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Article

Expression Patterns of Genes Involved in Sexual Development of Turbot (*Scophthalmus maximus*): A Histological Analysis by *In Situ* Hybridization on Differentiated Ovarian and Testicular Tissue

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Abstract

Genes involved in sexual development have been identified in many teleost fishes including the turbot, a flatfish with great commercial value for the aquaculture industry, where a principal Sex Determination (SD) Quantitative Trait Loci has been identified and *Sox 2* has been proposed as the SD gene. Although RT-PCR, qPCR, and transcriptomic analyses have been carried out in turbot gonads, histological studies are still scarce. We have studied the tissue-specific expression patterns of several genes involved in the sexual development in turbot by *in situ* hybridization on paraffin sections (SISH) of differentiated ovary and testis of juvenile and adult specimens. *Vasa*, *Foxl2*, *Amh*, *Sox2* and *Sox17* transcripts were detected both in male and female gonads while *Cyp19a1a* and *Sox19* expression were ovary specific, supporting previous data on gene expression analyses and suggesting a role for those genes in gonad development and/or in reproductive functions in this species. Therefore, SISH provides complementary information to molecular analyses identifying the cell types where sex-related genes are expressed in gonadal tissue and to obtain a more comprehensive view of the gonadal differentiation process in turbot. Comparison with the results obtained in the gonads of other teleost species showed a similar tissue-specific gene expression during sexual development.

Keywords: gonadal development; turbot; *Vasa*; sox genes; *Amh*; *Cyp19a1a*; *Foxl2*; *In situ* hybridization

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Key Contribution: *In situ* hybridization was performed to analyze the expression of genes involved in sexual development of turbot on differentiated ovarian and testicular tissue sections. *Vasa*, *Amh*, *Foxl2*, *Sox2*, and *Sox17* were expressed both in male and female turbot gonads while *Cyp19a1a* and *Sox19* expression was ovary-specific. *In situ* hybridization allows identifying cell types expressing sex-related genes in turbot gonadal tissue providing complementary information to molecular analyses.

1. Introduction

The turbot (*Scophthalmus maximus*) is the farmed flatfish with higher production worldwide (~ 72,000 tonnes) being Spain and PR China, the principal producers. Controlling reproduction and sex ratio is one of the main challenges for turbot producers along with increasing growth rate and disease resistance [1]. This teleost is a gonochoristic species, in which each mature individual possesses only

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ovaries or testes, and exhibits the highest sexual dimorphism among marine fish both in growth rate and sexual maturity [2,3]. In turbot females sexual maturity occurs about at a year earlier than in males, increasing somatic growth and reducing susceptibility to diseases and mortality, which have aroused the interest of the aquaculture industry in obtaining all-female populations [2].

Sexual development occurs in a similar way across teleost fishes and, as in all vertebrates, relies on two concatenated processes, sex determination (SD) and sex differentiation. SD occurs early during embryogenesis when sex is established by the determination of the developmental pathway of the bipotential gonadal primordium which is identical in males and females. Then sex differentiation involves the forming of the testis or the ovary throughout development from the undifferentiated bipotential gonadal primordium [4].

In contrast to mammals and birds, which shows a common fixed sex determining molecular machinery, teleost fishes display notably different sexual development mechanisms, even among closely related species [5]. In fact, in certain cases SD and sex differentiation is species-specific and can be regulated by a gene network, by environmental factors (temperature, population density, hypoxia, pH and social interaction [6]), or by both [4,5,7]. In the turbot, sex is considered as a quantitative threshold trait, where genetic and environmental factors, as the temperature at which the embryos develop, are involved at the beginning of sex differentiation for determining the fate of the undifferentiated primordium [8–11]. Moreover, size and growth of the turbot specimens could be critical elements that strongly influence SD [9] as it was reported in other teleost species such as the European sea bass [12].

As it was mentioned above, SD mechanisms are very variable among teleosts so many sex-related genes have been identified in these fishes [5], including the turbot [1,8–10,13], although the SD master gene can vary even among close species. Studies carried out in *S. maximus* have identified a ZZ/ZW sex chromosome system with limited environmental influence based on the sex ratio obtained in progenies from hormonally sex-reversed parents [14]. These results were confirmed by Martínez et al. [15] who further identified a major sex-related Quantitative Trait Loci (QTL) at linkage group (LG) 5. After identification of several candidate genes at LG5 using the whole genome sequence of this species [16] and a genome-wide association study [10] *Sox2* has been proposed as the SD gene in the sexual development of the turbot.

Understanding how sexual development occurs in the turbot and the genes involved in this process is an important challenge that requires studies following different approaches. Analyses with RT-PCR, qPCR, microarray, and transcriptome sequencing have revealed the expression of SD and sex differentiation key genes in the turbot throughout gonadal development [9,13,17–21]. Moreover, qPCR analyses of genes related to primordial germ cells development have reported signs of sex differentiation in postmetamorphic turbot larvae (75-90 days post fertilization (dpf), 5-6 cm length) [9]. However, in the same study using the classical haematoxylin and eosin (H&E) stain morphological gonad differences between sexes were not detected before 105 dpf (7,2 cm length, [9]). More recent histological studies by H&E stain in turbot reported that the ovaries and testis could be morphologically distinguished in earlier stages of development [11,21].

Although numerous molecular analyses (i.e. by RT-PCR, qPCR, microarray, transcriptome sequencing) about gene expression patterns in teleosts gonads have been performed, histological studies are scarce despite identifying the cell types where sex-related genes are expressed represents essential complementary information to understand the process of gonadal development. To the best of our knowledge, only *Vasa* [17,22,23] and *Dnd* [18,23] expression have been studied by *in situ* hybridization (ISH) in differentiated testis and ovary of juvenile and adult turbot. We have performed ISH on differentiated ovary and testis paraffin sections (SISH) of juvenile and adult turbot to evidence the tissue-specific and cellular sites of the expression of *Vasa*, *Cyp19a1a*, *Foxl2*, *Amh*, *Sox2*, *Sox17*, and *Sox19*. The reason for studying the expression patterns of these genes is that they are involved in sexual development in turbot and have been previously characterized in our lab by molecular analyses in turbot gonads [9,10,13,15,16,24,25].

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The aim of our research was to use SISH with two goals (1) to determine the tissue specific expression of *Vasa*, *Cyp19a1a*, *Foxl2*, *Amh*, *Sox2*, *Sox17*, and *Sox19* in turbot gonads, i.e. cell types where these genes were expressed, and (2) to confirm on tissue sections the data observed in previous molecular analysis. Thus, our results showed that SISH, which gives spatial information on RNA in a tissue sample, and previous qPCR analyses, which quantifies the average expression level within the entire sample, provide complementary information for understanding gene expression profiles in the gonads of the turbot. Moreover, we have compared our results with similar studies performed in other teleost species to find out if there were differences in the expression patterns of the genes studied here between male and female specimens or through the gonad development.

2. Materials and Methods

2.1. Experimental Animals and Tissue Preparation

Turbot (*Scophthalmus maximus*) of different developmental stages were provided by the Instituto Español de Oceanografía (Vigo, Spain). Six turbot 130 dpf (three males and three females; 10-13 cm length, 21-37 g weight), five 180 dpf (two males and three females; 12-15.1 cm length; 37.9-62.8 g weight) and six adults (2 years post fertilization (ypf); three males and three females; 25-31 cm length; 280-550 g weight) were used for the *in situ* hybridization study. Gonads are differentiated at every stages studied, although they are mature only in male adult specimens.

All specimens were deeply anesthetized in a 0.5% solution of MS-222 (tricaine methane sulfonate; Sigma-Aldrich, St. Louis, MO) in seawater and then perfused transcardially with Ringer's solution containing 0.1% procaine (Sigma-Aldrich), followed by perfusion with cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline pH 7.4 (PBS) and finally sacrificed. The gonads were then removed from each individual and immersed in the same fixative for 13-16 hours at 4 °C. After several PBS washes, the gonads were dehydrated in methanol at 4 °C and then embedded in paraffin wax. All buffers used in the experiments were made in RNA free distilled water. Parallel transverse serial sections (10-14 mm thick) were cut on a rotary microtome and mounted on Superfrost Plus glass slides (Menzel, Braunschweig, Germany).

To facilitate identification of the anatomy and cell types in the testis and the ovary parallel gonad sections were stained with hematoxylin-eosin (H&E) following standard protocols.

2.2. Riboprobe Synthesis

Total RNA was isolated from gonads using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA with AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA) following supplier recommendations. Several genes involved in sex determination and differentiation (*Vasa*, *Amh*, *Cyp19a1a*, *Foxl2*, *Sox2*, *Sox17*, and *Sox19*) were amplified from cDNA with the primers detailed in Table 1. These primers were designed prior to the publication of the turbot genome from the sequences in public data base. Gene sequences from different fish species were used. Finally, it was verified by sequencing if the primers pair amplified the right gene. PCR was carried out in a volume of 50 µl: 75 ng of cDNA, 20 pmol of each primer, 0.2 mM of each dNTP, 1× PCR reaction buffer, and 2.5 units of GreenTaq DNA polymerase (GenScript, Leiden, The Netherlands), in a MyCycler Thermal cycler (Bio-Rad, Hercules, CA) as follows: initial denaturation at 94 °C for 3 min; 30 cycles including 94 °C during 30 s, 50 s at their specific annealing temperature between 59-60 °C, and an extension time dependent on amplicon size (about 1 kb/min) at 72 °C; and a final extension step at 72 °C for 7 min. The PCR products were separated on 1% agarose gels, stained with SYBR gold (Invitrogen) and purified using DNA Clean & Concentrator kit (Zymo Research, Irvine, CA) for sequencing following the manufacturer's protocol.

These DNA fragments were cloned in a pGEM-T vector (Promega, Madison, WI)), purified with QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and sequenced following the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit protocol on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster city, CA) with the SP6 and T7 universal primers (SP6:

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ATTTAGGTGACACTATAG, T7: TAATACGACTCACTATAGGG). The orientation of the insert was analyzed by the homology sequence analysis through BLASTn (Basic Local Alignment Search Tool Nucleotide). Sense and antisense digoxygenin-UTP-labeled riboprobes were synthesized by means of SP6 and T7 RNA polymerase (Promega) following the manufacturer's protocol and stored at -80 °C.

Table 1. Primers used for cDNA amplification in riboprobe synthesis.

GENE	PRIMERS	AMPLICON SIZE (pb)
Vasa	F: GAGCCTGAAGCCATCATTGT R: ACGTTCTCCTGGCACATCAACG	634
Amh	F: CTGGGCGTATCCTCTAACGA R: CATCCGCACTCCTTTGCTAT	693
Cyp19a	F: GGAGGTTTGTGTCTCCTCCA R: GAACTCCGTGCGGTGCATGC	715
Foxl2	F: AGAAGCGGCTCACGCTGTC R: TTTGGTCTCGTGCTCCCAGT	712
Sox2	F: CTGTTGGACTACTTAACGTTGGA R: CAGATGAAAAGTGGGAGACG	729
Sox17	F: GAGCAGATGCACCACTCTGA R: GCAAAAAGTATAAAAACACCGTCA	539
Sox19	F: TGTGCCTTGCCTTTTGATTT R: GGTTTGCTGGAGATTTAGTCTGA	600

2.3. In Situ Hybridization

In situ hybridization on tissue sections (SISH) was performed following standard protocols. Gonad sections were treated with proteinase K (Roche; 10 mg/ml) for 30 min at room temperature and hybridized separately with sense and antisense probes overnight at 65 °C in a humid chamber.

The posthybridization washes included RNAse A treatment (Invitrogen; 100 mg/ml, 30 min at 37 ºC), and then, the samples were incubated overnight with the alkaline phosphatase-coupled anti-digoxigenin antibody (1:2000, Roche Applied Science, Penzberg, Germany) at room temperature in a humid chamber. The color reaction was performed in the presence of BM-Purple (Roche) until the signal was clearly visible. The sections were dehydrated and coverslipped.

No positive signals were detected in the control hybridization sections with sense probes in none of the analyzed genes.

2.4. Image Acquisition and Analysis

ISH images were acquired with an Olympus BX51 microscope fitted with an Olympus DP71 color digital camera. For presentation, bright-field microscopy images were sized and optimized for brightness and contrast with Photoshop CS2 (Adobe, San Jose, CA) and plate photomontage and lettering were also done with Photoshop CS2.

3. Results and Discussion

In this work, we used H&E stain to study the gonadal morphology of juvenile and adult turbot (*Scophthalmus maximus*) and performed SISH on paraffin sections of differentiated testis and ovary to analyze from a histological point of view the tissue-specific expression patterns of some genes

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involved in sexual development in this flatfish species. Thus, we investigated the expression patterns of *Vasa, Cyp19a1a, Foxl2, Amh, Sox2, Sox17,* and *Sox19* genes, all of which are involved in the sex differentiation of teleost fishes, including the turbot, as qPCR, microarray and transcriptomic analyses have previously proven [9,13]. The most noticeable of our histological study was that genes studied were expressed in male and female germ cells and that our results support on tissue sections previous data on gene expression analyses in the turbot gonads. These results proved the usefulness of *in situ* hybridization as a useful complement for transcriptomic and/or proteomic evaluations to better understand the molecular mechanisms of gonad differentiation in teleost fishes.

3.1. Histology of the Differentiated Turbot Gonads

With the aim of better identifying the positive *in situ* hybridization regions and cell types in the gonads of the turbot, H&E stain were performed on paraffin sections to study the anatomy of the ovary and testis of juveniles (130 and 180 dpf) and adults (2 years post fertilization (ypf)) specimens. As mentioned in the introduction, Zhao et al. [21] established by H&E stain on early development turbot stages the ovary differentiation in premetamorphic larvae (60 days post hatching (dph), 3,5 cm length) while testis remained undifferentiated until 90dph (6 cm length). However, a recent study also by H&E stain reported an earlier differentiation of the turbot gonads, i.e. the ovary was already distinguished at 45 dph (3,7 cm length) with both the ovarian cavity and the germinal epithelium clearly defined [11]. However, the testis remained undifferentiated until 65dph when the number of germ cells increased through mitosis and the spermatogonial cysts were formed in the edge of the gonads [11]. As total length of turbot specimens initiating gonadal sex differentiation was consistent in the different studies, Meng et al. [11] proposed that body size or length rather than age (measured as dph or dpf) is basic in determining the timing of sex differentiation in this flatfish species. Anyway, all data obtained until now show a delay in testicular differentiation compared to ovarian development in turbot and, moreover, confirm that juvenile and adult turbot specimens used in our study present already differentiated gonads.

Our results showed that the turbot ovary was surrounded by a layer of connective condensed tissue, the tunica albuginea, and presented a hollow sac-like morphology with an ovarian cavity into which numerous ovigerous lamellae extended (Figure 1). These lamellae consisted of ovarian stroma bordered by germinal epithelium supporting somatic (epithelial and follicular cells) and germ cells (oogonia and oocytes) (Figure 1). The ovigerous lamellae at 130 dpf (Figures 1a-d) and 180 dpf (Figures 1e-h) stages contained abundant oogonia (9-13 µm in diameter) which showed a basophile nucleus, and numerous oocytes of different size (14-37 µm in diameter) in different steps of the primary growth stage. Primary oocytes displayed a densely basophile cytoplasm and one nucleolus but occasionally multiple nucleoli in the nucleus (Figures 1b-d and f-h). In the ovary of 2 ypf specimens, still sexually immature females, a small amount of oogonia was observed while there was an evident increase in the number and size (50-115 µm in diameter) of primary oocytes (Figures 1i-l), as previously described in immature ovaries of turbot specimens [26]. At this stage, the primary oocytes showed an evident nucleus with irregularly distributed multiple nucleoli (Figures 1j-l) and a basophile cytoplasm during the earlier stages which turned more acidophilic and showed a more apparent Balbiani body (a non-membrane bound compartment packed with RNA, mitochondria and other organelles) as development progressed (Figures 1k-l).

The turbot testis displayed an elongated morphology surrounded by the tunica albuginea and a lobular inner structure formed of germ cells and somatic cells comprising the germinal and the interstitial compartments (Figure 2). The germinal compartment was formed of seminiferous or testicular lobules that extended to the periphery of the testis with germinal cells and somatic epithelial cells (Sertoli cells) along their wall, while the interstitial compartment surrounding the testicular lobules was composed of connective tissue and Leydig cells (Figures 2a, d and h). In juveniles abundant spermatogonia were arranged in cysts throughout the length of the lobules and showed a basophilic nucleus (Figures 2b-c and e-g). The testis of 2 ypf specimens, already sexually mature males, showed a similar histology to that previously described in mature testes of turbot

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specimens [23,26]. Thus, we observed seminiferous lobules more numerous than in juveniles which run parallel with spermatogonia around the edge of the gonad (Figures 2i-j), cysts of primary spermatocytes, some of them in leptotene/zygotene throughout the length of the lobules (Figures 2i and k), and spermatids near the lumen of the efferent ducts where spermiation will occur (Figures 2i and l).

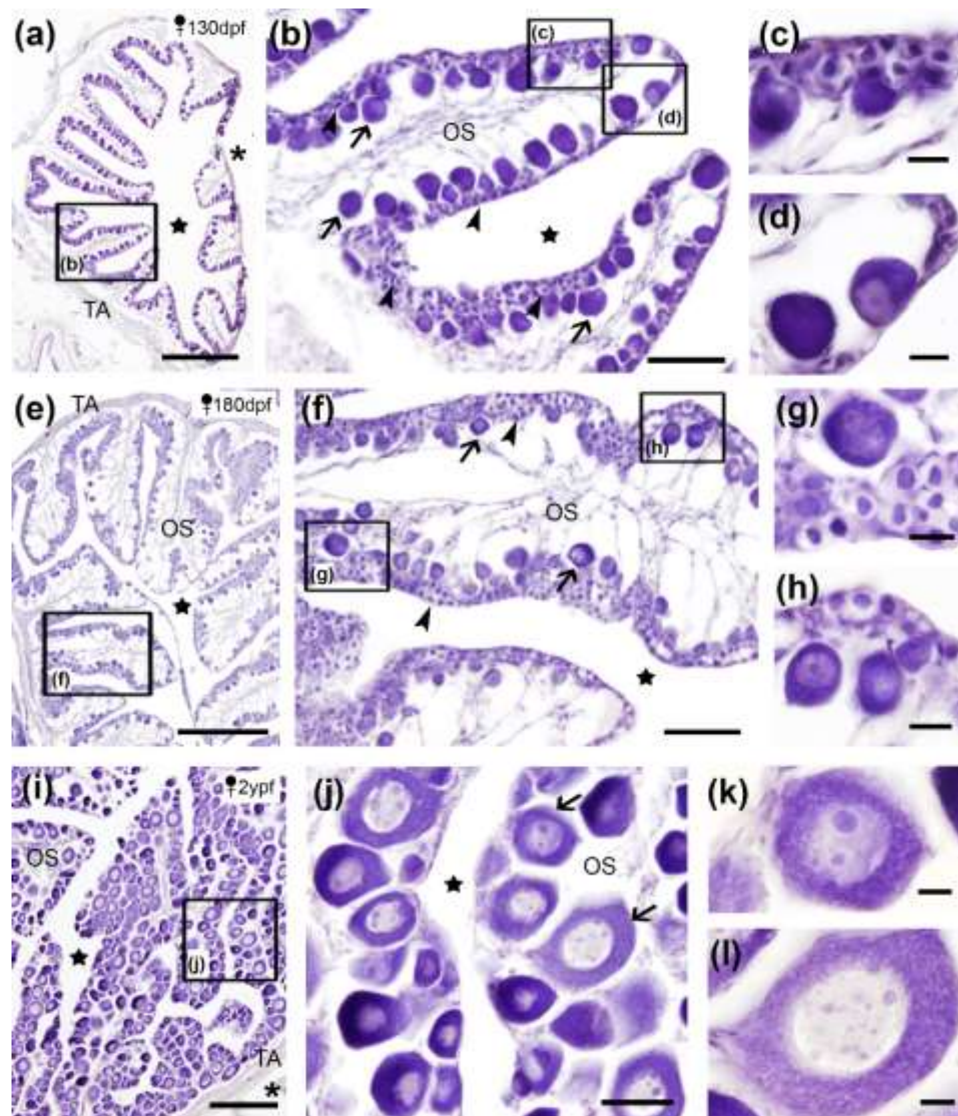

Figure 1. Cross sections of ovaries of juveniles and adult turbots stained with hematoxylin-eosin. (**a-h**) In juveniles, abundant oogonia (arrowheads) and primary oocytes (arrows) were observed (details in c-d and g-h). (**i-l**) In adults, numerous and larger primary oocytes were observed (arrows, details in k and l). TA, tunica albuginea; OS, ovarian stroma; stars, ovarian cavity; asterisks, celomic cavity. Squares in low magnification images (a-b; e-f; i) indicate the location of details (c-d; g-h; j). Scale bars: 500 µm (a,e,i); 100 µm (b,f,j); 20 µm (c-d, g-h, k-l).

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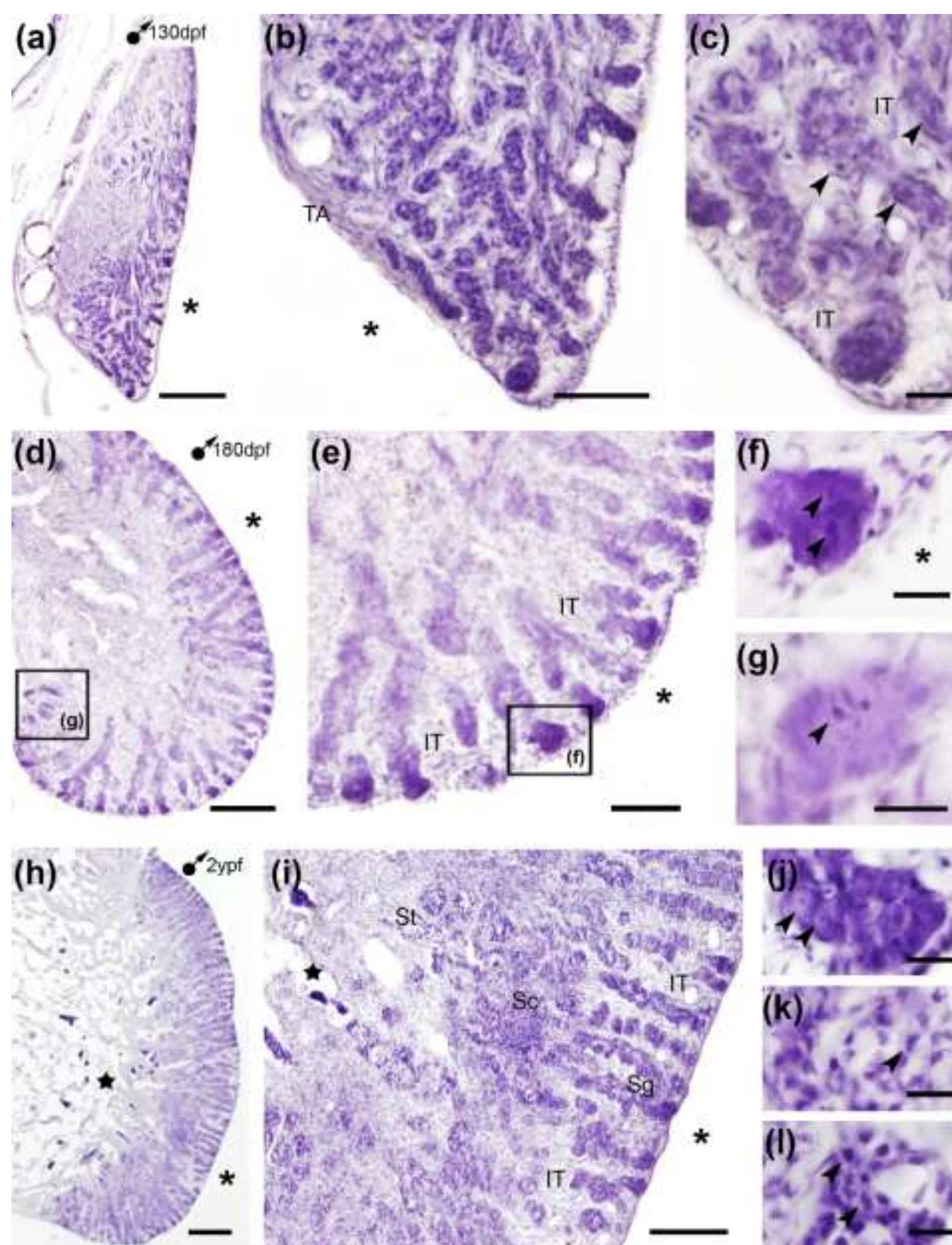

Figure 2. Cross sections of testis of juveniles and adult turbots stained with hematoxylin-eosin. (**a-g**) In juveniles, abundant spermatogonia (arrowheads) were observed. (**h-l**) In adults, spermatogonia (i: Sg, j: arrowheads), spermatocytes (i: Sc, k: arrowhead), and spermatids (i: St, l: arrowheads) were observed. IT, interstitial tissue; TA, tunica albuginea; stars, lumen of the efferent ducts; asterisks, celomic cavity. Squares in low magnification images (d-e) indicate the location of details (f-g). Scale bars: 500 μm (a, d, h); 200 μm (i); 100 μm (b, e); 25 μm; (c, f-g); 20 μm (j-l).

3.2. Histological Analysis of the Expression Pattern of Genes Involved in Sexual Development in Turbot

As mentioned in the introduction, the genetic cascade underlying the process of SD and gonad differentiation present a high diversity among teleosts, and even between closely related species, both in the master SD genes that trigger the male or female pathways and in the relative gene positions and their functions in testicular and ovarian differentiation [4,5,8,27]. Moreover, diverse expression patterns of the sex-related genes were observed between male and females of different teleost species over the course of development [5,7,28–31], including the turbot [9,13,19–21].

Regarding the expression of the genes studied in this work, previous data based on qPCR analysis in turbot gonads showed that *Vasa*, *Amh* and *Cyp19a1a* were the first sexual differentially expressed genes with a higher expression of *Vasa* and *Cyp19a1a* in females and *Amh* in males [9]. *Vasa*,

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Amh and *Cyp19a1a* increased their expression from 75 to 90 dpf stages (5-6 cm length), as well as *Foxl2*, which showed a significantly higher expression in turbot females after 90 dpf. Thus, 90 dpf was proposed as the development stage representing the onset of sex differentiation in the turbot based in molecular signs [9] although as it was mentioned in the introduction, more recent data showed morphological gonad differences at earlier developmental stages [11,21].

Finally, we have compared our results on tissue-expression pattern of genes involved in the sex differentiation of turbot with those obtained in other teleost species to try to elucidate possible histological or morphological differences which could underlay in gonad differentiation in this group of vertebrates.

3.2.1. Vasa

The identification of *Vasa* as a specific germ cell marker in the gonads of a multitude of vertebrate species, including fishes [32], has proved its conserved an essential role in germ cell development. Studies on this gene have facilitated the progress in knowing germ cell development and evolution of its localization in the animal kingdom. *Vasa* is the most characterized germ cell marker in teleostean species, including turbot, mainly by RT-PCR and/or qPCR analysis, but also by ISH or immunohistochemistry (IHC) techniques [32]. In fact, qPCR analyses in the turbot showed that *Vasa* expression is the first molecular signs (along with that of *gsdf* and *tdrd1* gene expression) of sex differentiation which started to increase between 75-90 dpf [9].

Our SISH results showed *Vasa* expression in germ cells on paraffin sections of turbot gonads. In the ovary, *Vasa* mRNA was detected in oogonia and the ooplasm of the primary oocytes (Figures 3a-c). In the testis *Vasa* expression was observed in spermatogonia and spermatocytes (Figures 3d-f) but no expression was founded in spermatids, indicating a decline in its expression with the testis differentiation. Our results aligned with those previously reported by SISH and/or IHC in germ cells of differentiated gonads of the same species [17,22,23] and in other teleosts [30,32–39]. Thus, *Vasa* is expressed in turbot primordial germ cells where it primarily participates in early gonadal differentiation [17,40] and remains active in differentiated gonads to regulate the development of germ cells [22, present results].

In addition, qPCR and transcriptome analyses showed a dimorphic sex-biased *Vasa* expression with higher expression in females from the onset of gonadal differentiation (90 dpf) onwards [9,20]. Nevertheless, turbot adults showed the highest *Vasa* expression levels in testes (3.5-fold more than ovary) probably due to the delay in primordial germ cells proliferation in males that occurs in this species [22]. However, our SISH results did not support this female-biased expression pattern maybe due to detection of different transcripts of *Vasa* homologues previously reported in the turbot [17,22]. Another reason for *Vasa* sex-biased expression could be environmental factors which alter gene expression [6] as it was reported in medaka which showed a decrease in *Vasa* expression under hypoxia which leading to a male-biased population [41].

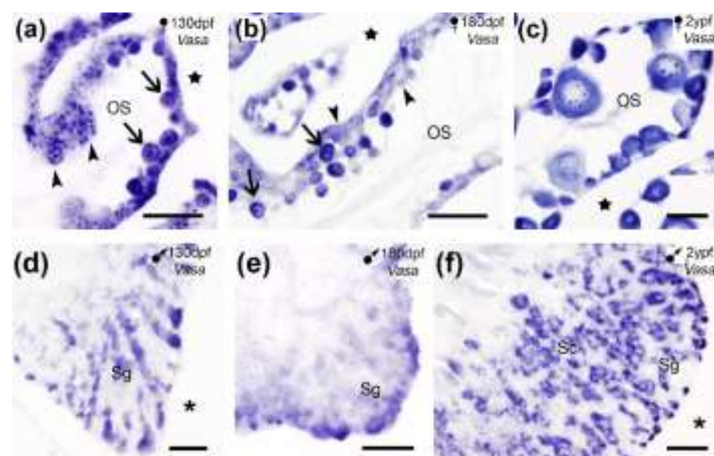

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Figure 3. *Vasa* expression in cross sections of the turbot gonads. (**a-c**) *Vasa* expression in oogonia (arrowheads) and primary oocytes (arrows) in juveniles (a-b) and adults (c). (**d-f**) *Vasa* expression in spermatogonia (Sg) in juveniles (d-e) and in spermatogonia and spermatocytes (Sc) in adults (f). OS, ovarian stroma; stars, ovarian cavity; asterisks, celomic cavity. Scale bars: 100 µm (a-e); 200 µm (f).

3.2.2. Cyp19a1a

Cyp19a1a gene encodes for aromatase, a key enzyme in ovary differentiation, necessary for the starting and maintenance of the ovarian fate throughout development since it catalyzes the transformation of androgens into estrogens and its repression leads to testicular development [42].

In our study, *Cyp19a1a* mRNA was detected in oogonia and the ooplasm of the primary oocytes in juveniles (Figures 4a-b) which increased in adults (Figure 4c), while no *Cyp19a1a* expression was observed in the turbot testis (Figures 4d-f). These results were in concordance with previous qPCR and transcriptomic analyses in turbot which reported a sex-dimorphic *Cyp19a1a* expression which allows discriminating males and females from 105 dpf until 435 dpf [9,13] and maintained in adults [20]. Thus, *Cyp19a1a* is a female-biased gene in turbot suggesting its key role in female differentiation and reproduction [9,13,20,present results].

The cellular localizations of *Cyp191a* transcripts were also investigated using SISH or IHC in the ovary of the killifish [43], the medaka [44], the catfish [45]) and the zebrafish [46], where they were detected in the primary oocytes suggesting a contribution of germ cells in the estrogen synthesis in some teleosts species, including the turbot [present results]. Moreover, numerous molecular analyses have revealed *Cyp191a* expression in the ovary of many other fish species so this gene have been considered as a reliable early marker of ovarian differentiation in these vertebrates [6].

Cyp19a1a is not expressed in the turbot males [present results] but its expression was detected by RT-PCR and/or SISH and IHC in the testis of other teleostean species [7,43,46–48] so *Cyp19a1a* seems to be an indispensable gene not only for female but also for male sex differentiation in some fish. Therefore, data obtained until now about the sex steroid pathway indicate that it is not conserved in fish, supporting the notion of flexibility in sexual determination and differentiation across species.

3.2.3. Foxl2

Foxl2 gene encodes the Forkhead Box (Fox) protein L2, a transcription factor involved and considered a good marker of ovarian differentiation which is highly conserved in vertebrates [49].

Our results in the turbot ovary showed *Foxl2* expression in oogonia and the ooplasm of primary oocytes in juveniles (Figures 4g-h) which increased in adults (Figure 4i). In the testes of juveniles *Foxl2* signal was detected in spermatogonia preferably located close to tunica albuginea (Figures 4j-k), and in spermatogonia, spermatocytes and spermatids in adults (Figure 4j).

Foxl2 expression in ovarian germ cells was also previously reported by IHC in the ovary of some teleots such as the zebrafish [46], the Atlantic salmon [48] and two medaka species [29,50] while its expression in testicular germ cells were only described in the zebrafish [46] and the Atlantic salmon [48]. On the other hand, SISH or IHC have revealed a *Foxl2* expression restricted to somatic ovarian (follicular/granulosa) cells in other teleostean species [45,51,52]. These data suggest a different role for this gene in the regulation of gonad differentiation depending on the species and evidence the need of more research in *Foxl2* characterization in fish to clarify its functions in developing and adult gonads.

In the turbot ovary, *Foxl2* showed a similar expression to *Cyp19a1a* [present results] in accordance with previous qPCR analyses which showed a high *Foxl2* expression in turbot females from 105 dpf onwards positively correlated with *Cyp19a1a* [9]. In fact, *Foxl2* has been described as a direct transcriptional activator of *Cyp19a1a* in teleosts [6,7], including the turbot [9,13]. However, although both genes are co-expressed during turbot sex differentiation until 90 dpf in both sexes, *Cyp19a1a* expression decays at later stages in males while *Foxl2* expression maintains through development [9,13,20] according to our SISH observations in juveniles and adult turbot. These results suggest other role for *Foxl2* not related to the activation of aromatase in turbot [9].

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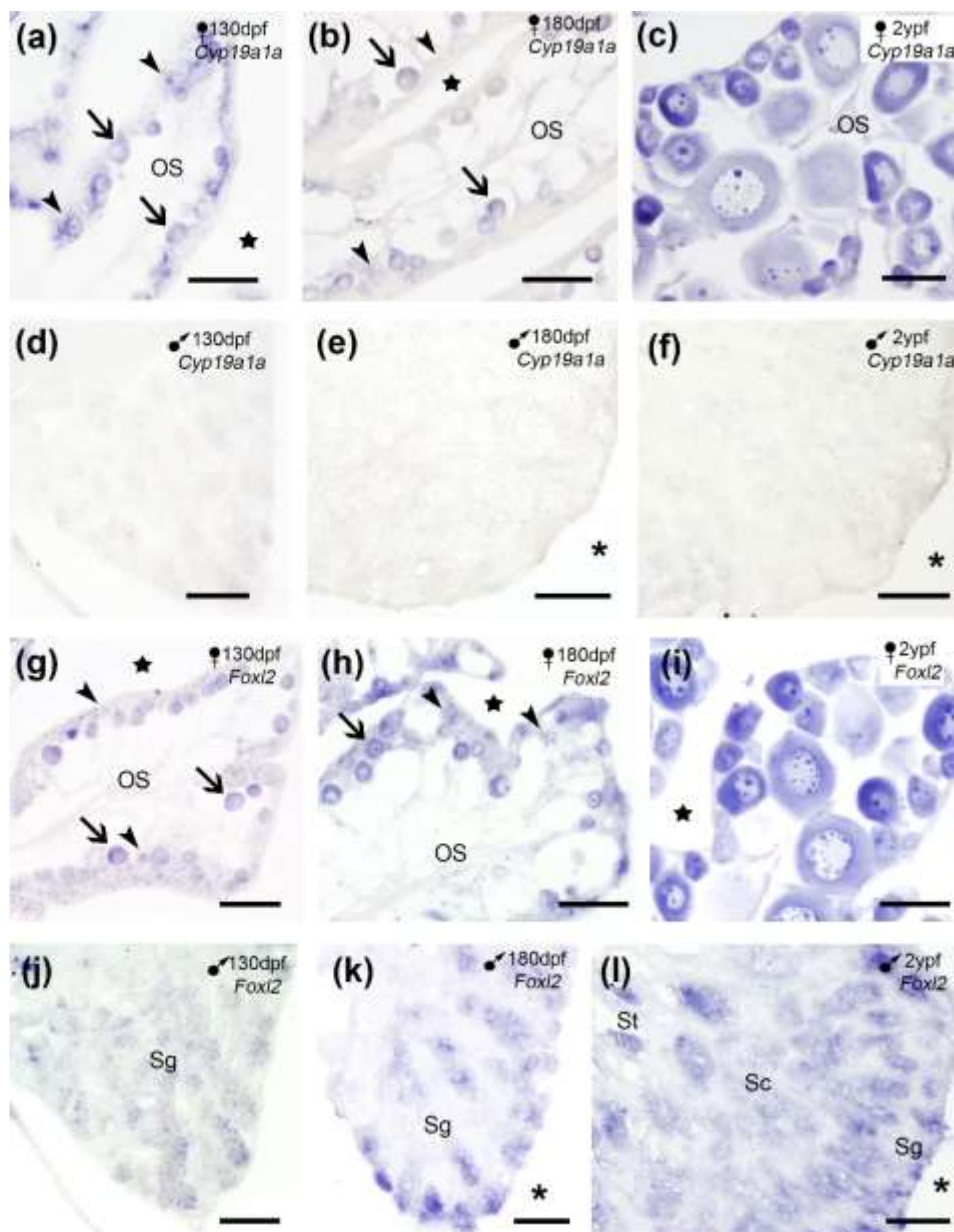

Figure 4. *Cyp19a1a* (a-f) and *Foxl2* (g-l) expression in cross sections of the turbot gonads. (**a-c**) *Cyp19a1a* expression in oogonia (arrowheads) and primary oocytes (arrows) in juveniles (a-b) and adults (c). (**d-f**) No *Cyp19a1a* expression was observed at any developmental stage in males. (**g-l**) *Foxl2* expression in oogonia (arrowheads) and primary oocytes (arrows) in juveniles (g-h) and adults (i). (**j-l**) *Foxl2* expression in spermatogonia (Sg) in juveniles (j-k) and in Sg, spermatocytes (Sc) and spermatids (St) in adults (l). OS, ovarian stroma; stars, ovarian cavity; asterisks, celomic cavity. Scale bars: 100 μm (a-d, g-k); 200 μm (e-f, l).

3.2.4. Anti-Müllerian Hormone (Amh)

Anti-Müllerian hormone (AMH) is mainly known by inducing the regression of Mullerian ducts, a characteristic feature of the female urogenital system in tetrapods [53]. Although Müllerian ducts are lacking in fishes, AMH homologs have been identified in many species of teleosts, indicating a different role for the *Amh* gene which acts a master-key gene of SD in some fish species [5,54]. Studies in turbot [9] and other teleosts species [54–56] have pointed to the inhibitory effect of *Amh* gene in proliferation of germ cells whose number may be crucial for gonadal fate in fish. In fact, a low cell number correlates with male development at least in the zebrafish and the medaka [57,58] and the same could happen in the turbot gonad due to the negative regulation of germ cell proliferation by *Amh* expression. On the contrary, high number of germ cells seems to be linked with ovarian

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differentiation, and proliferative germ cells might trigger *Cyp19a1a* expression in the somatic cells of the gonad [42,57,58].

Considered as a gonad specific factor for male sex differentiation, our results in turbot gonads showed *Amh* RNAm signal in both sexes. In the ovary a weak *Amh* expression was detected in oogonia and the ooplasm of primary oocytes in juveniles (Figures 5a-b) which increased in adults (Figure 5c), like *Cyp19a1a* and *Foxl2* expressions described above [present results]. In the testis, *Amh* mRNA was revealed in spermatogonia at the edge of the testes in juveniles (Figures 5d-e) and spermatogonia and spermatocytes in adults (Figure 5f), like *Foxl2* expression in turbot males [present results]. SISH revealed *Amh* expression in differentiated turbot ovary and testis in consistence with studies by qPCR which showed *Amh* expression from 90 to 135dpf, higher in males [9]. These results suggested that *Amh* has a role in turbot gonad differentiation with functions in both sexes.

However, in other teleosts species SISH has shown *Amh* expression restricted to ovarian and testicular somatic cells [54,55,59–62]. Thus, our results in turbot gonads deviate from this pattern, showing *Amh* expression in both male and female germ cells, as previously reported by IHC for the first time in a fish - in the ovary and testis of the Atlantic salmon [48] - and later in the ovary of the Nile tilapia [55] where somatic cells also exhibited *Amh* expression. Therefore, *Amh* expression is not restricted to the somatic cells in all fish suggesting a role for this gene (and likely also for *Foxl2* and *Cyp19a1a*) on germ cells probably related with regulation of the transcription and activity of specific genes in the surrounding cells through secretory signaling as it was proposed for the Atlantic salmon [48].

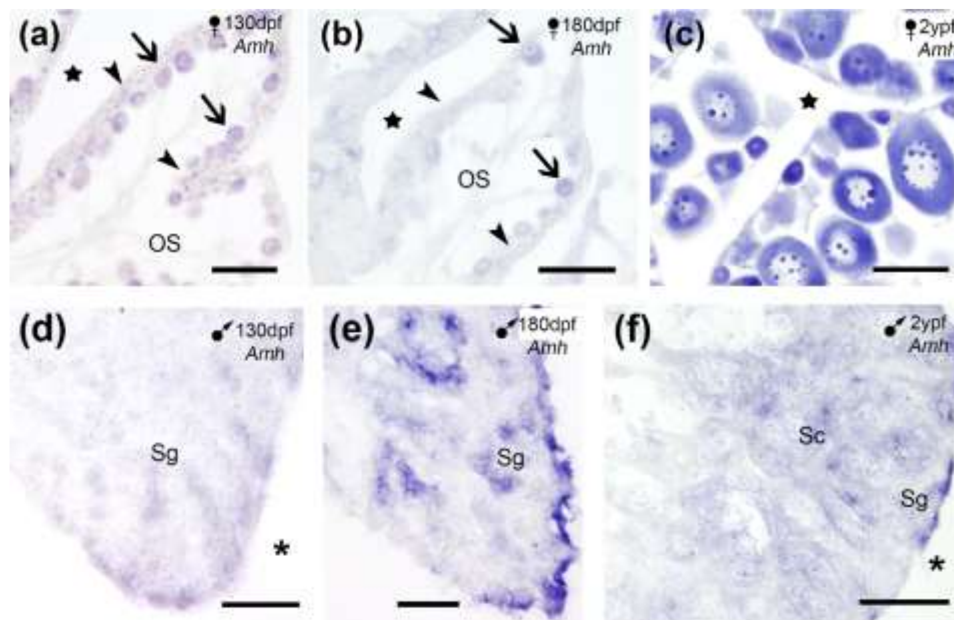

Figure 5. *Amh* expression in cross sections of the turbot gonads. (**a-c**) *Amh* expression in oogonia (arrowheads) and the ooplasm of the primary oocytes (arrows) in juveniles (a-b) and adults (c). (**d-f**) *Amh* expression in spermatogonia (Sg) in juveniles (d-e), and in Sg and spermatocytes (Sc) in adults (f). OS, ovarian stroma; stars, ovarian cavity; asterisks, celomic cavity. Scale bars: 100 µm (a-e); 200 µm (f).

Therefore, *Cyp19a1a*, *Foxl2*, and *Amh* expression were detected by SISH in germ cells of differentiated turbot gonads in accordance with that observed by IHC in the Atlantic salmon [48] and with previous qPCR analyses which showed the expression of these three genes in turbot gonads [9]. On the other hand, many molecular and histological analyses on *Cyp19a1a*, *Foxl2*, and *Amh* in teleost gonads [27,30,54,56,61] showed that their expression can vary depending on the species, developmental stage, sex, or temperature proving the huge variety of mechanisms sexual development present in fish.

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3.2.5. Sox Genes

We have performed SISH to detect the *Sox2*, *Sox17* and *Sox19* expression in the turbot differentiated gonads previously reported by qPCR and microarray in undifferentiated and differentiated gonads of this species [9].

The *Sox* gene family encodes a group of conserved transcription factors related to the mammalian testis-determining factor *sry* (sex determining region Y) and they are developmental regulators of multiple processes in vertebrates, including sex determination and reproduction in fish [63]. Sox genes are classified based upon their HMG box sequences into subgroups A to K found throughout the animal kingdom [64] most of them identified in teleosts (except soxA, soxG and sox J subgroups) in which Sox genes play essential roles in fish embryogenesis and ontogenesis [63]. *Sox2* and *Sox19* belong to the B1 subgroup and *Sox17* to the F group.

3.2.5.1. B1 Sox Genes: Sox2 and Sox19

In turbot, *Sox2* gene deserved special attention since it is located within the turbot main sex determination region [24] and it has been proposed as a candidate SD in this species [15]. Our results in the turbot ovary showed a weak *Sox2* expression in the oogonia which increased along the primary oocyte development (Figures 6a-c) while in testis, *Sox2* was exclusively expressed in spermatogonia-containing cysts, close to the tunica albuginea in juveniles and adults (Figures 6d-f), suggesting effects during the proliferative stage. These observations were in accordance with previous reported in qPCR and microarray analyses where no differences were observed in *Sox2* expression between turbot males and females [9]. As far as we know, there are no *Sox2* expression studies by SIHS or IHC in fish differentiated gonads but qRT-PCR analyses in the ovary or testes of the rohu carp [65], the channel catfish [66], and the Japanese flounder [67] suggesting a role for *Sox2* in gonadogenesis of fish.

On the other hand, a strong *Sox19* expression, a fish-specific gene, was detected by SISH in oogonia and in the ooplasm of the primary oocytes of juvenile and adult turbot (Figures 6g-i) while in testis its expression was much weaker (Figures 6j-l). Thus, *Sox19* can be considered as a female-biased gene in the turbot due higher expression levels were observed in the ovary which is maintained until adulthood suggesting a role in ovarian differentiation. Accordingly, previous qPCR analyses in turbot gonads reported an increase of *Sox19* transcription from 75 to 90 dpf in both sexes, but only in females from 105 dpf to 135 dpf [9], a dimorphic expression which is maintained at 400 dpf [13] and adult specimens [20].

To our knowledge, there are no histological studies on *Sox19* expression in fish except for the common carp where *Sox19* protein was observed in somatic (i.e. granulosa) cells of postvitellogenic oocytes suggesting a role in late ovarian differentiation in this species [68]. Moreover, RT-PCR analyses showed that this gene is more expressed in adult ovary than in the testis in the common carp [68] and in the European sea bass [69] and playing a role in the differentiation of the ovary but not the testis.

Furthermore, *Sox2* and *Sox19* have a crucial role in neurogenesis and nervous system development in vertebrates, including fish [70]. In fact, *Sox2* and *Sox19* present a similar expression pattern by SISH in the developing and adult turbot forebrain which suggest similar functions in brain development in this species [71]. Moreover, *Sox2* expression in subependymal cells of the preoptic and hypothalamic regions could indicate a role for this gene in the control of reproduction, onset of puberty and sexual maturation in turbot [71]. *Sox2* and *Sox19* expression were also reported in the brain of other teleostean species, so their involvement in the regulation of the reproductive system through the brain-pituitary-gonadal axis in these vertebrates [63,67,69,70,72,73]. A recent study by histological methods reported a distinct sex difference in the turbot brain during the gonadogenesis with a total brain volume and a pituitary gland size larger in mature males [26] as it was observed in the brain of other teleosts species [70]. However, how the brain regulates sex determination and differentiation in teleosts is still needs to be unraveled [7].

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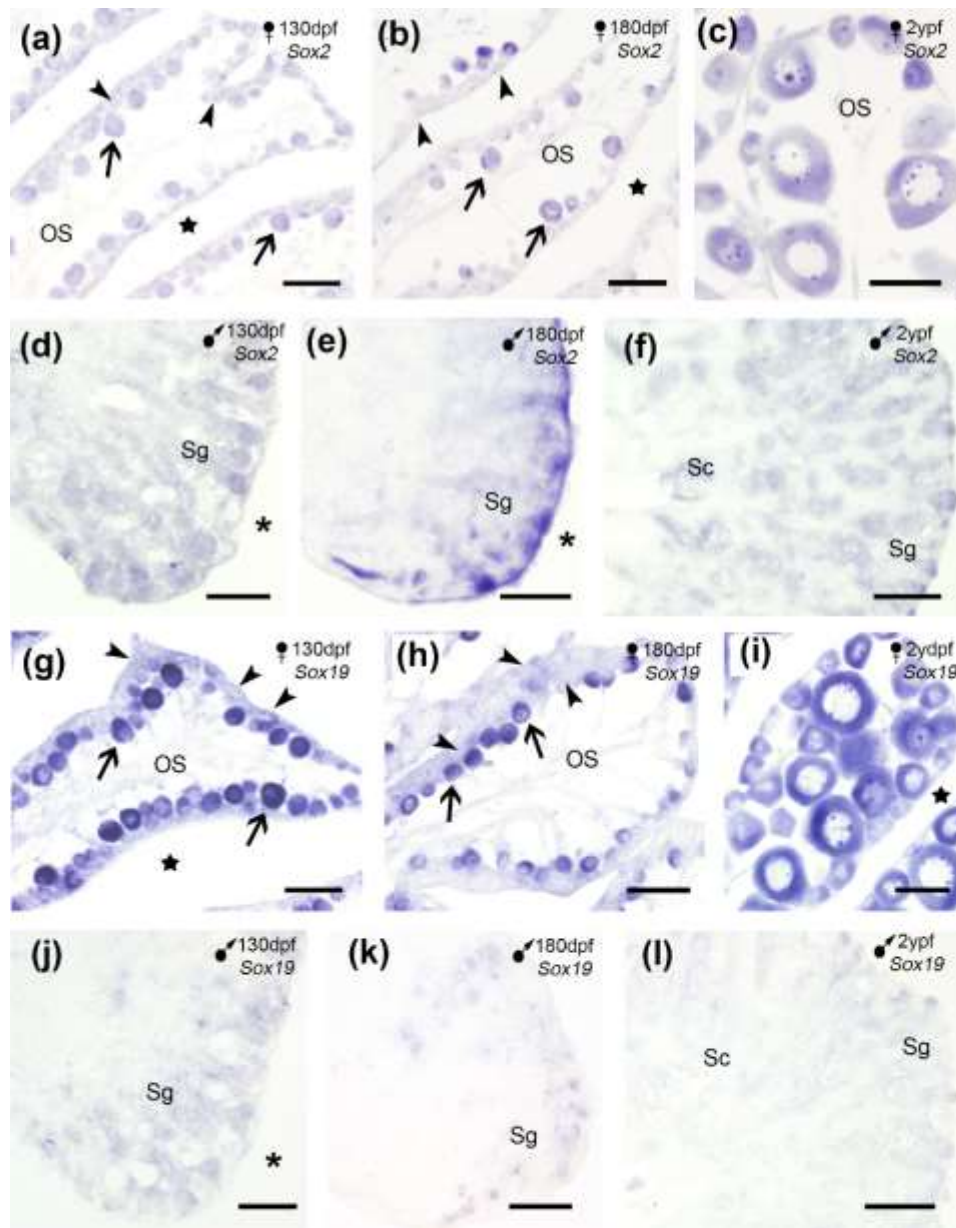

Figure 6. *Sox2* (a-f) and *Sox19* (g-l) expression in cross sections of the turbot gonads. (**a-c**) *Sox2* expression in oogonia (arrowheads) and primary oocytes (arrows) in juveniles (a-b) and adults (c). (**d-f**) In the testis, *Sox2* expression was observed in spermatogonia (Sg) in juveniles (d-e) and Sg and spermatocytes (Sc) in adults (f). (**g-i**) *Sox19* expression in oogonia (arrowheads) and primary oocytes (arrows) in juveniles (g-h) and adults (i). (**j-l**) Weak *Sox19* expression in spermatogonia of juveniles (j-k) and adults (l). OS, ovarian stroma; stars, ovarian cavity; asterisks, celomic cavity. Scale bars: 100 µm (a-e, g-k); 200 µm (f, l).

Therefore, *Sox2* and *Sox19*, as other SoxB1 genes (i.e. *Sox1* and *Sox3*), play an essential regulatory role in gonadal development, sex determination and differentiation, and in nervous system development of fish [61,68].

3.2.5.2. F Sox Genes: Sox17

Sox17 showed a very weak no sexual dimorphic expression in juveniles and adult turbot gonads, which was detected in oogonia and the ooplasm of the primary oocytes (Figures 7a-c) and in spermatogonia in the testis (Figures 7d-f). In accordance with our SISH observations, transcriptomic studies reported a weak *Sox17* expression in ovary and testis of the adult turbot [20]. In fact, qPCR and microarray analysis of *Sox17* expression in turbot reported a peak of expression around the onset

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of sex differentiation (90 dpf) in both sexes concomitantly with a raise in the expression of other germ cell markers such as *Vasa* [9,13]. These data suggest some function for this gene, previously mapped in sex-related QTL [25], in early gonadogenesis and germ cell proliferation [9].

Although as far as we know, there are no *Sox17* expression studies by SISH in fish differentiated gonads except for the rice eel where *Sox17* mRNA was detected in the germinal lamellae of ovaries (but not in the oocytes), ovotestis and testes as well as in developing germ cells of the testis during sex change from male to female which this species undergoes along its life [74]. Thus, most of *Sox17* expression data in teleost gonads were obtained by RT-PCR which detected *Sox17* transcripts in the gonads of the Japanese flounder [67], the Nile tilapia [72], the channel catfish [73], the rice eel [74], the sea bass [75], the medaka [76], and the Yellow River carp [77]. Although all these data was obtained by molecular analyses and avoid attribute a specific function to *Sox17* in fish reproduction, allow knowing that this gene is expressed in the testis and ovary of fish, suggesting its important role in sex differentiation of these vertebrates [63,70].

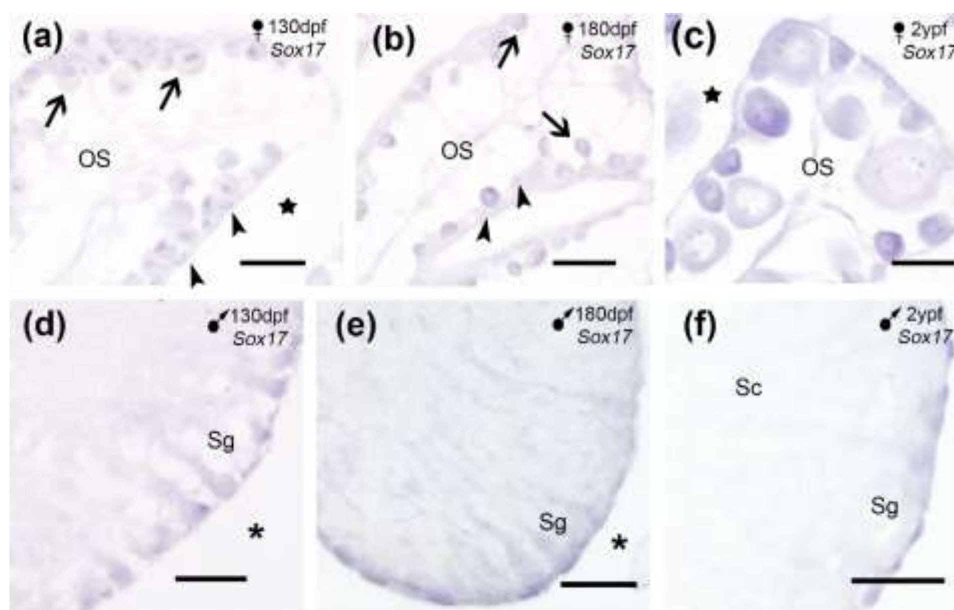

Figure 7. *Sox17* expression in cross sections of the turbot gonads. (**a-c**) *Sox17* expression in oogonia (arrowheads) and primary oocytes (arrows) in juveniles (a-b) and adults (c). (**d-f**) *Sox17* expression was observed in spermatogonia (Sg) in juveniles (d-e) and adults (f). OS, ovarian stroma; stars, ovarian cavity; asterisks, celomic cavity. Scale bars: 100 µm (a-e); 200 µm (f).

To our knowledge there is no still data on the *Sox17* expression in the turbot brain, but *Sox17* transcripts were detected in the brain of the Japanese flounder [67], the rice eel [74], the sea bass [75], and the Yellow River carp [77] suggesting that SoxF genes may be involved in the regulation of the nervous system as it was pointed for SoxB1 genes.

4. Conclusions

In the present study, several genes were analyzed for the first time by *in situ* hybridization (ISH) in turbot gonads at different developmental stages. We performed ISH on differentiated ovaries and testes paraffin sections to know the cell/tissue specific expression of sex differentiation and gonadal development related genes in this species. *Vasa*, *Foxl2*, *Amh*, *Sox2* and *Sox17* expression were observed in both ovary and testis while *Cyp19a1a* and *Sox19* expression was ovary specific, suggesting a role for these genes in gonad development or reproductive functions in turbot. Moreover, ISH results support previous data on these genes expression by molecular analyses in the turbot gonads and highlight the cellular types, i.e. germ cells, where they are expressed. Therefore, we consider ISH provides complementary information to the functional studies with transcriptomic or proteomic

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techniques so gives spatial information on RNA in a tissue sample, i.e. cellular localization, to better understand the molecular mechanisms of sex differentiation in vertebrates. However, further research in gonadal sections of early developmental turbot stages is needed to gain knowledge on the gonadal development in this flatfish species from a histological point of view. Finally, comparison with the results obtained in differentiated gonads of other teleosts species showed a similar tissue-specific gene expression but also revealed the notable variety of sexual development and differentiation molecular mechanism in fish.

Author Contributions: Conceptualization, A.V.; methodology, F.A and X.T.; supervision of the data collection and research activity execution, F.A and A.V.; analysis and interpretation of the data F.A and A.V.; writing-original draft and designing of the figures, F.A. and V.A; writing, review and editing, F.A., P.M. and A.V; funding acquisition, A.V. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of the University of Santiago de Compostela and all procedures were in accordance with the guidelines established by the European Union Directive (2010/63/UE) and by the Spanish Royal Decree 118/2021 for animal experiments.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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