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**Localization of steroidogenic enzymes and Foxl2a in the gonads of mature zebrafish (*Danio rerio*).**

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## ABSTRACT

In zebrafish, the identification of the cells expressing steroidogenic enzymes and their regulators is far from completely fulfilled though it could provide crucial information on the elucidation of the role of these enzymes. The aim of this study was to better characterize the expression pattern of steroidogenic enzymes involved in estrogen and androgen production (Cyp17-I, Cyp11c1, Cyp19a1a and Cyp19a1b) and one of their regulators (Foxl2a) in zebrafish gonads. By using immunohistochemistry, we localized the steroid-producing cells in mature zebrafish gonads and determined different expression patterns between males and females. All these steroidogenic enzymes and Foxl2a were detected both in testis and ovary. In the testis, they were all localized both in Leydig and germ cells except Cyp19a1b which was only detected in germ cells. In the ovary, Cyp17-I, Cyp19a1a and Foxl2a were immunolocalized in both somatic and germ cells while Cyp19a1b was only detected in germ cells and Cyp11c1 in somatic cells. Moreover, Cyp19a1a and Foxl2a did not display exactly the same patterns of spatial localization but their expressions were correlated suggesting a possible regulation of *cyp19a1a* gene by Foxl2a in this fish species. Comparative analysis revealed a dimorphic expression of Cyp11c1, Cyp19a1a, Cyp19a1b and Foxl2a between males and females. Overall, our study provides a detailed description of the expression of proteins involved in the biosynthesis of steroidal hormones at the cellular scale within male and female gonads, which is critical to further elucidating the intimate roles of the enzymes and the use of the zebrafish as a model in the field of endocrinology.

**Key-Words:** 11- $\beta$ -hydroxylase; 17- $\alpha$ -hydroxylase, 17-20 lyase; aromatases; Foxl2a; immunohistochemistry; ovary; steroidogenic enzymes; testis; zebrafish

## 1. Introduction

Zebrafish (*Danio rerio*) is a small freshwater fish that has been extensively used in developmental biology and is increasingly used as a model organism in the field of endocrinology (for review see Tokarz et al., 2013). Despite the progresses made in the past few years in zebrafish endocrinology, some key issues have received little attention so far. As

regards to steroid research, it was stated that an extensive functional characterization of zebrafish steroidogenic enzymes is desirable (Tokarz et al., 2013). Indeed, although tissue expression of steroidogenic genes is well characterized in this species (Chiang et al., 2001; Wang and Ge, 2004; Sawyer et al., 2006; Wang and Orban, 2007), the identification of the cells expressing steroidogenic enzymes and their transcriptional regulators is far from completely fulfilled though it could be a critical step in the elucidation of the intimate roles of these enzymes in the different cell types of the gonads.

Steroidogenic enzymes are responsible for the biosynthesis of steroid hormones that are key regulators of important biological processes including sexual differentiation and reproduction in many vertebrates (Baroiller et al., 1999). Steroidogenesis begins with the rate-limiting transport of cholesterol into mitochondria mediated by steroidogenic acute regulatory protein (Star) (Miller and Bose, 2011). Subsequently, this process is supported by numerous enzymes including several P450 cytochromes (CYPs) (Baroiller et al., 1999; Tokarz et al., 2013).

Among these CYPs, the 17- $\alpha$ -hydroxylase,17,20 lyase (Cyp17), the 11- $\beta$ -hydroxylase (Cyp11c1, formerly known as Cyp11b) and aromatases (Cyp19a1) are key enzymes involved in the biosynthesis of estrogens and androgens. In teleosts, there are two types of Cyp17, i.e. Cyp17-I possessing 17 $\alpha$ -hydroxylase and 17, 20-lyase activities, and Cyp17-II possessing only 17 $\alpha$ -hydroxylase activity; as well as two isoforms of aromatase, i.e. Cyp19a1a mainly expressed in the gonads and Cyp19a1b mainly expressed in the brain (Tchoudakova and Callard, 1998; Chiang et al., 2001; Kobayashi et al., 2004; Zhou et al., 2007a; Zhou et al., 2007b; Jin et al., 2012). Male and female gonads are one of the major sites of steroid production, both expressing these steroidogenic enzymes. Similar to mammals, these enzymes are strongly expressed in Leydig cells of the testes of several fish species including zebrafish (Wang and Orban, 2007; De Waal et al., 2009; Hinfrey et al., 2011; Hinfrey et al., 2013). Recently, we also demonstrated the presence of Cyp17-I and Cyp19a1 enzymes in testicular germ cells of the zebrafish (Hinfrey et al., 2013). In fish ovary, steroidogenic enzymes are expressed in follicular layer cells and/or in the cytoplasm of oocytes and interstitial cells. In zebrafish, *cyp19a1a* mRNA was detected in follicular layer cells (Rodriguez-Mari et al., 2005; Wang and Orban, 2007) while *cyp11c1* mRNA was never detected in the ovary (Wang and Orban, 2007).

Forkhead box 12 (Foxl2) is a putative winged helix/forkhead transcription factor involved in ovarian differentiation but also follicle development and maintenance in mammals (Baron et al., 2005; Boulanger et al., 2014), birds (Govoroun et al., 2004) and fish (Baron et al., 2004;

Yamaguchi et al., 2007; Ijiri et al., 2008; Dong et al., 2011; Sridevi and Senthilkumaran, 2011; Hattori et al., 2012). In various teleost species including zebrafish, two paralogs of *foxl2* named *foxl2a* and *foxl2b* (also known as *foxl2* and *foxl3*), have been described or predicted and considered as fish-specific duplicates (Baron et al., 2005; Jiang et al., 2011; Crespo et al., 2013). Moreover, *foxl2a* and *cyp19a1a* and/or *cyp19a1b* transcripts levels are positively correlated in catfish (Sridevi and Senthilkumaran, 2011; Sridevi et al., 2012), in rainbow trout (Baron et al., 2004; Vizziano et al., 2007), in medaka (Nakamoto et al., 2006) and in tilapia (Wang et al., 2007). *Foxl2a* has been shown to regulate the transcription of *cyp19a1* genes either directly or indirectly by interacting with Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) or fushi tarazu factor 1 (FTZ-F1) (Wang et al., 2007; Yamaguchi et al., 2007; Sridevi et al., 2012). *FOXL2* is also known to negatively regulate the expression of *CYP17* in mice (Park et al., 2010) and of *STAR* in human (Pisarska et al., 2004). Altogether, these data suggest that *Foxl2* might be able to regulate the entire steroidogenic process. In fishes, *foxl2a* displays a clear dimorphic expression in the gonads with higher expression in ovary as compared to testis (Baron et al., 2004; Ijiri et al., 2008; Jiang et al., 2011; Sridevi and Senthilkumaran, 2011; Hattori et al., 2012; Crespo et al., 2013), and seems to be mainly expressed in follicular cells surrounding previtellogenic and vitellogenic oocytes (Nakamoto et al., 2006; Wang et al., 2007; Nakamura et al., 2009; Kobayashi et al., 2010b; Herpin et al., 2013).

In this context, the aim of this study was to provide a comprehensive characterization of the expression patterns and localization of steroidogenic enzymes (*Cyp17-I*, *Cyp11c1*, *Cyp19a1a* and *Cyp19a1b*) and one of their regulator (*Foxl2a*) in ovary and testis of zebrafish. By means of immunohistochemistry, we precisely localized the cellular expression of steroidogenic enzymes and *Foxl2a* in zebrafish gonad tissues and we newly described the pattern of expression of *Cyp19a1b* in ovary. Furthermore, comparative analysis of the expression of these enzymes showed dimorphic patterns for the expression of *Cyp11c1*, *Cyp19a1a*, *Cyp19a1b* and *Foxl2a* between males and females, which is consistent with the known circulating steroids levels in this species.

## 2. Materials and Methods

### 2.1. Zebrafish rearing

Wild type zebrafish (AB strain) were bred in our laboratory facility at INERIS (Institut National de l'Environnement Industriel et des Risques, Verneuil-en-Halatte, France). Zebrafish were maintained in 3.5 L aquaria in a recirculation system (Zebtec, Tecniplast, Buguggiate, Italy) on a 14:10 light:dark cycle at a temperature of 27.0 +/- 2.0°C. Animal handling and experimentation were in accordance with the EU Directive 2010/63/EU for animal experiments.

## **2.2. Fluorescence immunohistochemistry**

Zebrafish were euthanized in ice-cold water (Wilson JM et al., 2009). Gonads were removed and fixed in modified Davidson's fixative for 48 hours at room temperature. This fixative has been selected based on previous experiments for its good ratio signal/noise in immunohistochemistry experiments. After fixation, samples were dehydrated in ethanol, cleared in toluene and embedded in paraffin, according to conventional procedures. Zebrafish gonads were sectioned at 5 µm. Sections were dewaxed and rehydrated, and antigens were unmasked for 3 hours at 80°C in EDTA buffer (1mM, pH 8.5). Immunohistochemistry was then carried out using an In situ Pro VSI, an Intavis robotic station. Tissue sections were washed three times in PBS and incubated for 1 hour in a blocking solution (PBS containing 0.2% Triton X-100 and 1% milk powder). Incubation with primary antibodies against Foxl2a, Cyp17-I, Cyp11c1, Cyp19a1a or against Cyp19a1b (Table I) was performed overnight (1:300 (anti-Foxl2a, anti-Cyp17-I, anti-Cyp11c1 or anti-Cyp19a1b) or 1:600 (anti-Cyp19a1a) with 0.5% milk powder in PBS) at ambient temperature. Sections were rinsed three times with PBS containing 0.2% Triton X-100 and incubated for 1h30 with a goat anti-rabbit or a goat anti-rat (anti-Cyp11c1) antibody coupled to AlexaFluorR 594 (Invitrogen, Praisley, UK) (1:200 with 0.5% milk powder in PBS). Nuclei were revealed with Hoechst 33342. The specificity of the immunoreactivity was controlled by processing adjacent sections without primary antibody, with primary antibody pre-adsorbed with the peptides (100 µg/ml) for Cyp19a1b and Cyp17-I or with the pre-immune serum for Cyp11c1 and Foxl2a. In these negative controls no cross-reactivity was seen for any of the antibodies (data not shown).

## **2.3. Immunohistochemistry image analysis**

Immunofluorescence was observed with a Zeiss AxioImager.Z1 fluorescence microscope equipped with an AxioCam Mrm camera and combined with an ApoTome (Zeiss GmbH,

Gottingen, Germany). All micrographs presented in this article were taken using the ApoTome system and the Axiovision Imaging software.

Semi-quantification of fluorescence was performed on micrographs taken with the Zeiss AxioImager.Z1 fluorescence microscope without the ApoTome system. For each antibody, the micrographs were acquired with the same magnification (x10 objective) and time of exposure for all individuals. Fluorescence was measured as integrated density (IntDen, ImageJ software), i.e. the sum of the grey values of all the pixels in the region of interest. For each targeted protein, three micrographs per individual and 5 to 7 individuals per sex were analyzed. For each individual, the background fluorescence of the gonadal tissue was measured (sections processed in IHC without primary antibodies) and deduced from the measured fluorescence of the targeted proteins.

#### **2.4. Data analysis and statistics**

For each targeted protein, data obtained were expressed as relative fluorescence intensity as compared to the mean value of IntDen of females which was set to 1.

For each targeted protein, data of fluorescence (Int Den of the gonad – Int Den of the control gonad without antibodies) were subjected to a non parametric Wilcoxon test to investigate sex differences using R<sub>TM</sub> (R software, R development Core Team).

The non parametric Spearman's Rho test was used to assess the rank correlations between the fluorescence intensities measured for each of these proteins both in male and female gonads. Differences between groups for each statistical test were considered to be significant if  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*).

### **3. Results**

#### **3.1. Immunolocalization of Cyp17-I, Cyp11c1, Cyp19a1a, Cyp19a1b and Foxl2a in zebrafish gonads**

By using immunohistochemistry methods, we were able to localize the cellular sites of expression of Cyp11c1, Cyp19a1a, Cyp19a1b and Foxl2a proteins in zebrafish ovary (Figure 1). Similarly, we identified the sites of expression of Cyp11c1 and Foxl2a proteins in zebrafish testis (Figure 2), and confirmed those previously found for Cyp17-I, Cyp19a1a and Cyp19a1b proteins in zebrafish testis (Hinfray et al., 2013).

In ovary, Cyp17-I, Cyp11c1 and Cyp19a1a were detected in the follicular layer cells (FC) (theca and/or granulosa cells) surrounding vitellogenic oocytes but not pre-vitellogenic oocytes (Figure 1 A-M) except for some Cyp19a1a-immunoreactive FC in late pre-vitellogenic oocytes (Figure 1 I). No Cyp19a1b positive FC was revealed (Figure 1 N, O). In contrast, Foxl2a was localized to FC surrounding pre-vitellogenic oocytes but not vitellogenic oocytes (Figure 1 P, Q). Interestingly, all the targeted proteins except Cyp11c1 were also localized in the cytoplasm of oocytes. Cyp17-I was detected in the ooplasm of pre-vitellogenic oocytes (Figure 1 A, C), while the two aromatases and Foxl2a were detected in the ooplasm of both pre-vitellogenic and vitellogenic oocytes (Figure 1 I-Q). Additionally, Cyp17-I, Cyp11c1 and Cyp19a1a were also labeled in the cytoplasm of some interstitial cells (Figure 1 D, H, M).

In testis, all steroidogenic enzymes and Foxl2a were detected (Figure 2 A-Q). Cyp17-I, Cyp11c1, Cyp19a1a but not Cyp19a1b were immunolocalized in the cytoplasm of Leydig cells (Figure 2 A, B, D, E, H, I, L). Interestingly, the four steroidogenic enzymes were also detected in the cytoplasm of germ cells (Figure 2 A, C, D, F, G, H, J, K, L). Cyp17-I and Cyp19a1a were localized in all types of germ cells; Cyp19a1b was detected in all types of germ cells except spermatozoa whereas only spermatogonia were immunolabeled for Cyp11c1. Foxl2a was found in the nucleus of Leydig cells (Figure 2 M-O) and all types of germ cells (Figure 2 M, P, Q).

### 3.2. Semi-quantitative analysis of the expression of the target proteins

The fluorescence intensity measured in the gonads for each target protein was analyzed to compare protein expression between ovary and testis (Figure 3). A significant variability, within and between gender, was noted for most of the proteins investigated. While Cyp17-I did not show significant dimorphism of expression between ovary and testis ( $p = 0.137$ ; Figure 3), strong differences were noticed for the other proteins. For instance, a marked difference was revealed as regard to Cyp11c1 expression with a 6.4-fold higher expression in testis as compared to ovary ( $p = 0.022$ ; Figure 3). For Cyp19a1a, Cyp19a1b and Foxl2a, a dimorphic expression was found, with the highest fluorescence intensities measured in ovary as compared to testis. Indeed, there was a significant 3.7-, 6.9- and 8.9-fold higher expression in ovary than in testis for Cyp19a1a, Cyp19a1b and Foxl2a respectively.

Interestingly, a positive and significant correlation was found between the transcriptional factor Foxl2a and Cyp19a1a in ovary (the Spearman's Rho value (R) was 0.78,  $p = 0.03624$ ,

n=7) and testis (R=0.88, p = 0.01885, n=6). Conversely, no significant correlation has been found between Foxl2a and Cyp19a1b, neither in ovary nor in testis.

## 4. Discussion

### 4.1. Gender specific localization of proteins encoding for steroidogenic enzymes in the gonads

#### 4.1.1. Testicular localization

Cyp17-I and Cyp11c1 are two steroidogenic enzymes involved in the biosynthesis of androgens and 11-oxygenated androgens. Both *cyp17-I* and *cyp11c1* genes are known to be expressed in zebrafish mature testis (Sawyer et al., 2006; Wang and Orban, 2007; Hinfrey et al., 2011; Baudiffier et al., 2012; Baudiffier et al., 2013; Hinfrey et al., 2013). Using immunohistochemistry, we confirmed the cellular localization of Cyp17-I and Cyp11c1 proteins in zebrafish testis. Cyp17-I and Cyp11c1 protein expression was detected both in Leydig cells and in germ cells (only in spermatogonia for Cyp11c1). Several studies have previously demonstrated the presence of these genes and/or enzymes in Leydig cells of different species including zebrafish (Table II). More recently, these two enzymes were also reported to be present in male germ cells in fish (Vinas and Piferrer, 2008; Sreenivasulu and Senthilkumaran, 2009; Zhang et al., 2010; Hinfrey et al., 2013). The expression of these enzymes in fish testis is consistent with the requirement of androgens for both *ex-vivo* and *in vivo* spermatogenesis (Miura et al., 1991; De Waal et al., 2009), and the regulative actions of androgens in tubule formation, germ cell differentiation, sperm secretion and steroid production (Rolland et al., 2013).

Cyp19a1a and Cyp19a1b that are responsible for the biosynthesis of estrogens were also localized to zebrafish testis. We showed that Cyp19a1a is expressed in both Leydig cells and germ cells while Cyp19a1b was only localized to germ cells, confirming our previous results (Hinfrey et al., 2013). Several studies reported no aromatase immunoreactivity in fish testis (Table II), while aromatase genes are known to be expressed in this tissue (Kwon et al., 2001; Kobayashi et al., 2004; Sawyer et al., 2006; Hinfrey et al., 2011). However, in the gobiid fish *Trimma okinawae* and in the protandrous black porgy, Cyp19a1a gene and/or protein have been detected in Leydig cells of the testis (Kobayashi et al., 2004; Guiguen et al., 2010). In sea bass, RT-PCR experiments revealed the presence of *cyp19a1a* gene in testicular germ cells (Vinas and Piferrer, 2008). Aromatase immunolabeling was also reported in both Leydig

and germ cells of the testis of rainbow trout and atlantic salmon (Kotula-Balak et al., 2008; von Schalburg et al., 2013). Moreover, estrogen receptors have been localized to testis somatic and germ cells of different fish species (Bouma and Nagler, 2001; Wu et al., 2001; Menuet et al., 2002; Vinas and Piferrer, 2008). Altogether, these data suggest a role for estrogens in steroids production and spermatogenesis. Indeed, several evidences revealed a role of estradiol in spermatogonial stem cell renewal in fish (Miura et al., 1999; Miura et al., 2003).

#### **4.1.2. Ovarian localization**

In the ovary, our results showed a positive immunoreactivity to enzymes involved in the biosynthesis of androgens, Cyp17-I and Cyp11c1. Cyp17-I was localized in the cytoplasm of follicular cells surrounding vitellogenic oocytes, of pre-vitellogenic oocytes and of interstitial cells. In our previous study, no Cyp17-I immunoreactivity was evidenced in the ooplasm (Hinfray et al., 2011). This difference is likely due to the use of different fixative agents between this study (modified Davison's) and the previous one where Bouin fluid was used. The rationale for using Davidson's instead of Bouin fixative in the present study was based on the fact that it gives comparable morphological gonad sections for histopathological purposes, is less hazardous, requires fewer rinses before transfer of samples into alcohol and is also a recommended fixative by OECD for histopathological investigations (OECD, 2010). Furthermore, the presence of picric acid in Bouin's fixative requires strong antigens retrieval to unmask the antigens that are not always efficient. In other fish species such as Gilthead seabream, Medaka, Tilapia, Eel or Catfish, Cyp17, Cyp17-I and Cyp17-II expressions were always detected in the follicular cells surrounding pre-vitellogenic and/or vitellogenic oocytes by IHC or ISH (Table III) (Ijiri et al., 2003; Zhou et al., 2007a; Zhou et al., 2007b; Nakamura et al., 2009; Sreenivasulu and Senthilkumaran, 2009; Tosaka et al., 2010; Zapater et al., 2012). Recently, both *cyp17-I* and *cyp17-II* mRNA were localized to the ooplasm of primary (only *cyp17-II*) and vitellogenic oocytes in Gilthead seabream (Zapater et al., 2012). In our study, Cyp11c1 was localized to the cytoplasm of follicular cells surrounding vitellogenic oocytes and of interstitial cells. In a previous study in zebrafish, Wang and Orban (2007) reported an absence of *cyp11c1* mRNA labeling in ovary by ISH. In the protogynous Honeycomb grouper and Malabar grouper, Cyp11c1 immunoreactive cells were only observed in the vicinity of blood vessels at the periphery of the ovary (Alam et al., 2005; Alam et al., 2006; Murata et al., 2011). Recently, in the ovary of a protandrous anemonefish, a

weak Cyp11c1 signal was detected in theca cells surrounding developed oocytes and *in vitro* experiments showed the ability of the female-phase ovary to synthesize a small amount of 11-ketotestosterone (Miura et al., 2008). In rainbow trout, ISH signals for 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) mRNA, which is also involved in 11-oxygenated androgens production in fish, were found in theca cells of early vitellogenic ovarian follicles and in theca and granulosa cells of mid-vitellogenic and postovulatory follicles (Kusakabe et al., 2003). In Japanese eel ovary, androgen receptors have been localized by ISH in the epithelial cells of the ovigerous lamellae and in the follicular cells, some of them being also steroid-producing cells (Cyp17-I immunolabeled cells) (Tosaka et al., 2010). Moreover, 11-oxygenated androgens have been shown to promote growth of late stage of primary follicles in Coho salmon and Eel (Lokman et al., 2007; Forsgren and Young, 2012). Our finding, together with previous studies in other fish species, argues for a local production and role of 11-oxygenated androgens in fish ovary.

In addition, the expression of Cyp19a1 proteins were also detected in female gonads which is in line with the known pivotal role of estrogens in these tissues (Guiguen et al., 2010). Cyp19a1a was detected in the cytoplasm of follicular cells surrounding late pre-vitellogenic and vitellogenic oocytes, in the ooplasm and in the cytoplasm of interstitial cells of the zebrafish ovary. It is well admitted that in fish Cyp19a1a gene/protein is expressed in granulosa and/or theca cells of the ovary (Table III). Studies in zebrafish localized *cyp19a1a* by ISH in follicular cells of the oocytes (Rodriguez-Mari et al., 2005; Wang and Orban, 2007), mainly in follicular cells surrounding vitellogenic oocytes (Rodriguez-Mari et al., 2005). These results are consistent with the role of estrogens in the endocrine regulation of oocyte growth during vitellogenesis. Several studies also reported the presence of Cyp19a1a gene/protein in the germinal compartment, i.e. the ooplasm, of different fish species (Park et al., 2008; Gohin et al., 2011; Raghuvver et al., 2011; Zapater et al., 2012). In rainbow trout, a significant ovarian aromatase expression and activity was measured in the ooplasm of late vitellogenic oocyte, demonstrating the presence and functionality of the aromatase enzyme in the germinal compartment (Gohin et al., 2011). The presence of aromatase in interstitial cells has been reported in few species, i.e. the Tilapia and the gobiid fish *Trimma okinawae*, but the role of this expression is currently not elucidated (Sunobe et al., 2005; Wang et al., 2007; Ruksana et al., 2010). As regards to Cyp19a1b, previously published studies in the Atlantic salmon and the gobiid fish *Trimma okinawae* reported an absence of Cyp19a1b gene/protein

labeling in the ovary of these species despite the fact that this gene is known to be expressed in this tissue (Kobayashi et al., 2004; von Schalburg et al., 2013). Interestingly, Cyp19a1b immunolabeling was found in zebrafish ovaries but only in the ooplasm of pre-vitellogenic and vitellogenic oocytes. To our knowledge, our study is the first one that localizes the sites of expression of Cyp19a1b in the fish ovary. The precise role of this enzyme in ovarian germ cells is not known but *cyp19a1b* transcripts have been shown to be maternally inherited in fish including zebrafish since they are detected in unfertilized eggs (Callard et al., 2001; Sawyer et al., 2006; Shanthanagouda et al., 2012). In zebrafish, unfertilized eggs are even 10-fold enriched in *cyp19a1b* transcripts as regards ovary, suggesting a preferential synthesis or accumulation in mature oocytes (Sawyer et al., 2006). This maternally inherited Cyp19a1b protein could play a role in the early biosynthesis of estrogens that are essentials for embryolarval development, notably in neural development (for review see (Diotel et al., 2010)). Therefore, the content of Cyp19a1b protein in mature oocytes and unfertilized eggs could significantly play a role in egg quality as already shown for some other maternally inherited mRNAs (for a review see (Lubzens et al., 2010)).

#### **4.2. Sex specific relative quantification of expression of steroidogenic enzymes in the gonads**

Cyp17-I did not show a significant dimorphic pattern of expression between testis and ovary in our study, which is consistent with the need of both estrogens and androgens in these tissues for gonadogenesis and the gene expression levels (Wang and Ge, 2004; Table S1). In contrast, Cyp11c1 exerted a higher expression in testis as compared to ovary. In zebrafish adult gonads, RT-PCR experiments showed a clear dimorphic pattern of *cyp11c1* gene expression in favor of male gonads (Wang and Orban, 2007; Table S1). Consistent with the pattern of expression, the circulating and gonadal concentrations of 11-ketotestosterone are known to be higher in male as compared to female in zebrafish (Wang and Orban, 2007; Liu et al., 2012; Liu et al., 2013). While the high concentrations of 11-oxygenated androgens are consistent with their crucial role in testis to support the whole process of spermatogenesis (Schulz et al., 2010), their roles in fish ovary remains to be explored.

Consistent with the important role of aromatase in ovary, the relative quantification of Cyp19a1a and Cyp19a1b protein expression in zebrafish gonads indicated a higher expression in ovary than in testis. In zebrafish, ovaries were shown to have a higher *cyp19a1a* gene

expression than testes (Trant et al., 2001; Sawyer et al., 2006; Table S1). These results are consistent with the higher aromatase activity measured in fish ovaries as compared to testis (Gonzalez and Piferrer, 2002; Jeng et al., 2005; Blázquez et al., 2008; Hinfrey et al., 2008) and the higher circulating concentrations of estradiol in female zebrafish (Deng et al., 2010; Liu et al., 2012; Ma et al., 2012; Ji et al., 2013; Liu et al., 2013; Lu et al., 2014; Yu et al., 2014; Table S1). These estrogens are known to play a role in hepatic vitellogenin synthesis and ovarian growth (Nagahama 1994). Interestingly, Cyp19a1b which is routinely named the “brain aromatase” is also expressed in zebrafish gonads, mainly in the ovary in our study. Conversely, *cyp19a1b* gene was shown to be expressed more intensely in testis than in ovary in zebrafish (Trant et al., 2001; Sawyer et al., 2006; Table S1). The difference between the Cyp19a1b protein expression analysis found in this study and the gene expressions analysis available in the literature might be explained by an accumulation of the Cyp19a1b protein in the ooplasm conducting to a higher Cyp19a1b protein content in ovaries than in testis. According to this hypothesis, this Cyp19a1b abundance in the ovarian germ cells could serve as a maternal inherited material necessary for the early synthesis of estrogens in embryos. Overall, the different cellular localizations of the Cyp19a1a and Cyp19a1b proteins argue for different roles of these enzymes within the gonads.

#### **4.3. Localization and expression of Foxl2a**

In addition to our study of steroidogenic enzymes, we further intended to characterize the expression of an aromatase regulator, i.e. Foxl2a. Foxl2a is a putative winged helix/forkhead transcription factor which is known to be involved in ovarian differentiation, follicle development and maintenance in different vertebrate species while its putative presence and role in male gonad is not known. In zebrafish testis we localized a low expression of Foxl2a in the nucleus of interstitial Leydig cells and in germ cells (Table II). In some species such as Catfish, Rare minnow, Honeycomb grouper, Rainbow trout and Tilapia, a low but significant expression of *foxl2a* gene has been detected in the testis by RT-PCR (Baron et al., 2004; Wang et al., 2004; Vizziano et al., 2007; Alam et al., 2008; Jiang et al., 2011; Sridevi and Senthilkumaran, 2011), but not by ISH (Wang et al., 2004). More recently, *foxl2a* mRNA has been found by RTq-PCR in the protogynous wrasse testis, and Foxl2a protein was localized in the interstitial Leydig cells by immunofluorescence indicating that the gene was translated into proteins (Kobayashi et al., 2010b). However, the role of Foxl2a in fish testis is still unknown.

In the zebrafish ovary, we localized Foxl2a protein in the ooplasm of pre-vitellogenic and vitellogenic oocytes and in the cytoplasm of follicular cells surrounding pre-vitellogenic oocytes. This cytoplasmic localization of Foxl2a protein was unexpected but was previously observed for Foxl2a gene and/or protein in some fish species such as catfish, medaka and willow minnow (Nakamura et al., 2009; Sridevi and Senthilkumaran, 2011; Ashida et al., 2013). Regarding cellular localization, Foxl2a gene and protein expression in the follicular layer cells surrounding the oocytes is now well demonstrated in numerous fish species (Table III). Recently, Foxl2a has been detected by IHC in the follicular layer of the pre-vitellogenic oocytes and in the ooplasm of these oocytes in Catfish ovary (Sridevi and Senthilkumaran, 2011). Altogether, these patterns of localization of Foxl2a in fish gonads reinforce the idea that Foxl2a play a crucial role in ovary as compared to testis. Consistently, the relative quantification of Foxl2a protein expression in zebrafish gonads indicated a higher expression in ovary than in testis. In other fish species the same pattern of expression was observed for *foxl2* gene (Baron et al., 2004; Ijiri et al., 2008; Jiang et al., 2011; Sridevi and Senthilkumaran, 2011; Crespo et al., 2013).

*foxl2a* and *cyp19a1a* mRNA levels were positively correlated in different fish species (Baron et al., 2004; Nakamoto et al., 2006; Vizziano et al., 2007; Wang et al., 2007; Sridevi and Senthilkumaran, 2011; Sridevi et al., 2012) and *foxl2a* has been shown to regulate the transcription of *cyp19a1* genes either directly or indirectly by interacting with Ad4BP/SF-1 or FTZ-F1 (Wang et al., 2007; Yamaguchi et al., 2007; Sridevi et al., 2012). In our study, a significant positive rank correlation between Foxl2a and Cyp19a1a proteins in both ovary and testis was highlighted, hence suggesting a possible regulation of *cyp19a1a* gene by Foxl2a in zebrafish gonads as demonstrated in other species. In zebrafish, Foxl2a and Cyp19a1a proteins were both expressed in the ooplasm of pre-vitellogenic and vitellogenic oocytes. These two proteins were also detected in the cytoplasm of follicular cells. However, Cyp19a1a immunoreactive follicular cells surrounded late pre-vitellogenic and vitellogenic oocytes while Foxl2a expressed only in follicular cells surrounding pre-vitellogenic oocytes. This suggests that follicular cells expressing Cyp19a1a do not always express Foxl2a. In the developing gonads of transgenic medaka, EGFP-expressing cells (expression under the control of the *cyp19a1a* gene promoter) were clearly distinguishable from the *foxl2*-expressing granulosa cells (Nakamura et al., 2009). In adult medaka ovary, some aromatase-positive cells of the theca layer were *foxl2*-negative (Herpin et al., 2013). Thus, the positive correlation between Foxl2a and Cyp19a1a protein expressions and their partially different

patterns of spatial expressions in zebrafish ovary suggest a possible but not exclusive regulation of *cyp19a1a* by Foxl2a.

## 5. Conclusions

Our study newly provides a precise and comprehensive sex-specific mapping of the localization of key proteins involved in gonadal steroidogenesis. We confirm previous data reported in other fish species and provide new data regarding the cellular distribution of these proteins, notably Cyp19a1b in fish ovaries. Moreover, we showed that in zebrafish gonads Foxl2a and Cyp19a1a protein expressions were correlated suggesting a possible regulation of *cyp19a1a* gene by Foxl2a in this species that need to be further study. In addition, semi-quantitative analysis of expression of these proteins in gonads revealed dimorphic expressions for Cyp11c1, Cyp19a1a, Cyp19a1b and Foxl2a between males and females. Overall, this immunohistochemical approach combined with fluorescence imaging on slice gonads allowed us to provide a detailed description of the expression of proteins involved in the biosynthesis of steroidal hormones at the cellular scale, which is crucial to further elucidate the intimate roles of these enzymes and also helps the characterization of zebrafish as a model in the field of endocrinology.

## Declaration of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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## Figure legends

**Table I: General Information on immunohistochemistry antibodies**

**Table II: Cellular localization of several steroidogenic enzymes and foxL2 in fish testis**

**Table III: Cellular localization of several steroidogenic enzymes and foxL2 in fish ovary**

**Figure 1: Immunolocalization of Cyp17a1, Cyp11c1, Cyp19a1a, Cyp19a1b and FoxL2 in adult zebrafish ovary.** Immunolabeling of targeted proteins appears in red. Nuclei labeling with Hoechst appears in blue. Cyp17a1 was labeled in the cytoplasm of pre-vitellogenic oocytes (A), in follicular cells surrounding vitellogenic oocytes (B) but not pre-vitellogenic oocytes (C) and in the cytoplasm of interstitial cells (D). Cyp11c1 was detected in the cytoplasm of follicular cells surrounding vitellogenic oocytes (E,F) but not pre-vitellogenic oocytes (G) and in the cytoplasm of interstitial cells (H). Cyp19a1a was labeled in the cytoplasm of pre-vitellogenic and vitellogenic oocytes (I), of primary oocytes in meiosis (J, surrounded by a dotted line), in follicular cells surrounding vitellogenic oocytes (K) but not pre-vitellogenic oocytes (L) and in the cytoplasm of interstitial cells (M). Cyp19a1b was detected only in the cytoplasm of pre-vitellogenic and vitellogenic oocytes (N, O: higher magnification of the white rectangle in N). FoxL2 was labeled in the cytoplasm of pre-vitellogenic oocytes and in the cytoplasm of the follicular cells surrounding pre-vitellogenic oocytes (P). A higher magnification of a pre-vitellogenic oocyte is presented in (Q). LPvo: Late pre-vitellogenic oocyte; Pvo: pre-vitellogenic oocyte; Vo: vitellogenic oocyte. Yellow

arrow head: vitellogenic oocyte follicular cell labeled for Cyp17a1; White arrow head: non labeled follicular cells surrounding pre-vitellogenic oocytes. White scale bars: 20  $\mu\text{m}$ ; Yellow scale bars: 10  $\mu\text{m}$ .

**Figure 2: Immunolocalization of Cyp17a1, Cyp11c1, Cyp19a1a, Cyp19a1b and FoxL2 in adult zebrafish testis.** Immunolabeling of targeted proteins appears in red. Nuclei labeling with Hoechst appears in blue. Cyp17a1 was labeled both in the cytoplasm of Leydig cells (A,B) and germ cells (A,C). Cyp11c1 was detected in the cytoplasm of Leydig cells (D,E) and spermatogonia (F,G). Cyp19a1a was labeled both in the cytoplasm of Leydig cells (H,I) and germ cells (H,J). Cyp19a1b immunoreactivity was only found in the cytoplasm of germ cells (K, higher magnification in L). FoxL2 was detected in the nucleus of Leydig cells (M, N, O) and of germ cells (P,Q). White arrow head and white dotted line: Leydig cells, IS: interstitial cells other than Leydig cells (myoid cells...); SC: spermatocytes; SG: spermatogonia; ST: spermatids; SZ: spermatozoa. White scale bars: 20  $\mu\text{m}$ ; Yellow scale bars: 10  $\mu\text{m}$ , Blue scale bars: 5 $\mu\text{m}$ .

**Figure 3: Quantification of Cyp17a1, Cyp11c1, Cyp19a1a, Cyp19a1b and FoxL2 fluorescence.** For each targeted protein, results are expressed as relative fluorescence intensity as compared to the mean value of females which was set to 1. For each targeted protein, three micrographs per individual and 5 to 7 individuals per sex were analyzed. Cyp17a1 fluorescence revealed no statistically significant differences between males (M) and females (F), Cyp11c1 was significantly more expressed in male gonads, while Cyp19a1a, Cyp19a1b and FoxL2 were significantly more expressed in the ovary as compared to testis (Non parametric Wilcoxon test, \* $p < 0.05$ ; \*\* $p < 0.01$ ). Open circles represent individual relative fluorescence intensity, black bars represent the mean of these relative fluorescence intensity data.

**Table I**

	<b>Cyp17-I</b>	<b>Cyp11c1</b>	<b>Cyp19a1a</b>	<b>Cyp19a1b</b>	<b>Foxl2a</b>
<b>Type</b>	Polyclonal, Immunopurified	Polyclonal, Serum	Polyclonal, Immunopurified	Polyclonal, Immunopurified	Polyclonal, Serum
<b>Host</b>	Rabbit	Rat	Rabbit	Rabbit	Rabbit
<b>Species</b>	Zebrafish	Trout	Zebrafish	Zebrafish	Trout
<b>Polypeptides aa sequences</b>	aa 126-139 : afadysstwkfhrk  aa 505-519 : kvradweksplmqhc	aa 145-159 : pwathretrqhskgv (93% identity with zebrafish)  aa 207-221 : ekdggkeerghslti	Synthetic peptide from the C-terminal region	aa 497-511 : cnsnetadnrtske	aa 2-16 : mdtyqnpeddalm (60% identity with zebrafish)  aa 33-47 : pvqekvsektbpsqk (73% identity with zebrafish)
<b>Dilution used for IHC</b>	1/300	1/300	1/600	1/300	1/300
<b>References / Supplier</b>	(De Waal et al., 2009; Hinfray et al., 2011; Baudiffier et al., 2012; Baudiffier et al., 2013; Hinfray et al., 2013)	(Baudiffier et al., 2012, Baudiffier et al., 2013)	(Hinfray et al., 2013) / AnaSpec	(Menuet et al., 2005; Vosges et al., 2010; Vosges et al., 2012; Hinfray et al., 2013)	/

IHC : immunohistochemistry

Table II

Target protein	Species	Interstitial cells (including Leydig cells)	Germ cells	Sertoli cells	Methodology	References
<b>Cyp17-I</b>	<b>Zebrafish</b>	+	+	-	<b>IHC</b>	<b>Our study</b> , Hinfray et al., 2013
	Zebrafish	+	-	-	IHC	De Waal et al., 2009; Hinfray et al., 2011; Baudiffier et al., 2012; Baudiffier et al., 2013
	<sup>a</sup> Nile tilapia, <sup>b</sup> Medaka	+	-	-	ISH	<sup>a</sup> Zhou et al., 2007a; <sup>b</sup> Zhou et al., 2007b
<b>Cyp17-II</b>	<sup>a</sup> Nile tilapia, <sup>b</sup> Medaka	+	-	-	ISH	<sup>a</sup> Zhou et al., 2007a; <sup>b</sup> Zhou et al., 2007b
<b>Cyp17</b>	Catfish	+	+	-	IHC	Sreenivasulu and Senthilkumaran, 2009
	Rainbow trout	+	-	-	IHC	Kobayashi et al., 1998b
<b>Cyp11c1</b>	<b>Zebrafish</b>	+	+	-	<b>IHC</b>	<b>Our study</b>
	Zebrafish	+	-	-	ISH	Wang and Orban, 2007
	Nile tilapia	+	+	-	ISH / IHC	Zhang et al., 2010
	<sup>a</sup> Japanese eel, <sup>b</sup> Protandrous anemonefish <i>Amphiprion clarkii</i> , <sup>c</sup> Rainbow trout	+	-	-	<sup>a,b</sup> IHC / <sup>c</sup> ISH	<sup>a</sup> Kobayashi et al., 1998a; <sup>c</sup> Kusakabe et al., 2002; <sup>b</sup> Miura et al., 2008
<b>Cyp19a1a</b>	<b>Zebrafish</b>	+	+	-	<b>IHC</b>	<b>Our study</b> ; Hinfray et al., 2013
	Zebrafish	-	-	-	ISH	Rodriguez-Mari et al., 2005; Wang and Orban, 2007; Dranow et al., 2013
	Atlantic salmon	+	+	ns	IHC	von Schalburg et al., 2013
	<sup>a</sup> Gobiid fish <i>Trimma okinawae</i> ; <sup>b</sup> Protandrous Black Porgy#	+	-	-	<sup>a</sup> ISH / <sup>b</sup> IHC	<sup>a</sup> Kobayashi et al., 2004; <sup>b</sup> Guiguen et al., 2010
<b>Cyp19a1</b>	Rainbow trout	+	+	+	IHC	Kotula-Balak et al., 2008
	<sup>a</sup> European Eel, <sup>b</sup> Gobiid fish <i>Trimma okinawae</i> , <sup>c,d</sup> Nile Tilapia, <sup>e</sup> Rainbow trout	-	-	-	IHC	<sup>c</sup> Kobayashi et al., 1998b; <sup>b</sup> Sunobe et al., 2005; <sup>d</sup> Wang et al., 2007; <sup>a</sup> Grandi et al., 2010; <sup>e</sup> Ruksana et al., 2010
<b>Cyp19a1b</b>	<b>Zebrafish</b>	-	+	-	<b>IHC</b>	<b>Our study</b> , Hinfray et al., 2013
	Atlantic salmon	+	+	ns	IHC	von Schalburg et al., 2013
	Gobiid fish <i>Trimma okinawae</i>	-	-	-	ISH	Kobayashi et al., 2004
<b>Foxl2</b>	<b>Zebrafish</b>	+	+	-	<b>IHC</b>	<b>Our study</b>
	Protogynous wrasse	+	-	-	IHC	Kobayashi et al., 2010b
	<sup>a</sup> Atlantic Salmon, <sup>b</sup> Japanese flounder, <sup>c,d</sup> Medaka, <sup>e,f</sup> Nile Tilapia	-	-	-	<sup>a</sup> IHC / <sup>b,c,d,e,f</sup> ISH	<sup>e</sup> Wang and Ge, 2004; <sup>c</sup> Nakamoto et al., 2006; <sup>f</sup> Wang et al., 2007; <sup>b</sup> Yamaguchi et al., 2007; <sup>d</sup> Nakamoto et al., 2009; <sup>a</sup> von Schalburg et al., 2013

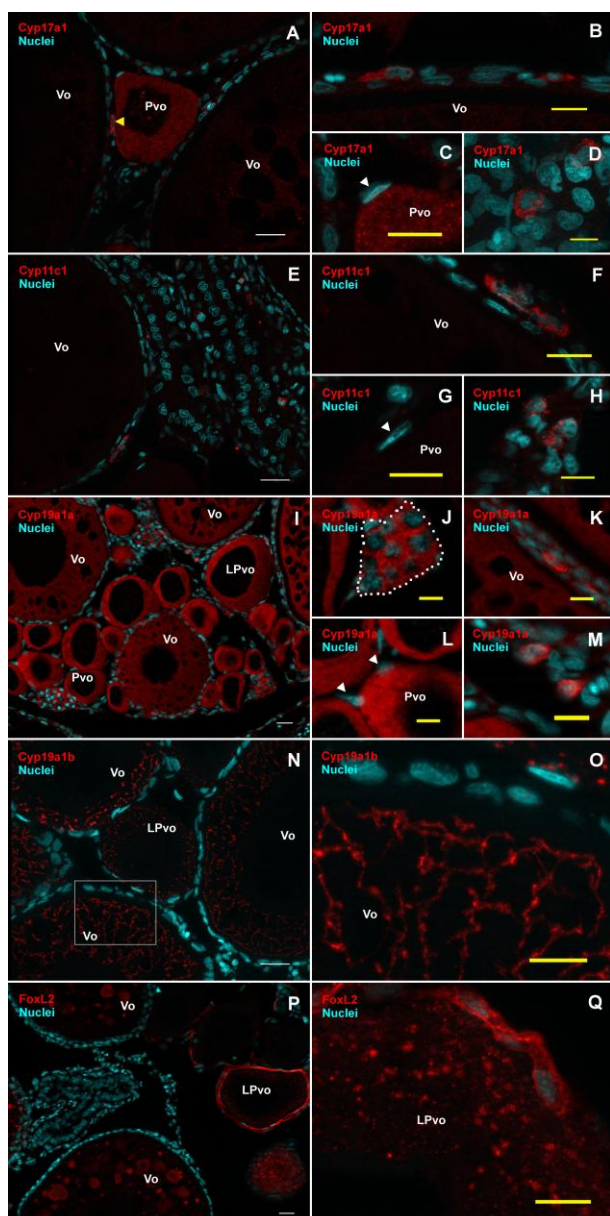
IHC: immunohistochemistry; ISH : *in situ* hybridization; ns: not specified; # testicular tissue in a bisexual gonad

Table III

Target protein	Species	Thecal and/or granulosa cells	Ooplasm	Interstitial cells	Methodology	References
<b>Cyp17-I</b>	<b>Zebrafish</b>	+	+	+	<b>IHC</b>	<b>Our study</b>
	Zebrafish	+	-	+	IHC	Hinfray et al., 2011
	Gilhead seabream	+	+	-	ISH	Zapater et al., 2012
	<sup>a,b</sup> Medaka, <sup>c</sup> Nile Tilapia	+	-	-	ISH	<sup>c</sup> Zhou et al., 2007a; <sup>b</sup> Zhou et al., 2007b; <sup>a</sup> Nakamura et al., 2009
	Japanese eel	+	ns	ns	IHC	Tosaka et al., 2010
<b>Cyp17-II</b>	Gilhead seabream	+	+	-	ISH	Zapater et al., 2012
	<sup>a</sup> Medaka, <sup>b</sup> Nile Tilapia	+	-	-	ISH	<sup>b</sup> Zhou et al., 2007a; <sup>a</sup> Zhou et al., 2007b
<b>Cyp17</b>	<sup>a</sup> Catfish, <sup>b</sup> Japanese eel	+	-	-	IHC	<sup>b</sup> Ijiri et al., 2003; <sup>a</sup> Sreenivasulu and Senthikumar, 2009
<b>Cyp11cl</b>	<b>Zebrafish</b>	+	-	+	<b>IHC</b>	<b>Our study</b>
	Zebrafish	-	-	-	ISH	Wang and Orban, 2007
	Protandrous anemonefish <i>Amphiprion clarkii</i>	+	-	-	IHC	Miura et al., 2008
	Honeycomb grouper	-	-	+*	IHC	Alam et al., 2005; Alam et al., 2006
	Protogynous Malabar grouper	-	-	+*	IHC	Murata et al., 2011
<b>Cyp19a1a</b>	<b>Zebrafish</b>	+	+	+	<b>IHC</b>	<b>Our study</b>
	Zebrafish	+	-	-	ISH	Rodriguez-Mari et al., 2005; Wang and Orban, 2007; Dranow et al., 2013
	<sup>a</sup> Air-breathing catfish, <sup>b</sup> Gilthead sea bream, <sup>c</sup> Medaka, <sup>d</sup> Rainbow trout	+	+	-	<sup>a</sup> IHC / <sup>b,c,d</sup> ISH	<sup>c</sup> Park et al., 2008; <sup>d</sup> Gohin et al., 2011; <sup>a</sup> Raghuveer et al., 2011; <sup>b</sup> Zapater et al., 2012
	<sup>a</sup> Medaka, <sup>b</sup> Gobiid fish <i>Trimma Okinawae</i> , <sup>c</sup> Nile Tilapia, <sup>d</sup> Yellowtail Clownfish	+	-	-	<sup>a</sup> Tg / <sup>a,d</sup> IHC / <sup>b,c,d</sup> ISH	<sup>b</sup> Kobayashi et al., 2004; <sup>c</sup> Zhou et al., 2007a; <sup>a</sup> Nakamura et al., 2009; <sup>d</sup> Kobayashi et al., 2010a
	Nile tilapia	+	-	+	IHC	Wang et al., 2007
	Atlantic salmon	-	-	-	IHC	von Schalburg et al., 2013
	Protogynous Malabar grouper	-	-	+	IHC	Murata et al., 2011
	Protandrous Black Porgy#	+	-	ns	IHC	Guiguen et al., 2010
<b>Cyp19a1</b>	<sup>a</sup> Gobiid fish <i>Trimma okinawae</i> , <sup>b</sup> Yellow Eels	+	-	+**	IHC	<sup>a</sup> Sunobe et al., 2005; <sup>b</sup> Grandi et al., 2010
	Medaka	+	-	-	ISH	Nakamoto et al., 2010
	Nile Tilapia	-	-	+	IHC	Ruksana et al., 2010
<b>Cyp19a1b</b>	<b>Zebrafish</b>	-	+	-	<b>IHC</b>	<b>Our study</b>

	<sup>a</sup> Atlantic salmon, <sup>b</sup> Gobiid fish <i>Trimma Okinawae</i>	-	-	-	<sup>a</sup> IHC / <sup>b</sup> ISH	<sup>b</sup> Kobayashi et al., 2004; <sup>a</sup> von Schalburg et al., 2013
<b>Foxl2</b>	<b>Zebrafish</b>	+	+	-	<b>IHC</b>	<b>Our study</b>
	Japanese flounder	-	-	+	ISH	Yamaguchi et al., 2007
	<sup>a</sup> Air breathing catfish, <sup>b,c,d,e</sup> Medaka, <sup>f</sup> <i>Oryzias</i> <i>Luzonensis</i> , <sup>g</sup> Protogynous wrasse, <sup>h</sup> Willow minnow	+	-	-	<sup>a,g,e</sup> IHC / <sup>b,c,d,f,h</sup> ISH	<sup>b</sup> Nakamoto et al., 2006; <sup>c</sup> Nakamura et al., 2008; <sup>f</sup> Nakamoto et al., 2009; <sup>d</sup> Nakamura et al., 2009; <sup>g</sup> Kobayashi et al., 2010b; <sup>a</sup> Raghuveer et al., 2011; <sup>h</sup> Ashida et al., 2013; <sup>e</sup> Herpin et al., 2013
	Air-breathing Catfish	+	+	-	IHC	Sridevi and Senthilkumaran, 2011
	Nile Tilapia	+	-	+	<sup>a</sup> ISH / <sup>b</sup> IHC	<sup>a</sup> Wang and Ge, 2004; <sup>b</sup> Wang et al., 2007

IHC : immunohistochemistry ; ISH : *in situ* hybridization ; ns: not specified; Tg: transgenic fish; \* specific clusters of cells distributed in the vicinity of blood vessels in the periphery of ovary; \*\* only at the early stages of gonadal development for Yellow eels; # Ovarian tissue in a bisexual gonad

**Figure 1**

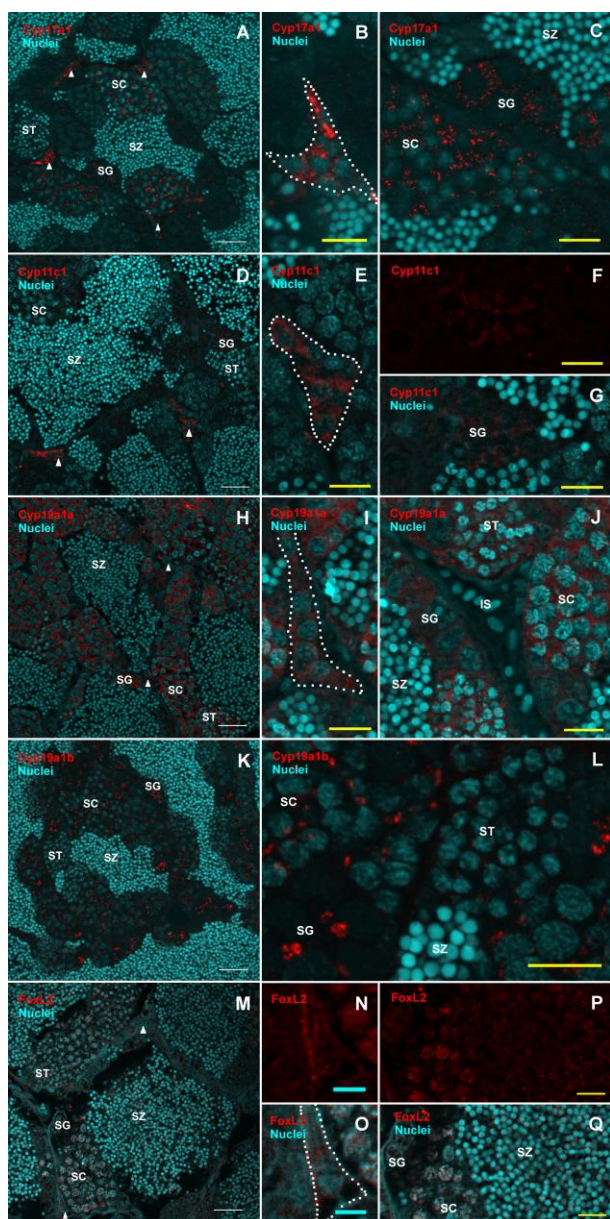


Figure 2

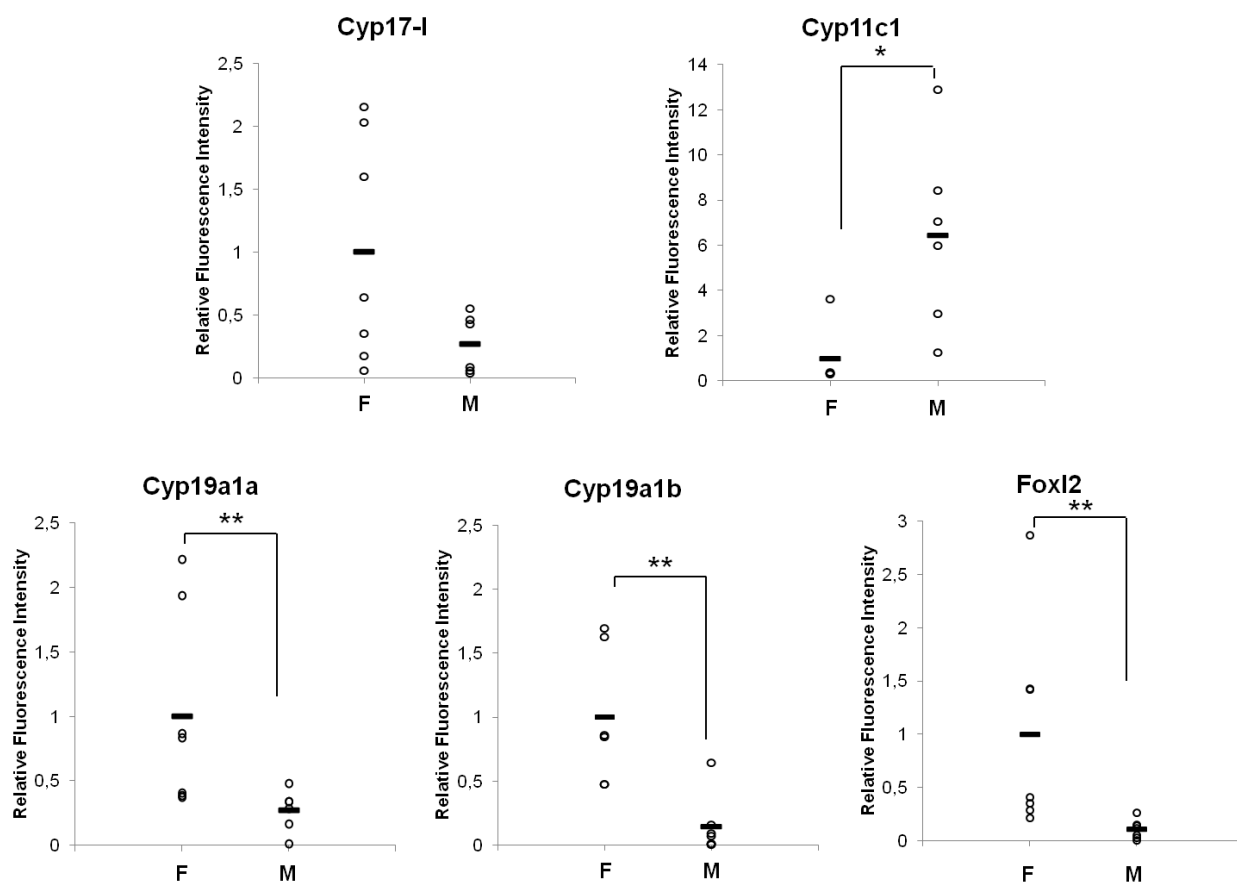


Figure 3