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Sex-Related Gene Expression and Morphological Changes During Early Ontogeny in Tropical Gar (Atractosteus tropicus, Lepisosteiformes: Lepisosteidae)

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Abstract

The expression levels of the genes *foxl2, sox9*, *cyp17a1*, *dmrt1*, and *cyp19a1* related to sexual determination and differentiation were determined during initial ontogenetic development in the tropical gar (*Atractosteus tropicus*). Samples were taken from the organisms from 5 to 30 days post-hatching (DAH), and gene expression were performed using qPCR. Gonadal histology allowed for a morphological understanding of the developmental changes and appearance of the gonads and their relationship to the expression of sex genes.The results show that during days 5 to 16 DAH of larval development, no differentiated gonads were observed, and no changes were detected in the expression profile of the analyzed genes. However, from 21 DAH (2.6 cm TL), the presence of previtellogenetic ovary was observed, and but no gonads with testicular arrangements were detected (undifferentiated gonads). This is consistent with the results of genomic expression, where *foxl2* and *cyp19a1* were overexpressed at 21 and 27 DAH, allowing for ovarian development. Meanwhile, the low expression levels of *dmrt1*, *sox9*, and *cyp17a1* may be related to differentiation into testes, although these may develop later (sizes) than females. This study allows for validate gene expression profiles as sex markers in early ontogeny for use in developing future technologies for early organism selection.

Introduction

The reproductive success of a species depends mainly on the proper development of cellular systems during early ontogeny (Tang et al., 2022). In this regard, the formation of gonads during sex determination (SD) depends on the action of genetic, endocrine, morphological, physiological, social, and environmental factors (Forconi et al., 2013; Sharma et al., 2022). In fish, this process begins with zygote formation, followed by the appearance of the germinal primordium. Subsequently followed by sexual differentiation (SDf), which is responsible for the appearance of secondary sexual characteristics from the ventromedial mesonephric tissue and the arrangement of the germinal compartment, which consists of spermatogonia (esp) or oogonia (oo) and the somatic compartment where Sertoli cells (cs) and Leydig cells (cly), blood vessels, and fibroblasts are found, which serve as support and nutrition structures for germ cells and sexual ducts (Forconi et al., 2013; Leet et al., 2011; Nagahama et al., 2021). SD is activated by chromosomal (CSD), genetic (GSD), environmental (ESD) mechanisms or a mixture between ESD and GSD (Nagahama et al., 2021) and begins with the action of one or more master genes, which are activated depending on a particular environment, establishing morphological and physiological changes in reproductive structures (Forconi et al., 2013; Sharma et al., 2022; Shoemaker 2007; Tang et al., 2022). In fish without sex chromosomes, SD points to a system of autosomal genes, which function in sequential steps known as genetic pathways, which interact with metabolic networks to control the functioning of the systems through feedback of positive or negative stimuli between genes (Berbejillo et al., 2012; Forconi et al., 2013; Mendoza-Pacheco et al., 2018; Tomás-Cobos et al., 2004).

Several genes have been identified that participate in sex determination. For example, *dmrt1* is a dominant factor in the development of testicular follicular cells, acts as a switch for sexual dimorphism, and regulates spermatogenesis (Guan et al., 2000). The *foxl2* gene is an important transcription factor

involved in ovarian formation by controlling estrogen production; it also acts as an antagonist of *dmrt1* during the development of the germinal epithelium (Bertho et al., 2016; Matson et al., 2012; Yamaguchi et al., 2007). The *sox9* gene is a transcription factor that plays crucial roles in fish physiology, primarily in craniofacial, skeletal, and neuronal development, as well as in the maintenance of stem, pre-Sertoli, and somatic cell phenotypes (Navarro-Martín et al., 2012; Sharma et al., 2022; Lee et al., 2011). On the other hand, the *cyp17a1* gene is responsible for steroidogenic synthesis in the interrenal tissue (the fish equivalent of adrenal glands) and Leydig cells, where it converts progestogens into androgens; this gene is also involved in brain sex differentiation (Shu et al., 2020; Uno et al., 2012; Zuber et al., 1986). Finally, the aromatase gene family (*cyp19a1*) synthesizes enzymes that catalyze the aromatization of androgens into estrogens during neurogenesis, thereby regulating growth, sex change, and reproduction (Karube et al., 2007).

In general, the expression pattern of these genes determines sex through the promotion or suppression of somatic cells, the establishment of primordial germ cells (PGCs), and the arrangement of sexual ducts (Barsoum *et al*., 2010; Leet et al., 2011; Shoemaker et al., 2007; Sun et al., 2013). However, studying these molecular mechanisms is complicated because not all genes can be sex regulators and their functions vary between species. For example, for the *sox9* gene is associated with testicular development in teleosts for in Nile tilapia (*Oreochromis niloticus*) lacks this function which is performed by *tdmrt1*, the latter is identified as a biomarker of masculinization during the formation of germ cells and intratesticular efferent ducts (Kobayashi et al., 2008). Numerous studies have identified sex-related genes in commercially important fish species (e.g., Berbejillo et al., 2012; [add other refs]); however, in ancestral fish species such as the tropical gar (*Atractosteus tropicus*), this information remains incomplete. In tropical gar, the expression of *fox9, soxl2, cyp19a1, cyp17a1* and *dmrt1* in various organs of adult females and males of *A. tropicus* has been studied (Méndez-Marín *et al.*, 2025). It is necessary to indicate that *A. tropicus* is a species of the Actinopterygii group with high cultural and ecological value in southeastern Mexico. Despite its importance, their populations are at risk due to overfishing and loss of their natural habitat, which has decreased their breeding and spawning sites (Aguilera et al., 2002; Mendoza-Alfaro et al., 2008; Márquez-Couturier *et al.*, 2015), resulting in a decrease in catch volumes and the possible loss of their genetic richness (Barrientos-Villalobos *et al.*, 2008). For this reason, it is necessary to characterize the functional genes in reproductive morphophysiology that allow generating biotechnological proposals for improvements in fish management in captivity and wild conditions.

Present study was aimed to characterize the expression pathway of the reproductive genes *dmrt1, foxl2, sox9, cyp17a1*, and *cyp19a1* that control the sexual phenotype and the appearance of reproductive morphological characters during initial ontogeny in *A. tropicus.* We hypothesized that female associated genes (*foxl2* and *cyp19a1*) would be overexpressed prior to ovarian differentiation, while male-associated genes (*dmrt1, sox9, cyp17a1*) would show delayed expression corresponding to later testicular development. From this research, bases will be generated for the management of the species that will contribute to sustainable use through the enhancement of cultivation to benefit the economic resources of southeastern Mexico.

Materials and methods

2.1 Fish Reproduction and Sampling

A batch of *A. tropicus* broodstock was selected from the breeding area of the Aquatic Resources Physiology Laboratory (LAFIRA) of the Academic Division of Biological Sciences (DACBiol) at the Universidad Juárez Autónoma de Tabasco (UJAT). Sexually mature females with average weight of 3.5 kg and minimum size of 36 cm standard length, were induced into reproduction by injection into the pelvic fin with the hormone aLHRH (Ovaprim®, 0.2 mL/kg) according to the proposal of Hernández (2002), it was placed in a 1500 L circular tank, where artificial grass had been previously placed as a substrate for the fertilized eggs (17 hours post-injection). Once spawning was achieved, the breeders were removed and a 100% water exchange was performed using dechlorinated water in a closed circulation system until the eleutherium embryos hatched (72 hours after injection, at 28°C). Once the larvae had absorbed the yolk (3 days after hatching, DAH), 900 larvae were pooled and haphazardly assigned, into three 70-L circular tubs with a temperature of 29°C ± 0.5, pH 7.3 ± 0.5 and a dissolved oxygen concentration of 5.5 ± 0.2 mg/L, which were monitored with a potentiometer (HANNA, HI 991001, Romania) and an oximeter (YSI 85, USA), respectively. The feeding scheme was based according to Frías-Quintana *et al*. (2010) and Márquez *et al*. (2004), the first 17 DAH feedings were to apparent satiation for larval culture, supplying *Artemia nauplii* (10–25 ind / mL). The day 18 DAH, the larvae were fed frozen commercial adult Artemia, which was subsequently replaced with 1.5 and 2.5 mm floating extruded feed, four times daily (8, 11, 15, and 20 hours) from Pedregal Silver Cup®, consisting of 45% protein and 15% lipids. Eight larvae were collected from 5 to 55 days post-hatching (DAH) to determine total length (TL) and total weight (TW). To identify the day of first expression of sexual genes, five larvae were collected per tub and three for histological analysis.

They were sacrificed with an overdose of clove oil (0.1 mL/L) and subsequently fixed in Davidson's solution for histological analysis and RNA-later in ultra-freezing at -80°C for genomic expression analysis. Sampling was carried out using the protocol established by the Ministry of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA NOM-062-Z00-1999, 2001). This research was approved by the Ethics Research Committee of the Universidad Juárez Autónoma de Tabasco under code UJAT-CIEI-2025-003.

2.2 Histological processing

270 larvae were collected throughout the experiment and fixed in Davidson solution. Larvae fixed in Davidson’s solution were dehydrated in increasing solutions of 50, 70, 80, 96, and 100% ethyl alcohol, cleared in 100% OH-xylene (1:1 solution), and xylene, paraffin-xylene (1:1 solution), individual larvae embedded in two changes of paraffin (1 and 2). Serial longitudinal sections of 7 µm were made with a microtome and stained with hematoxylin-eosin (Aguilar, 1996; Estrada *et al*., 1996; Humason *et al.*, 1979; Hinton, 1990). For morphological descriptions, a Zeiss microscope (Axiostar Plus), a Zeiss digital

imaging camera (Axiocam MRc 5), and Zen 2.3 Blue edition software were used for morphometric measurements.

Reproductive morphological changes in larvae were characterized based on Nakamura's (Nakamura et al. 1998) method, which identified germ cell proliferation (PGCs, spa, and oo), somatically differentiating cells (SScs and SLcs), ovarian cavity formation, measurement of gonad length (GL) and oocyte diameter (OD), establishment of the stages of sexual differentiation about days, and changes in gonadal development.

2.3 RNA extraction, cDNA synthesis, and gene expression analysis

A total of 162 whole larvae were suspended in Trizol (Invitrogen) for 48 hrs with renewal of the solution every 24 hrs. Total RNA from each larva was obtained according to the protocol suggested by Invitrogen, Waltham, MA, with a modification to the tissue processing consisting of two chloroform baths with two vortex agitations (Gusto, Heathrow Scientific R LLC. After this, the concentration and purity were evaluated by the absorbance ratio at 260 and 280 nm in a spectrophotometer (Jenway GenovaNano, Cole-Parmer, Staffordshire, United Kingdom). In a thermocycler (Mastercycle nexus GSX1, Eppendorf, Hamburg, Germany), one microgram of RNA was transcribed to cDNA using a reverse transcription kit with a volume of 20 µL (Maxima First Strand cDNA Synthesis Kit for RT-qPCR, ThermoScientific, Waltham, MA). The genes forkhead box protein L2 (*foxl2*), SRY-Box transcriber factor 9 (*sox9*), cytochrome P450 family 17 subfamily A member 1 (*cyp17a1*), doublesex and mab-3 related transcription factor (*dmrt1*) and cytochrome P450 family 19 subfamily A member 1 (*cyp19a1*) were selected, the design of specific oligonucleotides was from the transcriptome of *A. tropicus* (NCBI: PRJNA395289) National Center of Biotechnology Information (https://www.ncbi.nim.nih.gov/tools/primer-blast/) (Martínez-Burguete et al., 2021; Jiménez et al., 2021) (Table 1). The qPCR reactions were performed with 10 µL of Eva Green supermix (BioRad, Hercules, CA), 9 µL of cDNA (5 ng µL-1), and 1 µL of primer mix (3mM) in a 20 µL volume, with negative control by replacing the template cDNA with sterile water. Primer efficiency curves were generated with five dilutions (100 to 0.1 ng of DNA), using beta-actin (β-actin) as a reference gene. A CFX96TM real-time thermocycler (BioRad, Hercules, CA) was used with a denaturation cycle of 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. Gene expression was calculated using the -ΔΔCt method (Livak *et al.*, 2001).

Table 1 Sequence expression of primers *dmrt1, foxl2, sox9, cyp17a, cyp19a1, y β-actin* identified by qPCR en *A. tropicus.* The design of specific oligonucleotides was from the transcriptome of *A. tropicus* (NCBI: PRJNA395289).

Aim gene	Primer sequence (5´- 3´)	Reference
Foxl2	FW: AAC CTG AGC CTC AAC GAG TG RV: ACT GCA AGT ACT TCG GTG GG	NCBI: PRJNA395289
Cyp17a1	FW: AGC TAC AAC GAT GGC ACA CA RV: TGT TGT TCT CTG AGC TGC GT	NCBI: PRJNA395289
Sox9	FW: AGT GTC TTT CTG ATG CCC CG RV: ACG GGG AAT TTG TCG TCC TC	NCBI: PRJNA395289
Cyp19a1	FW: GTT ACT CGG CAG CTA CGT GA RV: TGA AGT GCG TTG CCT ACG AT	NCBI: PRJNA395289
Dmrt1	FW: CAG CAG GCC ATT ACCTCA GT RV: TGT CCA AAG AGG GGT TGCAG	NCBI: PRJNA395289
β-actin	FW: GAGCTATGAGCTGCCTGAGTGG RV: GTGGTCTCATGAATGCCACAGG	Jiménez-Martínez *et al.* [23]

2.4 Statistical Analysis

For comparative analysis of the relative expression of transcripts for each gene, data were analyzed using the normality test (Kolmogorov-Smirnov) and homogeneity of variance (Levine) tests. Changes in expression throughout ontogeny were compared using a one-way analysis of variance (ANOVA) and Tukey's post hoc test. A linear growth model was applied to analyze Pt vs. DAH and Lt vs. DHA. Statistical analyses were performed using STATGRAPHICS Centurion 16.1.03 software with a significance level (P) at 0.05 and SigmaPlot v 10.0.

Results

3.1 Growth assessment in A. tropicus

A total of 400 larvae were evaluated over 50 days to determine the association between total weight (TW) and days of development (DAH) and the linear model (R2 = 0.8085, P = 0.05) (Fig. 1A) explained it; the individual total length of LW and DAH was also described by the linear model (R2 = 0.8963, P = 0.05) (Fig. 1B).

3.2 Histological characterization

Analysis of morphological changes in larval gonadal structures revealed synchronous development, characterized by identical oocyte developmental features across all individuals examined (Figs. 2, 3). Accordingly, two distinct stages of sexual differentiation were identified between 5 and 55 DAH.

Table 2 Morphometric data during the development of *A. tropicus* larvae: days post hatching (DAH), gonad length (Ltg), oocyte diameter (Do), nucleus diameter (Dn).

	DAH	Ltg	Do	Dn
Undifferentiated	9	66.625	4.920	2.977
	10	49.056	4.742	3.264
	13	74.742	4.906	3.414
	15	48.761	4.757	2.028
	16	84.224	4.718	2.219
	21	87.907	4.154	2.512
	27	81.340	5.485	2.729
	30	80.100	5.959	3.198
	X ± SD	71.594±15.384	4.955±0.544	2.792± 0.507
Differentiated	21	174.844	6.365	3.039
	27	217.600	6.172	3.938
	30	213.557	6.437	3.105
	X ± SD	202±23.60	6.32±0.14	3.36± 0.50

Stage 1 (S1). Undifferentiated gonad: The gonadal primordium is developing (Ltg 71.594µm ± 15.384) and is composed of PGCs in mitosis and pre–Sertoli cells; it is located to the right and left of the dorsal wall of the coelomic cavity. The PGCs are spherical with average diameter ($\bar{x}$) 4.955 µm ± 0.544 with acidophilic cytoplasm, a large nucleus (2.792 µm ± 0.507), and a slightly basophilic nucleolus (Fig. 2a-e; Table 2). The somatic cell arrangement does not show morphological characteristics that allow sex differentiation.

Stage 2 (S2). Differentiated gonad in females: The gonadal primordia increase in length (Ltg = 202 µm ± 23.60), the right one close to the kidney and the left one in a cephalic direction. The PGCs grow to form oogonia in the early stages ($\bar{x}$ = 6.32 µm ± 0.14) with acidophilic cytoplasm and basophilic nucleoli, and the somatic cells with acidophilic cytoplasm ($\bar{x}$ = 3.36 ± 0.50); the primary indicator of ovarian formation is the presence of previtellogenic oocytes with slightly basophilic cytoplasm and nucleoli. Follicular cells, connective tissue, fibroblasts, and small capillaries are observed around them (Fig. 3a-f). Based on the increase in gonadal length (Lg) and oocyte diameter (Do) values, and the morphological pattern of sexual differentiation, differentiated female gonads (S2) were observed on day 21 DAH, since showed follicles with previtellogenic characteristics, suggesting that the period of sexual differentiation begins in day 21 DAH. However, undifferentiated gonads were observed, suggesting that these individuals were males that had not yet exhibited morphological changes, possibly representing undifferentiated testes.

3.2 Expression of sex genes in A. tropicus during its early ontogeny.

The expression of the sex genes was evaluated in parallel with the morphological sexual differentiation of *A. tropicus* larvae, based in the morphological pattern of sexual differentiation (21 DAH) the range of days (5, 10, 16, 21, 27 and 30 DAH) was es2lished to identify the master gene (*dmrt1, foxl2, sox9, cyp17a1*, and *cyp19a1)* that control the establishment of sex. The marked activity of all transcripts was observed during the increase in oocyte diameter, determining sex and stimulating the differentiation of secondary sex characteristics and their transformation into juveniles (Fig. 4A–E; Table 2).

The expression levels of the genes *dmrt1, sox9* and *cyp17a1*, related to male determination, show low levels of gene expression during days 5 to 15 DAH compared to the expression from 21 to 27 DAH where there is an increase (Fig. 4A, C, D); however, a reduction in their expression is observed on day 30 DAH. Besides, the genes responsible for ovarian formation, *foxl2* and *cyp19a1*, show high levels relative to genes testes determination, with a significant increase in their expression throughout the sampling period from 5 to 30 DAH (Fig. 4B, E).

The increase in the expression of the *cyp19a1* gene, responsible for regulating aromatase synthesis, is notable, as is the rise in *foxl2*, which regulates estrogen synthesis and ovarian formation. In general, low expression values of all genes were obtained from days 5 to 15, with an increase starting from day 21 Suggesting that the bipotential period is estimated to be between 5 and 15 DAH. After that day, the increase in *cyp19a1* is related to the formation of the ovarian genetic sex. This expression pattern suggests the larvae's feminization and the ovaries' formation.

Discussion

Present study characterized the expression of five sex-related genes (dmrt1, sox9, cyp17a1, foxl2, and cyp19a1) during early ontogeny of *A. tropicus*. The key finding was sustained upregulation of foxl2 and cyp19a1 from 21 to 30 DAH, coinciding with the appearance of previtellogenic oocytes and ovarian differentiation. No histological evidence of testicular differentiation was observed during this period, suggesting that ovarian development precedes male differentiation in this species, similar to pattern observed in some other teleosts (e.g., *Oreochromis niloticus*; Tao et al., 2013). Research on the relative expression of genes related to sexual differentiation processes in fish can explain the functioning and evolution of sexual systems, as pointed out by Symonová et al. (2017) and Amores et al. (2011). These auhtorscharacterized ancestral genes of the genera *Atractosteus* and *Lepisosteus* through comparing them with those of tetrapods. indicating that they share a common origin. Additionally, they showed some differences with other teleosts. Based on this, the identification of the *dmrt1, foxl2, Sox9, cyp17a1*, and *cyp19a1* genes during early ontogeny in *A. tropicus* suggests that they are conserved genes compared to other species and have the same functions as cytochrome P450 aromatases, which is a family of genes crucial for reproductive activity and whose different isoforms can be explained by the duplication of their genomes during evolution in many teleost fish species (Chen *et al.*, 2020; Di Nardo et al., 2021; Ferguson-Smith 2006). Likewise, knowledge of sex genes allows for the development of

biotechnological protocols for properly utilizing species. This is the case with *Acipenser baerii*, a species of high economic value due to caviar production. Identifying the *dmrt1, sox9, cyp17a1*, and star genes has improved the selection of organisms at appropriate ages to increase egg production (Berbejillo et al., 2012). Similarly, genes such as *dmrt1, sox9, cyp17a1, foxl2*, and *cyp19a1* have been found in several fish species and are related to sex determination and differentiation (Tang et al., 2022).

Present research found that during the development of *A. tropicus* larvae, elevated cellular activity is regulated by a gene pathway that controls morphophysiological changes in the gonad. In our study with *A. tropicus*, as the diameter of the oocytes increases, the expression of the evaluated genes increases, observing an expression pattern in the sex genes that are related to changes in gonadal morphology during the initial ontogeny, where at 21 DAH the activity of several genes are associated with the formation and regulation of Sertoli cells (dmrt1, sox9), Leydig cells (foxl2, cyp17a1), sex ducts (dmrt1) and the cyp19a1 isoform of the aromatase gene was observed, stimulating the development of secondary sexual characteristics (Ferguson-Smith et al., 2006; Nagahama et al., 2021).

In general, the changes in somatic and sexual cells allow us to indicate that the period of sexual differentiation begins between 21 and 27 DAH, since there is an increase in the overexpression of *cyp19a1a* and *foxl2*, which can cause the differentiation of the bipotential gonad into ovary. Our results agree with those found in *Monopterus albus* and *Oreochromis niloticus* where the *cyp19a1a* and *cyp19a1b* genes have an essential role in sex determination due to the control of estrogen by androgen (He et al., 2014; Tao et al., 2013). This aspect has been demonstrated in *Dicentrarchus labrax*, where hypermethylation of *cyp19a1* allows the control of ovarian differentiation (Enríquez-Valencia et al., 2022; Piferrer *et al.*, 2012). Likewise, in *Micropterus salmoides*, when 17-β-estradiol is incorporated into the diet, it has been shown that the expression of *cyp19a1b* causes sexual differentiation of ovaries and the development of the nervous system (Zhang et al., 2024).

In Nile tilapia (*Oreochromis niloticus*), the expression of *cyp19a1* and *cyp19a1b* in the gonad and brain plays a role in estrogen synthesis (Leet et al., 2011; Ljiri et al., 2008). In *A. tropicus*, *cyp19a1* is expressed early, so its role in the formation and development of the hypothalamus-pituitary-gonadal axis is highly relevant. This is related to the expression of *foxl2*, which is a transcriptional regulatory factor for aromatase family genes, allowing the formation of somatic cells in differentiated gonads during early ontogeny (Ljiri et al., 2008; Fan et al., 2014; Purcell et al., 2018). On the other hand, the under-expression of *dmrt1, sox9*, and *cyp17a1* in *A. tropicus* larvae may be related to delayed development of testes compared to the ovary, which depends on a feedback loop between these genes and the Sertoli and Leydig cells that achieve the production of androgens and consequently, the differentiation towards males (Leet et al., 2011). This aspect has been detected in other teleosts such as *Takifugu bimaculatus*, where through the culture of germ and somatic cells of testes, the regulatory mechanism of *dmrt1* was identified, where the knockdown of this gene decreases the expression of testes-related genes *amh, sox9*, and *cyp11a* and increases the expression of the female genes *foxl2, sox3*, and *cyp19a* (Zhong et al., 2022).

Similarly, *sox9* is present in Leydig cells in fish; its absence leads to the formation of ovaries due to increased androgen (cyp) levels. Thus, Chiang et al. (2001) point out that in the zebrafish (*D. rerio*), *sox9* orthologs with differentiated expression patterns have been identified, such as *sox9a*, which has been detected in the testes, and sox9b in the ovary. Likewise, *sox9* is a dysmorphic gene that participates in various metabolic pathways, so its expression level affects the development of reproductive ducts (Bertho et al., 2016; Karube et al., 2007; Matson et al., 2011; Nagahama et al., 2021). In general, there are essential differences between the different groups of vertebrates, where the variation in the expression of sexual genes does not seem to be the same for the development of the reproductive organs and which occurs during embryogenic development, where *foxl2* is determinant for males while *dmrt1* and *sox9* are determinant for females (Chang et al., 2005; Ljiri et al., 2008; Matson *et al.*, 2012; Navarro-Martín et al., 2012; Tanaka *et al*. 2016; Uno et al., 2012).

It should be noted that during the development of this research, the growth correlation models for weight and height obtained differed from those presented by Frías-Quintana et al., 2015, who worked with the ontogeny of the digestive tract in the same species. This difference may be explained by the different conditions present during larviculture, which may cause modifications during sexual determination and differentiation due to changes in DNA methylation levels related to the development of the gonadal phenotype. However, this suggests the development of new research using an epigenetic approach. Furthermore, growth differences are not central to the sex differenciatio findings of present study.

In *Atractosteus tropicus*, female sexual differentiation occurs before male differentiation, driven by the overexpression of *foxl2* and *cyp19a1* from 21 days post-hatching. The sex determination genes are conserved relative to other vertebrates. These results enable early sex identification in captive populations, supporting reproductive management and conservation efforts for this threatened species. Future studies should explore the role of environmental factors and epigenetics in sex determination. The findings allow for the establishment of early sex selection protocols in captive populations. By identifying the expression patterns of female-associated genes, it is possible to select organisms for reproductive improvement and sustainable management of the species. This is particularly valuable for *A. tropicus*, a species of cultural and ecological importance in southeastern Mexico that is threatened by overfishing and habitat loss. The study highlights the need for further research using epigenetic approaches (e.g., DNA methylation analysis) to understand how environmental factors may influence sexual determination and differentiation in this species (Pandian *et al*., 2003). Differences in growth patterns compared to previous studies suggest that larviculture conditions could modify DNA methylation levels and affect gonadal phenotype development. In conclusion, Future research should investigate: (1) the epigenetic regulation of *cyp19a1* via promoter methylation; (2) the effects of temperature or other environmental factors on sex ratios; and (3) functional validation of candidate genes using knockdown or knockout approaches.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Author Contributions

Conceptualization, O.M.-M., M.d.L.J.-B. and C.A.A.-G.; methodology, G.G.A.-A., L.D.J.-M., C.S.Á.-V., G.M.P.-J. and V.E.A.-F.; formal analysis, G.G.A.-A., R.M.-G., G.M.P.-J. and L.D.J.-M.; investigation, O.M.-M., M.d.L.J.-B., G.G.-C. and C.A.A.-G.; resources, R.M.-G. and C.A.A.-G.; writing—original draft preparation, O.M.-M., G.G.A.-A., G.G.-C., C.A.A.-G., L.D.J.-M., C.S.Á.-V., U.R.-E., G.M.P.-J. and G.H.P; writing—review and editing, O.M.-M., G.G.A.-A., R.M.-G., V.E.A.-F., U.R.-E. and C.A.A.-G.; visualization, O.M.-M., G.G.A.-A., R.M.-G., U.R.-E., G.M.P.-J., G.H.P., and C.A.A.-G.; super-vision, M.d.L.J.-B. and C.A.A.-G.; project administration, R.M.-G. and C.A.A.-G.; funding acquisition, R.M.-G. and C.A.A.-G. All authors have read and agreed to the published version of the manuscript.

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Author Contribution

Otilio Méndez-Marin, María de Lourdes Jimenéz-Badillo, Carlos Alfonso Álvarez-González, Gabriela Galindo-Cortés, Virgilio Eugenio Arenas-Fuentes, wrote the main manuscript text .Carina Shianya Álvarez-Villagomez , Luis Daniel Jiménez-Martínez, Uriel Rodriguez-Estrada, César Antonio Sepúlveda-Quiroz, Graciela María Pérez-Jiménez, genomic analysis.Guadalupe Hernández Piedra, Rafael Martínez-García, Gloria Gertrudys Asencio-Alcudia, prepared figures and tables.All authors reviewed the manuscript

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information. The design of specific oligonucleotides are provided in Supplementary Table 1, along with original reference describing the specific oligonucleotides used in this study.

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Figures

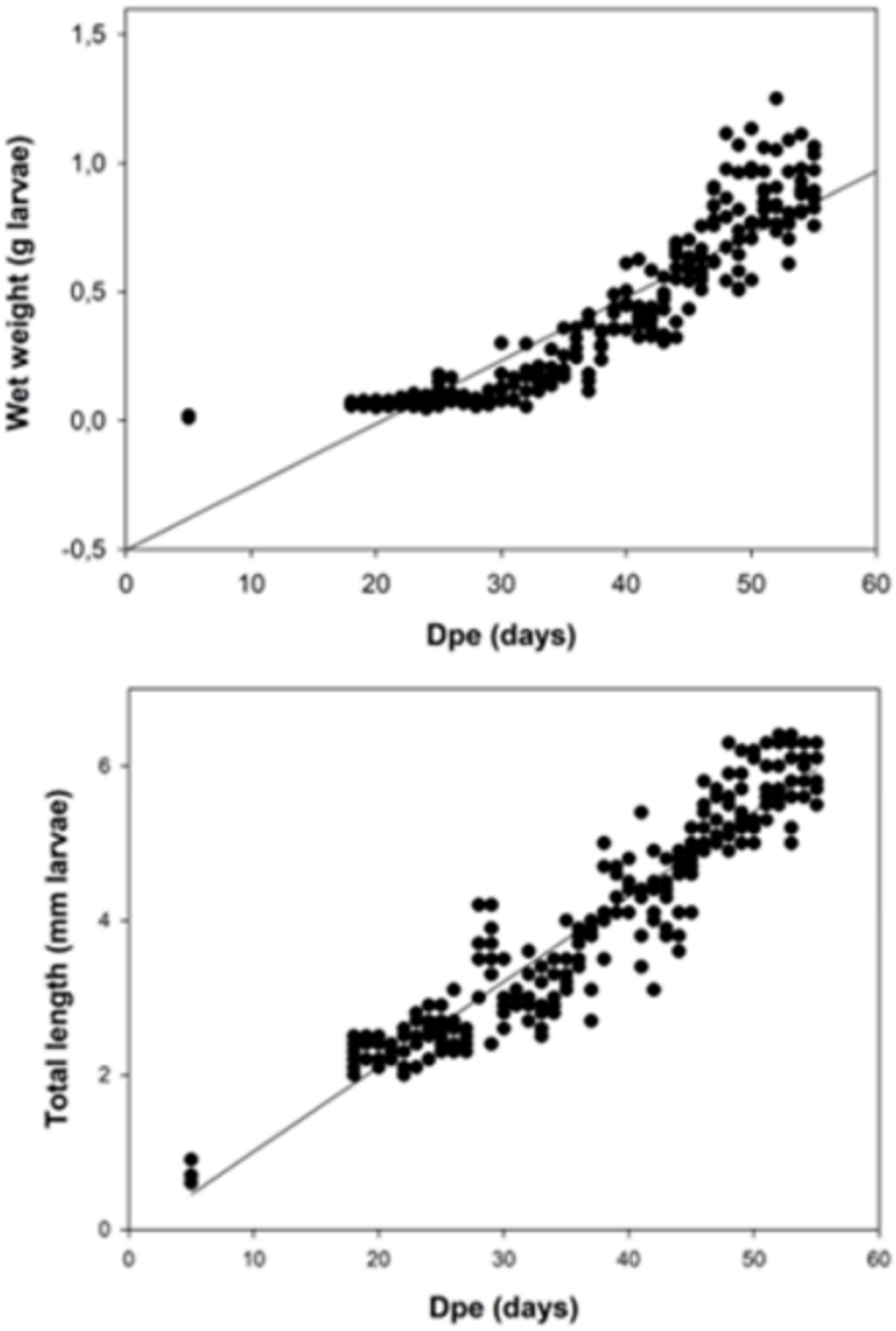


Figure 1

A)Relationship between total weight (TW) and post-hatch day (PHD); B) Total length (TL) and post-hatch day (PHD) of *A. tropicus* from day 5 to 55 of development.

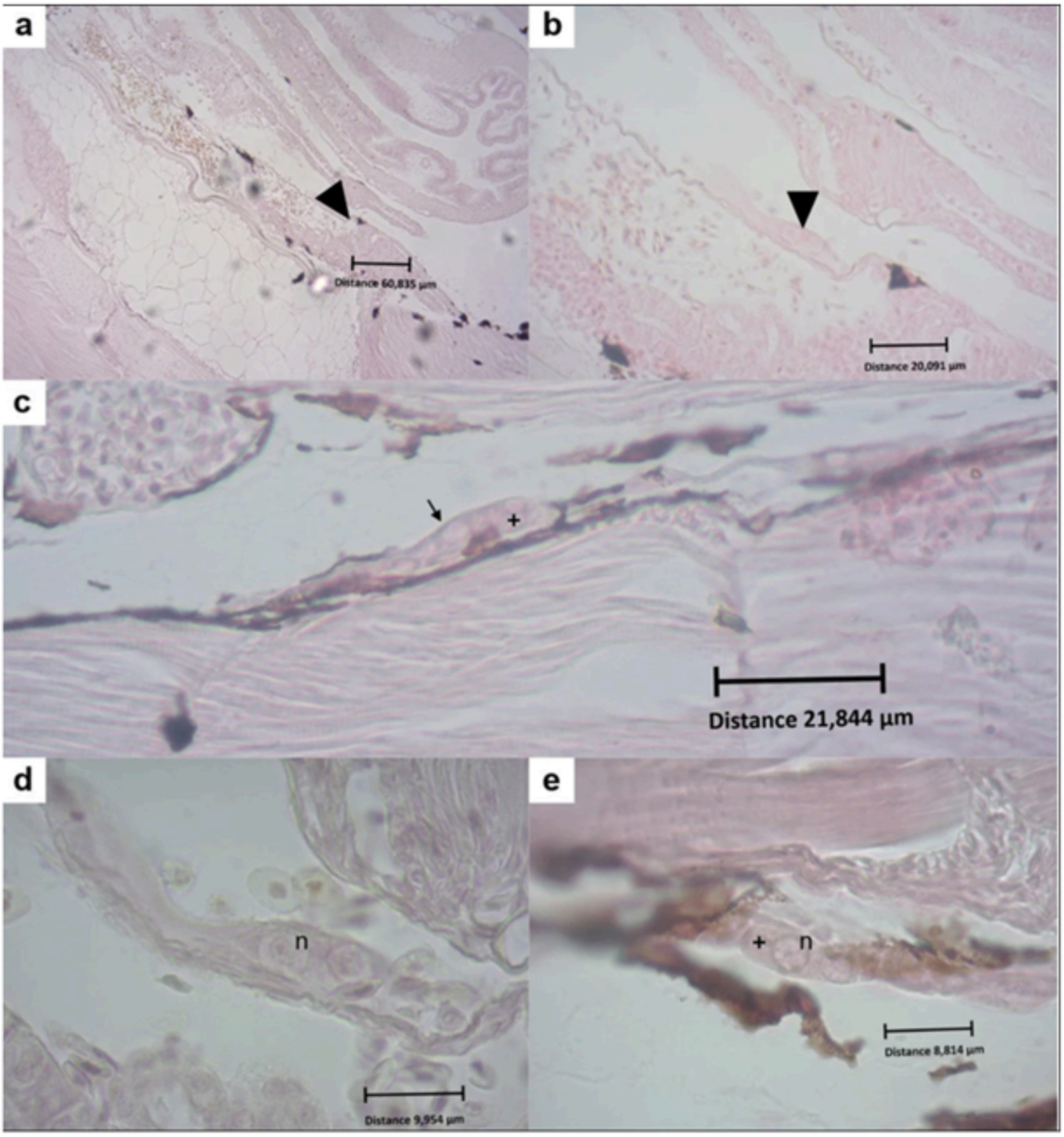


Figure 2

Morphology of the gonadal primordium in *A. tropicus* larvae. Stage 1 (S1) undifferentiated gonada. Hematoxylin and eosin (H-E). a) elongated gonadal primordium, 4x objective; b) CPG mitosis (10x); c, d, e) spherical CPG: gonadal primordium (□) Nucleus (N), acidophilic cytoplasm (+).

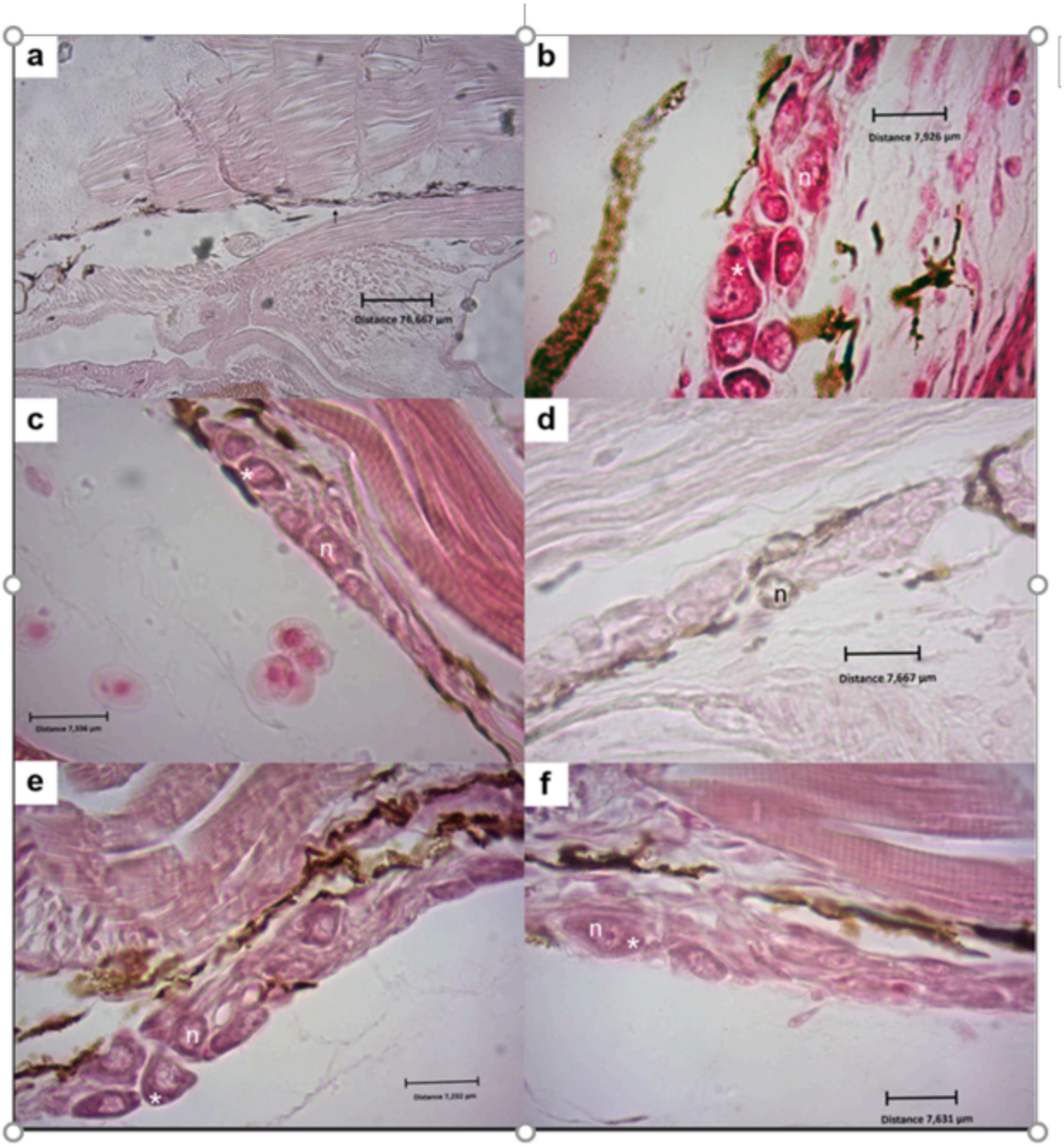


Figure 3

Morphology of previtellogenesis in *A. tropicus*ovary. Stage 2 (S2). Ovaries hematoxylin and eosin (H-E), a) elongated thin ovary (4x); b) elongated thin ovary (10x); c, d, e, f) previtelogenic ovary: ovaries (⟶) nucleus (n), basophilic cytoplasm (*).

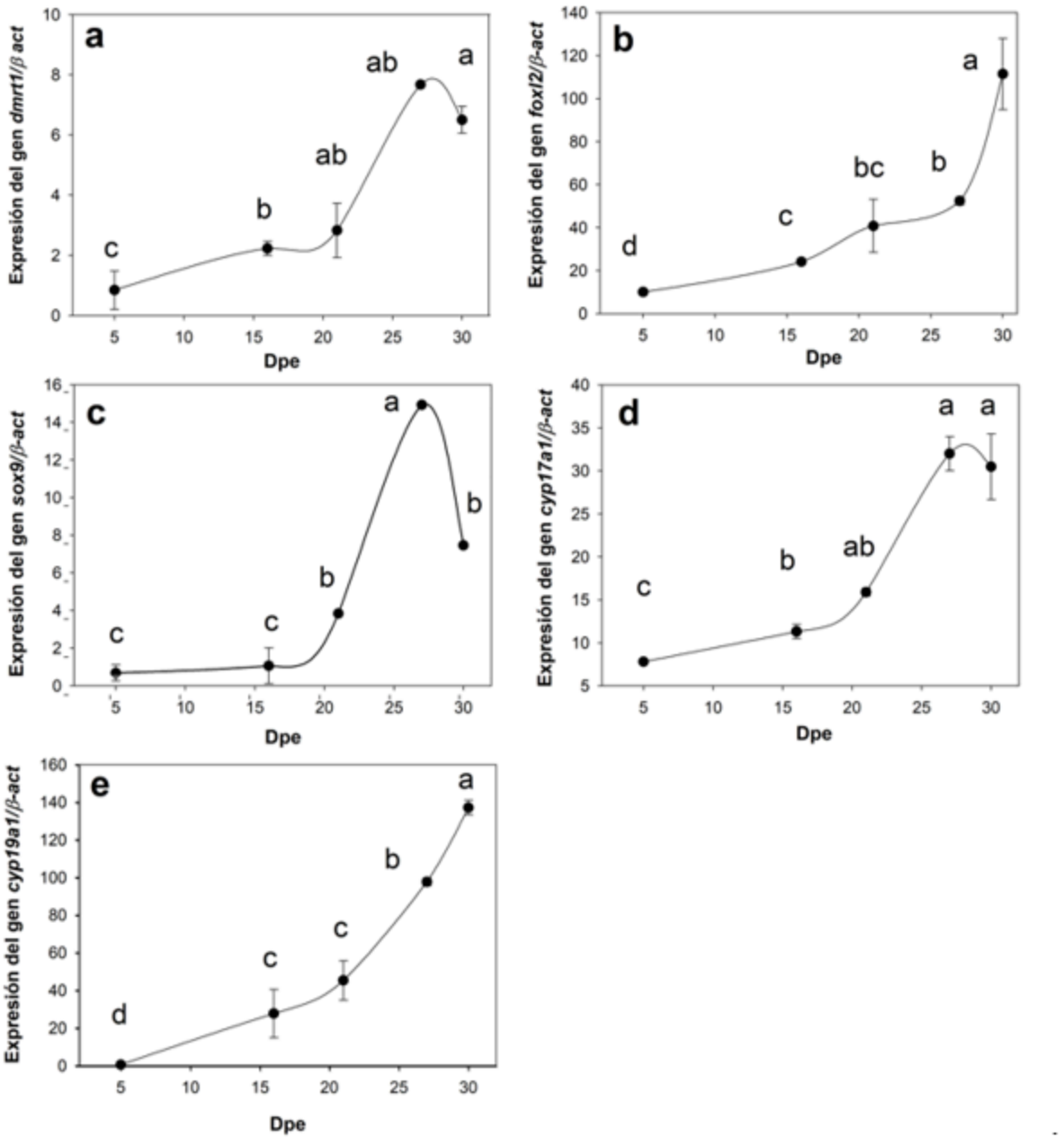


Figure 4

Expresión of A) dmrt1, B) foxl2, C) sox9, D) cyp17a1 and E) cyp19a1 transcripts in *A. tropicus* larvae. Relative quantification was performed by normalization of the relative values concerning those of the constitutive β-actin gene.