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Characterization of foxl2, sox9, and wt1a genes and their expression pattern as putative targets for sex identification in *Astronotus ocellatus* (*Agassiz*, 1831)

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Abstract: Using molecular markers is an effective method for sex identification in fish. In this regard, autosomal genes related to sex determination and differentiation may be of great interest. This study aimed to evaluate the expression pattern of Foxl2, Sox9, and Wt1a as molecular markers for sex identification in Oscar (*Astronotus ocellatus*). Molecular characterization and gene expression profiles for forkhead box l2 (Foxl2), SRY-box 9 (Sox9), and Wilm's tumor suppressor gene (Wt1a) were performed in male and female gonads. After euthanasia, histological and molecular analyses were conducted using ovarian and testis fragments of adult *A. ocellatus*. Histology was used for sex confirmation. The isolation of partial cDNA sequences encoding Foxl2, Sox9, and Wt1a from *A. ocellatus* was obtained by PCR using primers designed from conserved regions of the corresponding coding-domain sequence in other species. Sequencing of *A. ocellatus* Foxl2, Sox9, and Wt1a cDNA fragments allowed us to perform RT-qPCR assays in male and female gonadal tissue to analyze the gene expression profile. RT-qPCR revealed markedly gender-influenced gene expression patterns for all target genes. Foxl2 mRNA levels were significantly higher in the ovary than in the testes. At the same time, Sox9 and Wt1a were upregulated considerably, and mRNA levels for Sox9 and Wt1a were 8- and 9-fold higher in the testis compared to the ovary. Our findings indicate that Foxl2 expression may serve as a molecular marker for the identification of males. At the same time, the genes Sox9 and Wt1a are effective in identifying females in *A. ocellatus* gonads up to 12 months of age.
Keywords: Wilm's tumor suppressor gene, Sex determination, Sex identification, Molecular markers.

1. Introduction

Sex identification in fish is a matter of great importance in aquaculture, especially in breeding in captivity. Through appropriate sexing, it is possible to establish a broodstock fish with an adequate proportion of males and females. Therefore, fingerling production can be improved by reducing costs from unappropriated breeder maintenance (Rodríguez-Hernández et al., 2025). However, sex identification in species that do not present sexual dimorphism and heteromorphic sex chromosomes is limited, making it necessary to use new sexing approaches.

Molecular markers are a highly efficient method for sexing fish (Zhang et al., 2017; Dai et al., 2021). Autosomal genes related to sex determination and differentiation have great potential as helpful sex markers. This is because these genes are differentially expressed in a sex-specific manner, playing a determining role in testicular and ovarian formation (Bertho et al., 2016).

In fish, sex determination and differentiation involve a complex network of molecular events that are influenced by various genetic and environmental mechanisms (Nagahama et al., 2021). Many studies have reported the involvement of autosomal genes in sex determination in fish, including the forkhead box l2 (Foxl2) gene (Bhat et al., 2016b), SRY-box 9 (Sox9) (Bhat et al., 2016a), and Wilm's tumor suppressor gene (Wt1a) (Chen et al., 2015). The Foxl2 gene is a family member of the forkhead transcription factors characterized by the conserved DNA-binding forkhead box domain (Tucker, 2022). Among the functions of Foxl2 in sex determination and differentiation, Foxl2 controls Cyp19a1 expression, which encodes the P450 aromatase. Consequently, Foxl2, by regulating this steroidogenic enzyme, has a strong influence on ovarian differentiation (Tucker, 2022; Liu et al., 2015). In addition, Foxl2 is also responsible for suppressing testicular differentiation by repressing Sox9 (Tucker, 2022).

Sox9 and Wt1a have been associated with testicular development and differentiation. Sox9 is a member of the E group of the SOX family in which genes have a DNA binding domain called HMG-box (Sreenivasan et al., 2022). This gene encodes the transcription factor Sox9, a crucial factor in gonadal differentiation in vertebrates (Gonen and Badge, 2019). Considering the role of Sox9 in fish testicular differentiation, its positive regulation of the Amh gene has been emphasized. Amh is responsible for Müller's ducts regression, which, in another instance, would differentiate to form the uterine tubes and uterus (Wagner, 2023). Thus, Sox9, through the regulation of Amh expression, is a key factor for testicular differentiation.

Wt1a encodes a transcription factor that plays a key role in the urogenital development (Murugananthkumar and Senthilkumaran, 2016). In fish, Wt1 participates in the transcriptional regulation of genes involved in testicular differentiation, such as Sf1 and Gata4 (Chen et al., 2015; Murugananthkumar and Senthilkumaran, 2016). In addition, Wt1 is also an essential regulator of steroidogenesis (Wang et al., 2021), including negative regulation of Cyp19a (Meinsohn et al., 2019).

Foxl2, Sox9, and Wt1a expression in different fish species was detected mainly in gonads and other tissues (Bertho et al., 2016; Murugananthkumar and Senthilkumaran, 2016; Li et al., 2018). Furthermore, although they are typically expressed during sexual differentiation, elevated Foxl2, Sox9, and Wt1 gene transcript levels are also observed in adult animals (Caburet et al., 2012; Murugananthkumar and Senthilkumaran, 2016). *Astronotus ocellatus* is a fish species native to South America, initially found in the Amazon River and its tributaries. It is a species with great representativeness and increasing interest in breeding aquarium fish. Sex identification in this species is minimal because it does not present well-defined sexual dimorphism. To our knowledge, no studies are available addressing the use of molecular markers for sex identification in *A. ocellatus*. Therefore, the present study aims to evaluate the expression pattern of Foxl2, Sox9, and Wt1a gene expression profiles in male and female gonads.

2. Materials and Methods

2.1. Animals and tissue samples

The animals used in this study were obtained from the Multiuser Laboratory – Vivarium of Federal University of Lavras, MG, Brazil. Adults of *A. ocellatus* aged approximately 12 months, with average weight and length of 435 ± 72 g and 26 ± 3 cm, respectively, were individually maintained in 40 L tanks under a recirculation system for 12 months. The photoperiod was maintained at 12 h of light and 12 h of darkness. The fish were fed ad libitum twice daily at 8:00 h and 16:00 h with extruded commercial feed (Fri-Aqua juvenile) containing 32% crude protein in pellets approximately two millimeters in diameter.

After 24 h of fasting, 10 animals were anesthetized with eugenol (250 mg/L of water) and euthanized by medullary section. After opening the coelomatous cavity, gonad fragments were dissected, rapidly frozen in liquid nitrogen at -196 °C, and stored at -80 °C until analysis. In addition, a fragment of the gonad of each fish was fixed in Bouin's solution to determine the sex and stage of gonadal development through histological analysis. All procedures performed were approved by the Animal Research Ethics Committee of UFLA, under protocol number. 052/2013.

2.2. Gonad histology

Routine histological techniques were used on gonad samples, and the slides were stained with hematoxylin-eosin. The slides were analyzed under light-field optical microscopy with a 40x objective, and the sex was identified based on the gonadal histological characteristics. The gonadal stage of development and the sexual maturity of the animals were determined based on the morphological characteristics of the ovary and testis (Godinho et al., 2005; Wallace and Selman, 1981).

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from the frozen ovary (n = 5) and testes (n = 5) using the commercial Total SV RNA Isolation kit (Promega, Madison, Wisconsin), following the manufacturer's recommendations. Isolated RNA was quantified using NanoDrop ND-1000 (Thermo Scientific, Waltham, Massachusetts). Reverse transcriptase assays were performed using oligo-dT to obtain single-strand cDNA templates by the commercial GoScript ™ Reverse Transcription System kit (Promega, Madison, Wisconsin). The cDNA obtained for each sample was used for further molecular cloning and real-time quantitative PCR (RT-qPCR).

2.4. Molecular cloning of *A. ocellatus* Foxl2, Sox9 and Wt1a cDNA fragments

Gene-specific primers were designed based on conserved regions of Foxl2, Sox9, and Wt1 coding-domain sequences in other fish species and vertebrates (Center for Biotechnology Information (NCBI)). GenBank entries of cDNA sequences used for multiple alignments are shown in Table 1. For the selection of conserved regions, selected sequences were aligned with the online tool EMBL-EBI Clustal Omega – Multiple Sequence Alignment Tool (https://www.ebi.ac.uk/Tools/msa/clustalo). The primer quality was evaluated using OligoAnalyzer (Integrated DNA Technologies, Coralville, USA). The primers and the amplicon size obtained by PCR for each gene of interest are shown in Table 1. Ovary and testis cDNA from adult *A. ocellatus* were used for PCR amplification of the target genes (Foxl2, Sox9, and Wt1) (Table 1). The PCR reaction consisted of PCR buffer 1X, 2 mM of MgCl2, 200 µM of dNTPs, 4 µg of template cDNA, 0,5 µl of Taq DNA Polymerase (5 U/µl), and 0,2 µM of gene-specific primers. PCR conditions included one initial denaturation step at 95°C for 2 min, followed by 40 cycles of 94 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 100 sec. A final extension step of 1 min at 72°C was used. PCR products were applied to a 1 % agarose gel, stained with Gel Red™ 1X, in an electrophoresis chamber using TBE as a running buffer. The DNA fragments of A. ocellatus Foxl2, Sox9, and Wt1 amplified by PCR were purified from the agarose gel using the commercial High Pure PCR Product Purification kit (Roche) and quantified in NanoDrop ND-1000 (Thermo Scientific, Waltham, Massachusetts). The fragments were then ligated into pGEM-T Easy Vector Promega, using T4 DNA ligase for 1 h at room temperature. The transformation was performed by heat shock using competent *Escherichia coli* cells (XL1-Blue). Subsequently, aliquots of 100 to 200 µl of each cloning transformation reaction were seeded on plates with LB-agar containing ampicillin (50 µg/ml) and incubated overnight at 37 °C. For cloning confirmation, 20 bacterial colonies were selected. Colonies were individually removed from the plates and deposited in PCR tubes for colony PCR using the primers described in Table 1. Positive colonies were then selected and cultured in LB with ampicillin. Plasmid DNA was isolated from bacterial cultures using the commercial kit GenElute Plasmid Miniprep Kit (Sigma, St.

Louis, Missouri) according to the manufacturer's specifications. Three independent clones for each amplified product were fully sequenced. The cDNA sequences obtained were used to design the primers, which were subsequently used in RT-qPCR assays.

Gene	Primer	Sequence (5'-3')	*Amplicon* (bp)	Aligned sequenced (GeneBank access)
Foxl2	*Forward*	GGTTGGCAGAACAGCATCAG	259	*Sebastes schlegelii* (JN998083); *Danio rerio* (NM_001045252); *Oreochromis niloticus* (NM_001279778); *Dicentrarchus labrax* (KF208536); *Gobiocypris rarus* (JN200819); *Epinephelus merra* (EU555180); *Homo sapiens* (NM_023067); *Xenopus laevis* (AB372218); *Gallus gallus* (JF708868)
	Reverse	CCAGGAGTTGTTCATAAAGC		
Sox9	*Forward*	GGTTCGGACACTGAGAACAC	242	*Danio rerio* (NM_131643); *Epinephelus akaara* (AY676309); *Scatophagus argus* (JQ740598); *Oncorhynchus mykiss*(AB006448); *Epinephelus coioides* (GQ232762); *Homo sapiens* (Z46629); *Gallus gallus* (AB012236.1)
	Reverse	CCCAGGGTTTTGCTGAGTTC		
Wt1	*Forward*	CAGTACCGCATCCACACA	279	*Oryzias latipes* (AB070576.1); *Danio rerio* (NM_001039634.2); *Salmo salar* (NM_001173778.1); *Epinephelus coioides* (JX564599.1); *Oncorhynchus mykiss* (NM_001124295.1)
	Reverse	AACTTTCTCTGACACGTCTC		

Table 1 – Primers and amplicon size were obtained by PCR, and aligned sequences were used for the primer design.

2.5. Sequence analysis

The nucleotide sequences of each DNA fragment were translated into amino acid sequences using a translation program (http://web.expasy.org/translate) to identify the open reading frame (ORF). After ORF identification, the deduced amino acid sequences of the target genes were checked using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. RT-qPCR

RT-qPCR assays were developed to evaluate differences in the gene expression profile of A. ocellatus Foxl2, Sox9, and Wt1a in gonads. Table 2 shows specific primers (forward and reverse) designed for each gene. Standard curves with serial cDNA dilutions (1:1 to 1:40) were performed to evaluate primer linearity and efficiency. The Foxl2, Sox9, and Wt1a mRNA levels were determined in the StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts). The reaction consisted of 1X Power SYBRGreen (Applied Biosystems, Waltham, Massachusetts), 0.5 ng of cDNA, and 0.4 µM of each primer.

The mRNA levels for target genes were normalized according to the geometric mean of two reference genes, β-actin and elongation factor 1 alpha (Ef1α) (Table 2). Agarose gel electrophoresis was routinely used to control the amplicon size for β-actin, Ef1α, Foxl2, Sox9, and Wt1a. For each sample, RT-qPCR was performed in duplicate, and the variation in gene expression was calculated by the standard method of ΔΔCt. The data obtained were confirmed in at least two independent RT-qPCR assays.

Gene	Primers	Sequence (5'-3')	Amplicon size (bp)
Housekeeping genes			
B-actina	*Forward*	AGGATGCAGAAGGAAATCACAGC	124
	Reverse	GGAAGGTGGACAGAGAGGC	
Ef1α	*Forward*	GCTGGTTCAAGGGATGGAAGA	114
	Reverse	GGAGGGGCTTGTCAGTGG	
Target genes			
Foxl2	*Forward*	GGCGGGGAGAGAAAGGGAAA	123
	Reverse	GAAGTGCGTTGGTGGGGG	
Sox9	*Forward*	AGGTGTTGAAGGGTTATGACTGGA	161
	Reverse	GTTGTGCAGATGTGGGTATTG	
Wt1a	*Forward*	TCAGGGGAATACAGGATGTGCGG	185
	Reverse	GAACTCACACTGGTAGGG	

Tabela 2 – Primers used in qRT-PCR and amplicon size.

2.7. Statistical analyses

The Shapiro-Wilk test was used to verify the normality of the gene expression data. The non-parametric Kruskal-Wallis test was then applied to determine sex-dependent differences in expression profile. Data were expressed as mean ± standard error. Differences with $p<0.05$ were considered statistically significant. For statistical analyses, software R 3.2.2 was used.

3. Results

3.1. Gonad histology

Histological analysis of gonadal tissue revealed that five of the 10 animals evaluated were females and five were males. In relation to the stage of gonadal development, all the female fish were in stage III of ovary development, which means they were in

advanced maturation and characterized by large numbers of vitellogenic oocytes. Similarly, males were in stage III of testicular development, with a large number of spermatozoa in the seminiferous lumen (Figure 1).

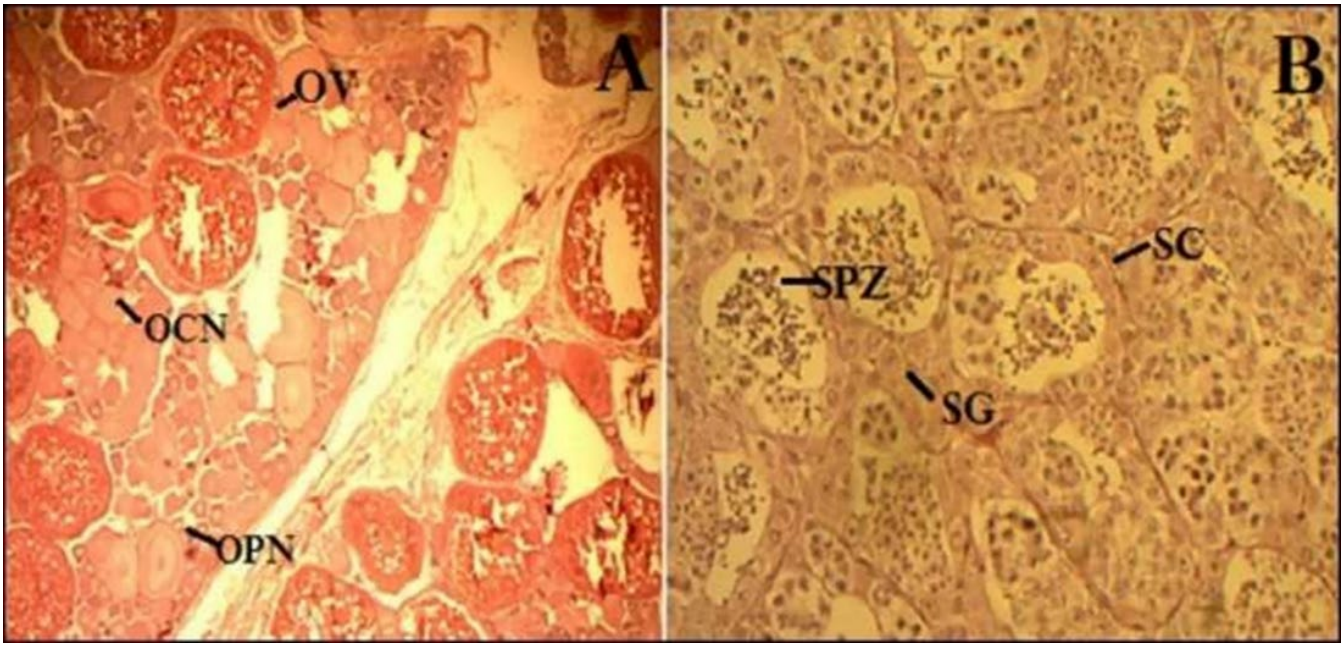

Figure 1 – Micrographs of the histological sections of the ovary (A) and testis (B) in adult *A. ocellatus*. In A, the presence of ovigerous lamellae with oocytes at different stages of development (OCN, chromatin-nucleolus oocytes; OPN, perinuclear oocytes; OV, vitellogenic oocytes). In B, spermatic cysts with the presence of sperm cells at different stages of development (SG, spermatogonia; SC, spermatocytes; and SZ, spermatozoa). Images at 40x.

3.2. Characterization of partial cDNA sequences of Foxl2, Sox9 and Wt1a from *A. ocellatus*

Multiple alignments of Foxl2, Sox9, and Wt1 cDNA sequences from other species allowed us to design oligonucleotides to isolate cDNA fragments of these genes from *A. ocellatus* by RT-PCR. Nucleotide sequences of *A. ocellatus* Foxl2, Sox9, and Wt1a genes were deposited in the GenBank database (GenBank accession n. KT337395, KT337394, and KT337396, respectively).

Foxl2's partial coding-domain fragment of 252 bp was obtained, with an open reading frame (ORF) encoding 80 amino acids. The deduced amino acid sequence contains the conserved DNA-binding domain, forkhead box (FH), between amino acid residues 1 and 40. For Sox9, a fragment of 259 bp encoding 86 amino acids was obtained. An HMG-box domain is conserved between amino acid residues 57 and 86. For Wt1a, we isolated a fragment of 279 bp encoding 93 amino acids. The deduced amino acid sequence of Wt1a contains the conserved WT1 domain at residues 1 to 29.

3.3. Gene expression profile (RT-qPCR)

Sequence analysis of *A. ocellatus* Foxl2, Sox9, and Wt1a cDNA isolated fragments made it possible to design oligonucleotides to analyze the pattern of gene expression in gonadal tissue of adult fish by RT-qPCR. RT-qPCR assays revealed a gene expression profile markedly influenced by sex for Foxl2, Sox9, and Wt1a in the gonadal tissue of *A. ocellatus*. Foxl2 mRNA levels were 3-fold significantly higher in the female ovary than in the male gonads ($p = 0.033$) (Figure 1). In contrast, an opposite expression profile was found for the Sox9 and Wt1a genes, which were significantly upregulated in males ($p = 0.020$ and $p = 0.014$, respectively). The mRNA levels for Sox9 and Wt1a were 8- and 9-fold higher in the testis compared to the ovary (Figure 2). Indicating that Foxl2 expression may serve as a molecular marker for identifying males, while the genes Sox9 and Wt1a are effective for identifying females in *A. ocellatus* gonads up to 12 months of age.

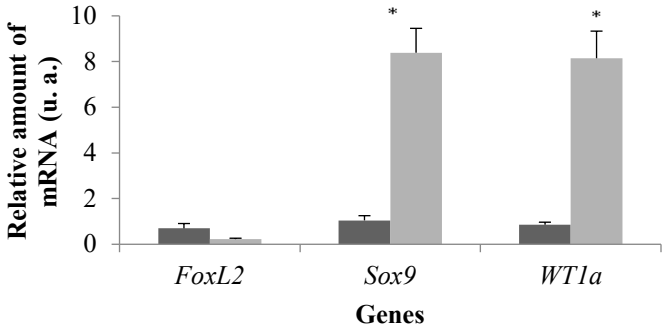

Figure 2 – Expression profile of Foxl2, Sox9, and Wt1a in gonads of females (grey bars) and males (dark grey bars) of adult A. ocellatus. The level of expression for each gene was normalized using the reference genes β-actin and ef1α (standard method of ΔΔCt), considering the geometric mean of the Ct values of the reference genes. Results are presented as mean and standard error (n = 5 per group). u.a: arbitrary units. Asterisks mean significant differences between groups for each gene ($p < 0.05$).

4. Discussão

4.1. Characterization of the partial genomic sequence of Foxl2, Sox9, and Wt1a genes

The present study addressed cloning and characterization of partial cDNA sequences from three different *A. ocellatus* genes: Foxl2, Sox9, and Wt1a. These genes were previously characterized in other fish species, and their functions were associated with sex determination and differentiation (Bhat et al., 2016a; Chen et al., 2015; Jiang et al., 2020). The literature reports the coexistence of two paralytic Foxl2 genes, Foxl2a and Foxl2b, in the fish genome with different functions (Bertho et al., 2016). Foxl2a has been associated with ovarian differentiation (Castro et al., 2019), while Foxl2b plays a significant role in regulating specific genes involved in testis development and final maturation (Yang et al., 2017). Although our findings allowed us to isolate a unique cDNA fragment of Foxl2 from the gonadal tissue of *A. ocellatus*, we cannot discard the presence of more isoforms in this species. However, considering the expression pattern observed in males and females, the isolated cDNA fragment is more likely to correspond to the Foxl2a gene due to higher mRNA levels in the ovary than in the testes.

The gender-dependent expression pattern of *A. ocellatus* Sox9 suggests that the isolated cDNA corresponds to the testicular type already described in other fish species, such as *Danio rerio*, *Clarias batrachus* and *C. gariepnus* (Bhat et al., 2016a; Raghuveer and Senthilkumaran, 2010; Rodríguez-Marí et al., 2005).

Two variants of the Wt1 gene, Wt1a and Wt1b, were identified in fish species such as *Cyprinus carpio* (Chen et al., 2015), *Oryzias latipes* (Klüver et al., 2009), and *D. rerio* (Bollig et al., 2006). Regarding the function of Wt1 genes in *O. latipes*, Wt1a and Wt1b are co-orthologous genes, with evidence of functional redundancy in the maintenance and primordial germ cell survival (Klüver et al., 2009). In *D. rerio*, although the proteins encoded by Wt1a and Wt1b present more than 90% of identity, different and non-redundant functions in gonadal development have been proposed (Bollig et al., 2006). The present study's single Wt1 cDNA fragment isolated from *A. ocellatus* corresponds to the Wt1a gene.

4.2. Gene expression profile

The present study addressed the expression profiles of Foxl2, Sox9, and Wt1a in *A. ocellatus* for the first time. Expression analysis in *A. ocellatus* gonads showed a significantly different sex-dependent pattern for the three genes. Therefore, given the sexual dimorphism profile for these genes, it can be inferred that these are suitable targets for sex identification in this species.

Our findings showed that higher levels of Foxl2 mRNA were detected in the ovaries than in the testes. Such an expression pattern was also observed for other fish species. In *Dicentrarchus labrax*, the gonadal expression of Foxl2 also showed strong sexual dimorphism, with elevated levels of mRNA in the ovary. In contrast, Foxl2 expression in the testis remained low throughout the reproductive cycle (Crespo et al., 2013). Similarly, Foxl2 was highly expressed in the ovary, while moderate levels were detected in the testis of *C. batrachus* (Bhat et al., 2016a).

Consistent with these results, a similar expression pattern was observed in *Anoplopoma fimbria* (Smith; Guzmán; Luckenbach, 2013), *Gobiocypris rarus* (Jiang et al., 2011), *Paralichthys olivaceus* (Si et al., 2016), *Oreochromis niloticus* (Wang et al., 2004), and medaka *O. latipes* (Nakamoto et al., 2006). Therefore, the results in the literature emphasize Foxl2's crucial participation in ovarian differentiation in fish (Liu et al., 2015).

Although deferred to males, Foxl2 expression in *A. ocellatus* females showed relatively low mRNA levels in the ovary. The fact that in the present study, all animals, including males and females, were at an advanced stage of gonadal development may have contributed, at least in part, to the observed expression profile. The results of the present study suggest that for *A. ocellatus*, Foxl2 could exert more significant activity in the early stages of gonadal development. Indeed, although still controversial, reports from other fish species indicate that the expression pattern of Foxl2 is markedly influenced by the stage of gonadal development, with a differentiated profile for each species. For example, ovarian levels of Foxl2 are higher in the early stages of gonadal development, decreasing with gametogenesis advancement in *Scatophagus argus*, *O. latipes*, and *A. fimbria* (Liu et al., 2015; Nakamoto et al., 2006; Smith; Guzmán; Luckenbach, 2013). In females, higher Foxl2 expression at the beginning of gonadal development may be

associated with its role in granulosa cell differentiation and follicle membrane formation (Liu et al., 2015). In contrast, for other species, such as *D. labrax*, high levels of Foxl2 were observed in the late stages of gonadal development, vitellogenesis, and final maturation (Crespo et al., 2013). According to these authors, Foxl2 may be involved in regulating steroidogenesis, growth, and ovarian maturation for this species.

Higher mRNA levels for Sox9a and Wt1a were observed in males of *A. ocellatus* than in females. These results agree with the literature for other fish species (Bhat et al., 2016a; Luo et al., 2010; Rodríguez-Marí et al., 2005), such as Sox9 and Wt1, which were previously reported as important transcription factors regulating sex determination and differentiation in males (Bhat et al., 2016a; Vidal et al., 2001). We detected extremely high levels of Sox9 transcripts in the adult testis of *A. ocellatus* using data available for other fish species. For example, in *D. rerio*, elevated levels of Sox9a were detected in undifferentiated gonads and juvenile and adult testes (Rodríguez-Marí et al., 2005). Similarly, in *C. batrachus*, Sox9a was highly expressed in developed gonads, especially in the preparatory and pre-spawning phases of the reproductive cycle (Bhat et al., 2016a). In contrast, for other species, such as O. latipes (Nakamoto et al., 2005), *A. fimbria*, and *O. niloticus* (Ijiri et al., 2008; Luo et al., 2010), Sox9 does not play a fundamental role in sexual differentiation, given the similarity between genders during sexual differentiation.

In this context, it can be inferred that Sox9a is a key factor in regulating testicular function for *A. ocellatus*. Possible roles of Sox9a in testicular function include positive regulation of the Amh gene, development of tubular structures in the testis after gonadal differentiation (Hu; Binzhong; Du; 2021), proliferation of primordial germ cells in the testis and ovary (Begun et al., 2022), and differentiation and maintenance of Sertoli cells (Hu; Binzhong; Du; 2021).

The available studies corroborate the results obtained for *A. ocellatus* regarding Wt1a. Wt1a was also found to be differentially expressed regarding sex in other species. For example, in C. batrachus, higher Wt1 expression was detected in the developing testis compared to the ovary (Murugananthkumar and Senthilkumaran, 2016). Similarly, in *C. carpio*, higher Wt1 expression was also detected in the testis, possibly associated with testicular differentiation (Chen et al., 2015).

In the present study, very high levels of Wt1a transcripts were observed in the adult testis of *A. ocellatus*. This finding is consistent with that found for other species and seems directly related to the advanced gonadal maturation stage. For example, in addition to high levels of Wt1 during testicular ontogenesis, significant expression of Wt1 was also observed in the testicles of adult *C. batrachus*, being directly correlated to the reproductive cycle phase. In this species, higher Wt1 expression was detected in the pre-spawning and spawning stages, indicating this gene's participation during the active spermatogenesis (Murugananthkumar and Senthilkumaran, 2016). Thus, the high level of Wt1a in the testis of *A. ocellatus* can be explained, at least in part, by the advanced stage of gonadal maturation.

The expression pattern of Wt1a indicates that it may be an essential factor in regulating testicular function in adult *A. ocellatus*. Among the tasks of Wt1a in differentiation and gonadal development can be highlighted: participation in spermatogenesis (Murugananthkumar and Senthilkumaran, 2016), support of Sertoli cells (Jiang et al., 2017), maintenance and survival of primordial germ cells (Jiang et al., 2017), regulation of the transcriptional network of genes involved in sexual differentiation (Wilm and Muñoz-chapuli, 2016), and regulation of gonadal steroidogenesis (Chen et al., 2014).

In the present study, Foxl2, Sox9a, and Wt1a expression analyses were restricted to tissues previously known to be the primary expression sites of these genes: testis and ovary (Tucker, 2022; Gonen and Badge, 2019; Murugananthkumar and Senthilkumaran, 2016). Future studies will evaluate the expression of these genes in extra-gonadal tissues of *A. ocellatus* since the presence of these transcripts in several tissues was evidenced in other fish species (Bhat et al., 2016b; Chen et al., 2015; Wei et al., 2016). This approach could facilitate sex identification in tissues that are easier to collect, such as fins and gills. In addition, the differential expression of these genes throughout sexual determination and differentiation, as well as during various stages of gonadal development, would be noticeable.

5. Conclusion

It is concluded that Foxl2, Sox9, and Wt1a can be used as molecular markers for sex identification in A. ocellatus, given the strong sexual dimorphism achieved for the expression pattern of the three genes. In addition to contributing to sex identification in A. ocellatus, our findings provide insight into the molecular mechanisms involved in sex determination and differentiation.

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