Table 1). PCR reactions were carried out in a total volume of 25 μL containing: 1 μL of the RT reaction, 0.2 mM of each dNTP, 0.5 μM of each primer, 1.5mM $MgCl_2$ and 1.25 U Taq polymerase (Promega, Madison, WI, USA). Initial denaturation was performed at 94 °C for 5 min and followed by 30 cycles using a model PTC-150 Minicycler (MJ Research, Watertown, MA, USA). Each subsequent cycle consisted of a 94 °C (1 min) melting step, a 52 °C (for GH) or 57 °C (rRNA) (1 min) annealing step, and a 72 °C (1 min) extension. A final extension of 5 min at 72 °C was performed. The products were analyzed by electrophoresis through 2% agarose gels after ethidium bromide staining.

### 2.3. GH cloning and sequences analysis

The amplified GH was cloned by the T-vector method (Harrison et al., 1994), using pBlueScript as a vector. Its nucleotide sequence was determined from three independent clones using the Li–Cor DNA system (LiCor, Lincoln, CA, USA). The rRNA sequence was determined directly from the purified PCR product. Alignments of nucleotide or amino acid sequences were done using the CLUSTAL W program and manually checked to achieve the best alignment. Phylogenetic analyses were carried out using the maximum parsimony (Protpars program) from the PHYLIP package (Felsenstein, 1993).

### 2.4. Semi-quantification of GH transcripts

The relative amounts of GH transcripts were determined by two methods: semi-quantitative RT-PCR and RNA nuclease protection assay (RNPA). RT-PCR assays for GH and rRNA were performed using the same protocol above described. The PCR conditions were adjusted to avoid saturating the amplification. Both PCR products were separated by electrophoresis through a 2% agarose gel. The relative intensity of the GH band *versus* that of RNA was determined by densitometry using the image analysis software of the Gel-Doc 1000 (Bio-Rad, Hercules, CA,. USA). Each value was obtained from three independent PCR experiments. The resulting data were subjected to a one way-ANOVA.

Additionally, GH and rRNA transcripts were also quantified by nuclease protection using MultiNPA (Ambion, Austin, TX, USA) conditions. Briefly, a GH probe (474 nt) and an rRNA probe (278 nt) were generated by asymmetric PCR using only the antisense corresponding primer and the GH clone or the rRNA amplified product, as template, respectively. During the PCR reaction, they were radioactively labeled using $^{33}$P–dATP as substrate, and gel purified after a 6% polyacrylamide–50% urea electrophoresis. After incubation of 5 μg of the RNA samples with both probes, non-protected fragments were digested with nuclease and the protected probes were resolved by electrophoresis in a 6% polyacrylamide–50% urea gel, according to the manufacturer's instructions. The relative amounts of GH transcripts versus rRNA were determined for each larval stage, by densitometry analysis of the autoradiography (using the same equipment as above described).

## 3. Results

### 3.1. Partial sequences of GH and rRNA from the alligator gar

Alignments of the GH coding sequences reported for ancestral fish (longnose gar, *L. osseus* and bowfin, *A. calva*) as well as European eel, allowed us to design a primer set upon conserved sequences located within the 2nd and 5th exons (Fig. 1). Once standardized, PCR allowed the amplification of the expected 491-pb fragment (Fig. 1A). The PCR product was enzymatically characterized and cloned using pBlueScript as vector before being sequenced.

The analysis of the fragment sequence confirmed that it corresponded to a GH gene, sharing 99% of nucleotide similarity with the GH previously reported for *L. osseus* (GenBank access number: S82528, Rubin et al., 1996). A

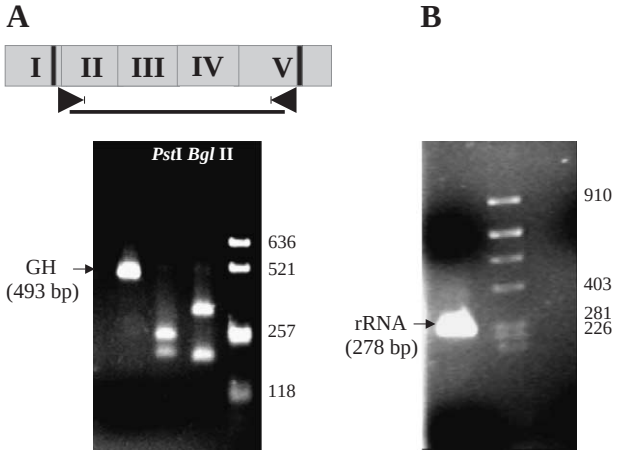


Fig. 1. Amplification of the GH cDNA and rRNA from the total RNA of the alligator gar. A—RT-PCR reactions were performed to amplify the GH coding sequence using a primer set designed on the second exon for the sense oligonucleotide and on the fifth exon for the antisense one. The dark bars indicate the position of the first and last codons within the coding sequence. The amplified product obtained was digested with *Bgl*II and *Pst*I and analyzed by electrophoresis in a 2% agarose gel. B—Amplification of the rRNA fragment of the alligator gar.

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A. spatula GH   CACCAATGCACTGATAAGAGCACAGCACCTCCACCAGCTTGCGGCTGATATTTACAAAGATTTTGAGCGCACCTATGTGCCAGAAGAG
L. osseus GH    CACCAATGCAGTGATAAGAGCACAGCACCTCCACCAGCTTGCGGCTGATATTTACAAAGATTTTGAGCGCACCTACGTGCCAGAAGAG
                                  A  Q  H  L  H  Q  L  A  A  D  I  Y  K  D  F  E  R  T  Y  V  P  E  E

CAGAGACAGTCAAGCAAAAGTTCTCCATCTGCCATATGCTATTCTGAGTCCATTCCTGCACCCACTGGCAAAGATGAAGCTCAGCAGAGATCTGATGTGGAGCT
CAGAGACAGTCAAGCAAAAGTTCTCCATCCGCCATATGCTATTCTGAGTCCATTCCTGCACCCACTGGCAAAGACGAAGCTCAGCAGAGATCTGATGTGGAGCT
 Q  R  Q  S  S  K  S  S  P  S  A  I  C  Y  S  E  S  I  P  A  P  T  G  K  D  E  A  Q  Q  R  S  D  V  E  L

GCTGAGATTCTCCCTGGCTCTCATCCAGTCCTGGATCAGCCCTCTGCAGACTCTGAGCCGGGTGTTCTCCAACAGCCTAGTCTTCGGCACTTCCGACAGGATCT
GCTGAGATTCTCCCTGGCTCTCATCCAGTCCTGGATCAGCCCTCTGCAGACTCTGAGCCGGGTGTTCTCCAACAGCCTAGTCTTTGGCACTTCCGACAGGATCT
  L  R  F  S  L  A  L  I  Q  S  W  I  S  P  L  Q  T  L  S  R  V  F  S  N  S  L  V  F  G  T  S  D  R  I

TTGAGAAGCTGCAGGACCTTGAGCGGGGGATCGTGACCCTCACAAGGGAAATTGATGAGGGAAGCCCCAGAATTGCAGCCTTCCTGACTCTCACGTATGAGAAG
TTGAGAAGCTGCAGGACCTTGAGCGAGGGATCGTGACCCTCACAAGGGAAATTGATGAGGGAAGCCCCAGAATTGCAGCCTTCCTGACTCTCACGTATGAGAAG
F  E  K  L  Q  D  L  E  R  G  I  V  T  L  T  R  E  I  D  E  G  S  P  R  I  A  A  F  L  T  L  T  Y  E  K

TTTGACACCAACCTGAGAAATGACGACGTTCTGATGAAGAACTACGGGCTTTTAGCTTGTTTCAAGAAAGACATGCTCAAAGTGGAGACTT
TTTGACACCAACCTGAGAAACGACGACGCTCTGATGAAGAACTACGGGCTTTTAGCTTGTTTCAAGAAAGACATGCACAAAGTGGAGACTT
 F  D  T  N  L  R  N  D  D  V  L  M  K  N  Y  G  L  L  A  C  F  K  K
                           A
```

Fig. 2. Lepisosteidae GH nucleotide sequences' alignment. The GH partial sequence obtained for alligator gar was compared with that previously reported for *L. osseus* (Genbank S82528). The nucleotide differences are set off, as well as the amino acid change. The primers' sequences are indicated in italics.

single amino acid change (Val/Ala) in the fifth exon was observed between the two lepisosteids (Fig. 2). The reduced residue changes observed between the GH of these two primitive fish, that diverged 180 million years ago, sustain the fact of a very slow evolution of the hormone within this fish family, in contrast with the burst of changes observed in the euteleosts (Fig. 3).

Using the same strategy, a second primer set was designed to amplify the alligator gar 18S rRNA (Fig. 1B). The oligonucleotides allow the amplification of a unique band of 278 bp. Once purified the PCR product, its nucleotide sequence was determined (Fig. 1B), showing no differences with the corresponding sequence reported for *L. osseus* (AF188369).

### *3.2. Expression of GH transcripts through larval development*

The GH transcripts were detected and semi-quantified from eggs to late larval stages by means of RT-PCR and RNPA. In both cases, a ribosomal RNA probe was used as internal control. The same pattern was obtained in both strategies, but as RT-PCR results were more reproducible, they were used for the analysis (Fig. 4). GH mRNA was detected in eggs as well as in all larval stages. High amounts of GH mRNA were found in fertilized and unfertilized eggs, whereas through the larval stages GH mRNA relative level was maintained constant showing its lowest expression at days 3 and 8. The GH expression was concentrated mainly in the heads of larvae since no GH transcripts were detected under the same conditions in the total RNA extracted from the rest of the body, although the rRNA was still detectable (data not shown).

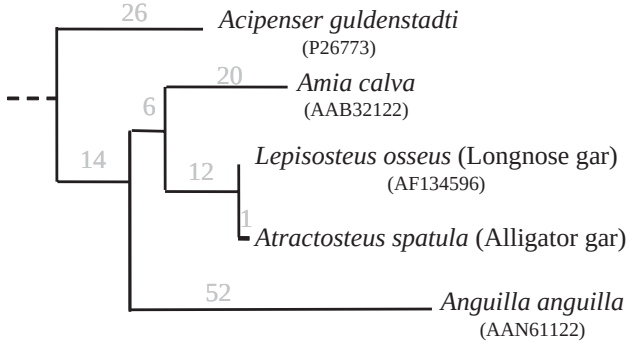


Fig. 3. Protein-based phylogenetic tree for primitive fish GH. The tree was constructed using a matrix based on protein sequences and the Fitch program. The numbers of amino acid substitutions are indicated on the branches.

## 4. Discussion

Despite the numerous GH coding sequences reported for teleosts, only a few nucleotide sequences for primitive fish are available in the GenBank, such as Amiiformes (*A. calva,* S73969), Semionotiformes (*L. osseus*,S82528) and agnathans (lamprey, AB081461). In this study, the GH partial coding sequence (GenBank accession number: AY738587) of another Semionotiform *A. spatula* was obtained, as well as part of its 18S rRNA sequence (GenBank accession number: AY738588). Few nucleotide substitutions were observed between the GH sequences of both Semionotiformes and none within the rRNA sequences obtained. Contrarily to the burst of changes that occurred within the GH gene in teleosts, the coding region within the gar has been highly conserved, although both species diverged 180 M years ago (Wiley, 1976).

Only one amino acid change (A to V) was found between the two Semiontiformes; curiously this substitution coincides with one of the three amino acid differences reported for the 2 isoforms (GenBank accession numbers: P26774 and P26773) of the sturgeon GH. Only one sequence could be obtained in this study for the alligator gar and slot–blot

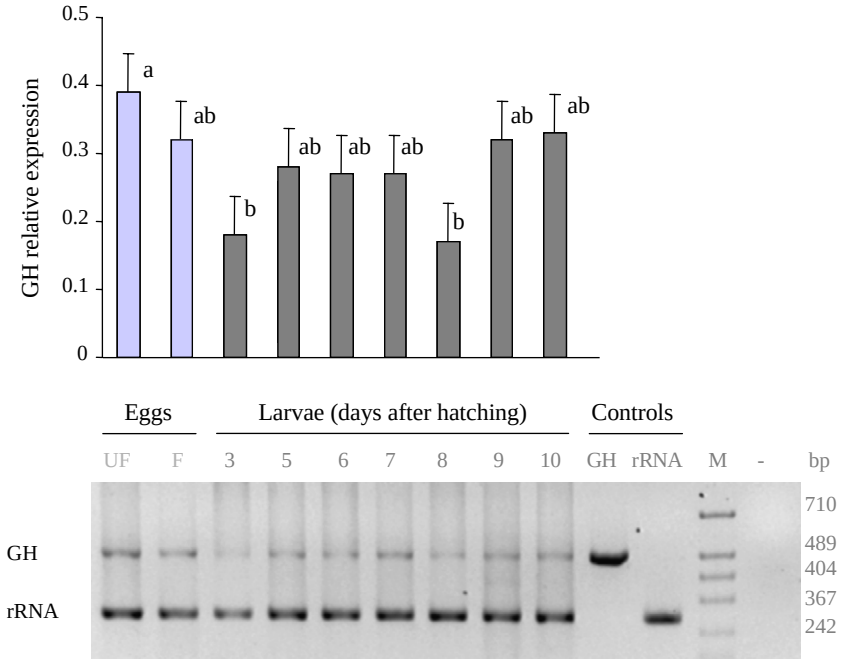


Fig. 4. Relative amounts of GH transcripts through larval development. Semi-quantitative RT-PCR assays were performed using RNA extracted from unfertilized eggs (UF), eggs after fertilization (F) and larvae at different days after hatching. Amplification of rRNA was used as internal control. The amplification products were analyzed by electrophoresis through 2% agarose gel. An example of the gel is shown in the bottom of the figure. The relative intensity of each GH band was determined by densitometry and the results of three independent assays are reported in the graph as well as the standard deviation. Significant differences between groups ($p < 0.05$) are indicated by letters.

hybridization assays suggest that its genome contains only one GH copy (data not shown); nevertheless, the existence of two GH genes for this species cannot be discarded.

The gar GH sequences present a higher amino acid similarity with that reported for *A. calva* (83%, GenBank: AAB32122.1) than with the sequences reported for other primitive fish such as sturgeon (78%, GenBank: P26773, P26774) and lamprey (27%, GenBank:BAC15763). It must be pointed out that the amino acid changes that affected specifically both gar GH sequences are located within the third helix (H3) and the loop H3–H4, which are important for the hormone–receptor interactions (Cunningham and Wells, 1993).

GH mRNA was detected in alligator gar eggs, even before fertilization, indicating their presence in the maternal gametes. Consequently, expression of GH in oocytes seems to occur in a wide range of vertebrates, since it has also been previously reported for the rainbow trout, an euteleost, as well as for mammals (Bevers and Izadyar, 2002). Furthermore, GH mRNA was detected all along the larvae stages by RT-PCR and hybridization experiments. Although GH mRNA was in lower concentration than rRNA, the results of the semi-quantification showed that the relative expression of the GH in relation to the rRNA was maintained.

These results corroborate those observed in trout, although larval development differs between the two species. Indeed, in the case of alligator gar, hatching occurs only two days after fecundation of the eggs and the larvae grow using their yolk reserves until 5 DAH when they begin to feed, although yolk reserves are still present until 8 DAH. Development during this first week is characterized by organogenesis and growth at a rate of 1 mm/day. After 10 DAH, when metamorphosis is completed, the larvae reach their highest growth rate at 5–6 mm/day (Mendoza et al., 2002b). The relative expression of GH through larval stages indicates the existence of substantial expression of this hormone in unfertilized eggs, declining thereafter until 3 DAH, then increasing around 5–7 DAH larvae, corresponding to the organogenesis. In 8 DAH larvae the expression diminishes again, this is consistent with the time when yolk reserves are depleted and with the onset of exogenous feeding. Lastly, the expression increases in 9–10 DAH larvae, probably corresponding to the formation of pituitary gland. At present, it is not possible to attribute this amazing growth rate only to the GH–GH receptor axis, since thyroid hormones (T3) have also been evidenced in the early stages with an increasing concentration until day 10 (Mendoza et al., 2001).

Although there is no clear evidence that GH expression is limited to the anterior pituitary gland, no PCR product could be obtained from RNA extracted from the headless body. Further experiments of in situ hybridization may allow corroborating GH expression specificity. Additionally, further histological studies will be necessary to detail the still unknown pituitary gland ontogeny for this species.

The fact that GH may act as an autocrine–paracrine factor during early embryogenesis of bovines (Bevers and

Izadyar, 2002), and its presence in the early embryogenesis of the alligator gar as well in other fish species suggests that this function may have appeared early during vertebrates evolution and that GH may play an important role during early embryogenesis in fish. Cloning of GH and the elucidation of its role during larval stages will certainly contribute to the better understanding of alligator gar larval physiology and will facilitate the culture of larvae and juvenile gar in order to repopulate their natural environment. On the other hand, gene technology provides the means for a commercial supply of the recombinant GH in large quantities (Moriyama and Kawauchi, 1990), which could be used to improve growth in a close related species with an important aquaculture value, such as the tropical gar (*Atractosteus tropicus*).

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