Sex differentiation of barramundi *Lates calcarifer* – Understanding male sexual development and its manipulation through exogenous steroids and non-steroidal aromatase inhibitor

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Abstract

Barramundi, or Asian seabass (*Lates calcarifer*), is considered one of the most important commercial tropical aquaculture species in Southeast Asia and Australia. Despite its importance, efforts to improve barramundi production via selective breeding have been challenging, primarily due to difficulties in controlling sex change. Barramundi is a protandrous hermaphrodite, where fish first mature as male and then, a few years later, sex change into female. The gonadal development of barramundi provides a good model to study the genetic mechanisms underlying natural sex change in fish; however, this natural sex change process poses significant challenges for barramundi broodstock management as high quality male broodfish change sex, which requires the constant recruitment of new male broodstock. Alternatively, and relevant to selective breeding programs, the sequential sex change of barramundi results in an age difference between the sexes in spawning groups, where females are one generation older than the males. As the rate of genetic gain is contingent on the generation interval, this inter-generation breeding thereby halves the annualised rate of genetic progress that could otherwise be made through a single-generation selection program. Therefore, sex control of barramundi in hatcheries through either inducing precocious females, or preventing natural sex change from male to female, is of great importance to the industry.

Sex control for fishes (including teleosts) is feasible as the course of gonadal development is a flexible process and may be subject to modification by extrinsic factors, especially during early gonadal development when animals are most sensitive to environmental stimuli (widely referred to as the labile period). Manipulation of environmental factors (e.g. addition of sex steroids, changing water temperature or pH) during early gonadal development may override genetically pre-programmed sex determination mechanisms, thus altering the resultant

phenotypic sex of the fish. Yet, the process of testicular development in barramundi is unknown. In particular, there is no information on when and how testicular differentiation commences in the species and what happens when exogenous steroids are subsequently administered. To address these knowledge gaps, the research that is reported in this thesis was conducted in three steps. Firstly, the timing of sexual development and differentiation in the protandrous barramundi was determined through tracking morphological differentiation and gene expression of key sex genes from early embryogenesis through to spermatogenesis. This provided an understanding of the timing of initial gonadal differentiation into the male testis state and an indication of the likely labile period whereby sex could be manipulated through the addition of exogenous factors such as hormones. Once the timing and process of testicular differentiation was established, the research then went on to examine the effect of applying exogenous steroid and non-steroidal hormones for inducing precocious feminisation and/or retaining masculinisation. This was examined through a feeding trial of exogenous steroid to juveniles and use of implants with estrogen and fadrozole in male barramundi.

The labile period of sex determination in barramundi was identified by examining the gonadal morphology of fish from newly hatched larvae till 9 months post-hatch, when barramundi gonads were previously reported as differentiated testes. As primordial germ cells (PGCs) of male fish remain morphologically unchanged for longer periods and are differentiated as the final step of testicular differentiation, early histological indicators for testicular differentiation, such as the slit-like lumen in stromal tissue, appearance of blood vessels, and invagination of the epithelial tissues into lobular structures, are often used. Results showed that the barramundi proto-testes originates as a strip of cells attached to the dorsal coelemic cavity at 4 days post hatch (dph), with the formation of a slit-like lumen by 44 dph (fish total length (TL) 25.8 ± 3.3 mm), which is the first morphological indication of testicular differentiation in the species. This slit-like lumen later develops into the testes

efferent duct. Other major indicators of testicular development were invagination among epithelial cells and the formation of capillary vessels at 60 dph, the organization of primary germ cells into lobules at 90 dph and differentiation of the somatic cells into Sertoli cells at 120 dph. Final testicular differentiation was histologically discernible at 140 dph when the somatic cells had differentiated into Sertoli cells and germ cells into spermatocytes. Noticeably, the male-associated gene *dmrt1* (double-sex and Mab-2 related transcription factor 1) was detected exclusively to be expressed in Sertoli cells by *in situ* hybridisation using an mRNA probe. Additionally, gene expression of the important male-related genes, *dmrt1* and *cyp11b* (cytochrome P450 11β-hydroxylase gene), were examined using RT-qPCR. *Cyp11b* and *dmrt1* were expressed highest in the gonad at 90 and 120 dph, respectively, with *dmrt1* expression observed to rapidly increase from 70 to 120 dph. The beginning of sex differentiation to the finalisation of testicular development in barramundi was determined in this study to occur from 44 to 140 dph (TL 169.4 ± 40.3 mm).

The next steps in this study were to assess the feminising effect of 17 β-estradiol (E_2) and 17 α-ethinylestradiol (EE_2) on barramundi during their determined labile period. Fish were fed pellets containing 10 mg E_2 kg^{-1} food, 20 mg E_2 kg^{-1} food, 5 mg EE_2 kg^{-1} food and 10 mg EE_2 kg^{-1} food from 30 to 160 dph, which covers the period of initial male gonad differentiation identified in the first study. The effect of E_2 and EE_2 treatment on gonadal morphology and *dmrt1* and *cyp19a1a* gene expression was analysed by histology and RT-qPCR. Results showed that orally delivered E_2 and EE_2 induced observable changes in gonad morphology. Previtellogenic oocytes (PO) were observed in 33% and 50% of the fish fed with 20 mg E_2 kg^{-1} food at 160 dph and 12 months post hatch (mph), respectively, while PO were not observed in control fish. The 10 mg E_2 kg^{-1} food treatment did not induce feminisation, but significantly suppressed testicular development. Treatment with EE_2 resulted in fibrosis within gonad tissues at a dose-dependent rate. E_2 administration resulted in upregulation of

the gene *cyp19a1a* and down-regulation of *dmrt1*. EE_2 significantly suppressed expression of *dmrt1* at 160 dph and 12 mph; while *cyp19a1a* was not significantly different at 160 dph and was significantly downregulated at 12 mph. The result of this study showed that E_2 is a more suitable hormone than EE_2 to induce precocious feminisation of barramundi, and when delivered through the feed it has a dose-dependent feminising effect.

Whilst feeding of estrogen steroids to juvenile barramundi results in sex change of some individuals in a selective breeding program, from a consumer awareness perspective it may be better to only administer hormone to males selected for use as female broodstock to allay any fears about hormone treatment of fish that may be destined for human consumption. Because of this, it was deemed vital to assess if there is an alternative route to sex change males. The literature shows that sex plasticity of some teleost species remains after sexual differentiation, therefore a trial was conducted to produce precocious female barramundi after fish had differentiated as males. Here, the efficacy of two dosages of E_2 delivered via implants to induce the early feminisation of barramundi were evaluated. Six-month-old male barramundi (405 ± 50 g body weight (BW)) were given a single cholesterol-based pellet implant containing either 0 mg E_2 kg^{-1} BW (untreated control), 4 mg E_2 kg^{-1} BW ('low dose'), or 8 mg E_2 kg^{-1} BW ('high dose'). At 4 and 9 weeks post-implantation, gonads were histologically assessed for morphological changes, liver condition histologically examined and expression profiles of key male (*dmrt1*, *cyp11b* and *esr1*) and female (*cyp19a1a* and *foxl2*) sex-related genes examined using RT-qPCR. At 9 weeks post-implantation, significant gonadal morphological changes were observed in E_2-treated fish, while all control fish remained as male. In the 'high dose' E_2 treatment group, 77.8 % (7/9) of fish sex-changed completely to female, signified by gonads containing oocytes (20-30 µm) and no observed residual sperm. Comparably, 44.4 % (4/9) of fish in the 'low dose' E_2 treatment group had sex-changed, while remaining fish showed complete testicular regression with gonads

containing only undifferentiated germ cells. In the 'high dose' E_2 treatment, increased expression of female-biased genes (*cyp19a1a* and *foxl2*) was observed, while downregulation of male-biased genes (*dmrt1*, *cyp11b* and *esr1*) was induced. The success of artificially-induced sex change in barramundi provides an important tool that is critical to improving selective breeding of this species.

Efforts to control sex of barramundi also means having the ability to maintain high quality broodfish as males. The results from the feeding and implant trials illustrated the important role of the *cyp19a1a* aromatase gene in barramundi feminisation, suggesting a possible way to block natural sex change in barramundi may be to reduce aromatase activity (estrogens are synthesised from androgens through a reaction catalysed by cytochrome P450 aromatase). Accordingly, a final trial was conducted to assess the impact of a non-steroidal aromatase inhibitor, Fadrozole (FAD), administered solely or with E_2 cholesterol pellet implants, on the gonadal development of mature male barramundi. Fifteen-month-old male barramundi (2102 ± 126 g BW) were divided into four groups of 12 individuals each. Fish in the first group were implanted with a cholesterol pellet without FAD or E_2 as control (n = 12). The three treated groups of fish were implanted with 8 mg E_2 kg^{-1} (n = 12), 8 mg FAD kg^{-1} (n = 12), or 8 mg E_2 and 8 mg FAD kg^{-1} were both implanted into individual fish (n = 12). Survival rates were 100% for all groups during the experiment with no damage observed in liver and kidney of any fish. At the final sampling 9 weeks after treatment, 8 % (1/12) of the untreated control fish were found in early stages of sex reversion (stage T1), whereas 100% of FAD-treated fish were male and 100% of E_2-treated fish were female. Binary treatment with FAD and E_2 resulted in 42 % (5/12) males, 42 % (5/12) females and 16% (2/12) transitional fish. In the FAD-treated fish, expression of male genes (*dmrt1* and *cyp11b*) were significantly upregulated, whilst female-biased gene (*foxl2*) downregulated. Exposure to E_2 resulted in significantly higher expression of *cyp19a1a* and *foxl2;* and lower expression of *dmrt1*,

cyp11b and *esr1* than control fish. FAD neutralised the effects of E_2, in terms of gonad morphology and gene expression.

In summary, this PhD research has revealed the important process of sex differentiation of barramundi, a sequential hermaphroditic teleost species. It unveiled the morphological changes at cellular levels of barramundi gonads from undifferentiated to maturity of testes, while revealing the expression patterns of the important sex-related genes during the process. The timing of this process provided the time window for testing the responsiveness of barramundi gonads to the exogenous steroids for sex control purposes. This research has determined a suitable hormone treatment for barramundi feminisation, including hormone types and doses, timing and administration route. It also filled our knowledge gap in gene expression of the important male- and female-biased genes in barramundi gonad exposed to exogenous steroids and non-steroid hormones. Although the effect of masculine fadrozole was investigated in this thesis, it is necessary to optimise the dose for better results. The process of obtaining young females by means of hormonal treatment in this study also require further research of their reproductive potential, as the ultimate goal is to produce dams for selective breeding programs.

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Chapter 1. General introduction

By 2030, the human population is expected to reach 9 billion people, with a resultant increase of 70% in the amount of animal-derived protein required from that of today (FAO, 2009). Aquaculture, or the farming of aquatic organisms, is considered as the fastest growing food production sector, with 47% of total global seafood consumed derived from farmed sources (FAO, 2018b). As the human population grows, the aquaculture sector is also expected to expand and provide an increasing proportion of humanity's future animal protein requirements (Cunningham, 2005). Despite this, for aquaculture to fulfil its destiny as a major supplier of future protein, the species that are farmed also need to become more biologically productive and, accordingly, selective breeding is considered an important and sustainable approach that needs to be implemented for most species (Gjedrem et al., 2012).

Selective breeding, also known as artificial selection, is defined as the targeted process of breeding individuals with desired characteristics to obtain a population of progeny with genetically improved traits of interest, compared to previous generations. The use of selective breeding has been documented for 10,000 years and has resulted in domestication of most current crop and livestock species (Zeder and Smith, 2009). While the terrestrial livestock sector, including bovine, swine and poultry, have seen increased productivity through genetic improvement over eons, selective breeding of most aquaculture species is generally still in its infancy. For the few aquaculture species that production has been based on genetically improved stock, high selection response on harvest weight of around 12% per generation have been achieved (Gjerde, 1999), whilst annual genetic gain for bovines and poultry is now only around 1% to 2% (Janssen et al., 2017).

Despite the obvious benefits of selective breeding programs to aquaculture production, only ~10% of farmed fish production is contributed by genetically improved stocks (Gjedrem et al., 2012). Apart from the huge and long-term financial investment, other challenges restrict the implementation of genetic selection in the aquaculture industry, including biological limitations such as environmental effects on genotype stability, ability to induce breeding in captive stocks, and, in some cases, difficulties created by a lack of sex control (Gjedrem, 1997).

1.1. Sex control for aquaculture purposes

An ability to control sex of farmed aquaculture species is critical for effective production. For broodstock management particularly, a well-balanced ratio between dams and sires ensures the success of many breeding schemes (Gjedrem, 1997; Robinson et al., 2010). In some species where one sex is preferable than the other, due to sexual dimorphism in growth rate, shape, colour or precocious maturation, monosex production of the desirable sex is targeted (Hunter et al., 1983; Pongthana et al., 1999; Beardmore et al., 2001; Luckenbach et al., 2017).

Fish represent the most diverse group of vertebrates and encompass more than 28,000 species (Nelson et al., 2016). Sexual differentiation in fish is not uniform, with species exhibiting an almost complete range of various types of reproductive strategies from gonochorism (sex remains unchanged once it is determined), unisexuality (all female individuals) to fluctuating or unidirectional hermaphroditism (Yamamoto, 1969). A species is defined as hermaphrodite when a substantial proportion of individuals in a population function as both sexes, either simultaneously or sequentially, at some time during their life. Sequential hermaphrodites are species in which some or all individuals function first as males then later differentiate into functional females (protandrous); or sex change from female to male (protogynous); or serial (bidirectional) sex change. Simultaneous hermaphroditic individuals function as both male and female at the same time of life.

Regardless of reproductive modes, sex of organisms including teleosts, is determined either by genetic, environmental, or a binary combination of these two factors. While sex determination in humans and other mammalian species is genetic and relatively stable, its diversity in lower organisms like reptiles and teleosts provides valuable models for understanding sex determination in general. The study of sex determination in fish, in particular, plays an important role in aquaculture, as understanding reproductive biology for sex control is essential in hatcheries to ensure efficient propagation. Specifically, the ability to induce synchronous and reliable maturation of broodstock is necessary for breeders to plan for production. Sexually different dimorphism in growth, appearance, flesh quality and puberty in some farmed species drives the need for manipulating the desired sex.

1.2. Sex differentiation of teleosts and methods of its study

The morphology of fish gonads at a cellular level is similar to that of other vertebrates, which is defined by the presence of germ cells and associated supporting somatic cells. Prior to sexual differentiation, gonads are undifferentiated and called primordial or primary gonads. The germ cells in undifferentiated gonads are called primordial germ cells (PGCs). In differentiated gonads (ovary and testis), PGCs are differentiated into stages of spermatogenesis (in testes) or oogenesis (in ovary) while the somatic cells are differentiated into associated structural and endocrine cell types.

The primordial gonad of fish, as is the case for many vertebrate and non-vertebrate organisms, shows a relatively simple pattern of cellular association and no signs of somatic or germ cell sex differentiation can be recognized at this time (Meijide et al., 2005). Once PGCs are formed they remain closely associated with endodermal tissues and migrate via the dorsal gut mesentery to the region of presumptive gonad, or germinal ridge (Nakamura and Takahashi, 1973; Hamaguchi, 1992; Nakamura, 2013). PGCs can generally be recognised by

their relatively large size, low ratio between nucleus and cytoplasm, well-defined cellular and nuclear borders, finely granular nuclear chromatin and, in some instances, the presence of one or two prominent nucleoli (Hardisty, 1978). In undetermined gonads, PGCs are similar in the two sexes and they remain undifferentiated and undetermined until exposed to hormonal and other influences from the developing gonad, which transforms them into spermatogonia, or oogonia. Surrounding the PGCs are the supporting somatic cells, which are generally smaller. Prior to differentiation, histological evidence does not reveal distinction between male and female gonadal cell types; differences would indicate that sex determination has occurred and that sex differentiation is proceeding.

Following migration of PGCs into the germinal ridge, differentiation of somatic cells into different types of functional cells is initiated depending on whether the gonad is differentiating into testis or ovary. In the testis, somatic cells differentiate into seminiferous tubules, supporting connective tissue and functional cells, such as Leydig and Sertoli cells (Paul-Prasanth et al., 2011). For ovarian development, the somatic cells and PGCs begin to differentiate to form follicles, comprised of oocytes surrounded by an inner granulosa and outer thecal layer (Nagahama et al., 1982). In most species, ovarian development in females is first detectable by the proliferation of somatic cells, oogonia, and early oocyte differentiation, which is then followed by the formation of the ovarian cavity.

The timing of sex differentiation is different between species and sexes. Testicular development usually occurs later than ovarian differentiation, for example, some weeks or months after the onset of gonad development in females of some species (Guraya, 1994; Nakamura et al., 1998). In salmonids, at the time of hatching, primordial gonads can be identified as paramedian ridges on the dorsal coelomic wall (Nakamura, 1974). The first evidence of change from an undifferentiated gonad in salmonids is observed in females, with the differentiation of oocytes in prophase at 3 weeks post-hatching (Nakamura and

Nagahama, 1993). Similarly, in coho salmon *Oncorhynchus kisutch*, differentiation of ovarian tissue was observed as soon as 27 days post hatch (dph) (Piferrer and Donaldson, 1989), whereas differentiation of testicular tissue occurred several weeks later.

The embryonic gonad is the only organ in vertebrates that takes two mutually exclusive differentiation pathways and, hence, gives rise to two different adult organs: testes or ovaries. Teleosts display a wide variety of sex determination and sex differentiation mechanisms, perhaps to a larger extent when compared to other vertebrates, due to the interplay of genetic and environmental influences. From a practical point of view, it is crucial to identify the essential biological mechanisms to understand and control fish reproduction in aquaculture.

The process of fish sex differentiation is well studied, both morphologically and endocrinologically (Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002; Piferrer et al., 2005). In the pejerrey, *Odontesthes bonariensis*, the process of sex differentiation of male and females displayed both a cephalocaudal and left-to-right gradient which was suggested as a mechanism to prevent the inconsistency within the same gonad between male and female (Strüssmann and Ito, 2005). According to Quillet et al. (2004), developmental abnormalities caused by exogenous steroid administration in the rainbow trout *O. mykiss*, were more stable in the left gonad than the right gonad. Maack et al. (2003) reported discrepancy in the timing of sex differentiation signs in zebrafish *Danio rerio*. Uchida et al. (2002) also detected the involvement of oocytes apoptosis as a mechanism of testicular and ovarian differentiation of this species. These results demonstrated that the increasing diversity of gonadogenesis in fish has been boosted as more studies in new species or deeper description of the sex differentiation were reported. Thus, given the diversity in teleost modes of sex differentiation, studies in gonadal morphology and formation in other unstudied fish species will definitely benefit from investigations that look at the role of germ cells during the early stages of the gonadal development (Slanchev et al., 2005).

1.3. Genetic mechanism of teleost sex differentiation

Genetic mechanisms of sex differentiation in vertebrates have mainly been studied in humans and model terrestrial species like mouse *Mus musculus*, chicken *Gallus gallus domesticus* (Koopman et al., 1991; Chaboissier et al., 2004; Smith and Sinclair, 2004; Smith et al., 2009), or model aquatic species such as zebrafish and medaka *Oryzias latipes* (Herpin et al., 2013; Webster et al., 2017).

The process of sex differentiation is regulated by the complex cascade of gene expression. In spite of genetically determined sex, phenotypic sex in teleosts is the result of an antagonistic interaction between feminising and masculinising gene networks, where the prevailing network acts to continuingly suppress the opposing sexual network. Genes central to the underlying feminising network are cytochrome P450 aromatase (*cyp19a1a*) and forkhead box protein L2 (*foxl2*), and those key to the masculinising network are doublesex and mab-3-related transcription factor 1 (*dmrt1*), cytochrome P450 11β-hydroxylase gene (*cyp11b*) and estrogen receptor 1 (*esr1*) (Liu et al., 2015; Todd et al., 2016). Specifically, the key female gene *cyp19a1a* is activated by the transcription factor encoded by the gene *foxl2; cyp19a1a* encodes the enzyme aromatase, which catalyses the conversion of androgens to estrogens (Kazeto et al., 2004; Guiguen et al., 2010). For males, the transcription factor *dmrt1* regulates the expression of male promoting genes (e.g. *cyp11b*, SRY-box containing gene 9 (*sox9*), Anti-Müllerian Hormone (*amh*)) and downregulates the female pathway by suppressing *foxl2* and, subsequently, reducing *cyp19a1a* expression and the presence of endogenous estrogen (Wang et al., 2010; Herpin and Schartl, 2011; Kobayashi et al., 2013).

Functional genomics has been supporting the research of sex differentiation, especially in the examination of the underlying molecular mechanisms. In fish, although numerous studies focused on monosex populations and effect of exogenous steroids on gonadal states, limited

results have been published using high-throughput expression profiling. Despite of numerous genomic tools are available for model fish species, up-to-date, complete genome sequences have published mostly for model species such as medaka (Kasahara et al., 2007), zebrafish (Howe et al., 2013) and fugu *Fugu rubripes* (Aparicio et al., 2002). However, small body size of most model species is a shortcoming for using them to conduct the biochemical or molecular studies directly on their gonads, especially during the early life stages.

Estrogens play key roles in development and maintenance of normal sexual and reproductive function. Estrogen action is mediated by three estrogen receptors (ERs), named *er1*, *erβ1*, and *erβ2* (*erα*, *erβ*, and *erγ*, in some cases), which have been identified in several fish such as zebrafish (Menuet et al., 2002), European sea bass *Dicentrarchus labrax* (Halm et al., 2004), fathead minnow *Pimephales promelas* (Filby and Tyler, 2007), and rainbow trout (Nagler et al., 2007). The ERs display discrepancies in tissue distribution, gene expression, gene regulation and nucleotide sequence as well as different ligand-binding properties. As evidenced by RT-PCR analysis, the three ERs of seabream showed a partly overlapping but different tissue distribution in males and females (Pinto et al., 2006).

In the European sea bass, expression of *er1* was significantly higher in the pituitary and gonads at 200 dph, whereas the peak expression of erβ1 and erβ2 were detected in gonads at 250 dph (Halm et al., 2004). In the Nile tilapia *Oreochromis niloticus*, sex differences in the gene expression of androgen and ERs were not detected, though *er1* expressed earlier than *erβ* (Sudhakumari et al., 2005). In this respect, transgenic medaka overexpressing ERs show usual sex differentiation and then similar sex inversion to wild-type non-transgenic individuals after estrogen or androgen exposure (Kawamura et al., 2003). These results, collectively, convey the difference in timing and effect of ERs in fish sexual differentiation. Fascinatingly, the ERs were more expressed in the sexually differentiating fish than in non-

reproductive adults (Halm et al., 2004). Therefore, specific role of the estrogen receptors in teleost sex differentiation should be determined with more evidence from further research.

Dmrt1 plays an important part in testicular differentiation in vertebrates and displays comparable role in male differentiation of gonochoristic and hermaphrodite fish (Marchand et al., 2000) like black porgy *Acanthopagrus schlegelii* (He et al., 2003), platyfish *Xiphophorus maculatus* (Veith et al., 2003), medaka (Kobayashi et al., 2004), zebrafish (Guo et al., 2005), European sea bass (Deloffre et al., 2009), and orange-spotted grouper *Epinephelus coioides* (Xia et al., 2007). Duplication of *dmrt1* resulted in higher expression of the sex-determining gene in medaka (Matsuda et al., 2002; Nanda et al., 2002). Noticeably, two types of DM genes, *dmrt1* and a related gene *dmrt4*, were detected in the Nile tilapia males and females, respectively (Guan et al., 2000). The two different transcripts of *dmrt1* in the European seabass were encoded by the same gene, and both transcripts were more abundant in testis than in females (Deloffre et al., 2009). Overall, these studies highlight the relationship between the gene *dmrt1* and the pathway of testicular differentiation. Accordingly, two key genetic factors of testicular and ovarian sex differentiation in fish are emerging, the role of *dmrt1* and *cyp19a1a*, respectively.

Foxl2 is a putative winged helix/forkhead transcription factor gene and a sexually dimorphic marker of ovarian differentiation in vertebrates. It is one of the earliest determined sex dimorphic markers of female differentiation (Wang et al., 2004). Duplicated copies of *foxl2* were reported in rainbow trout (Baron et al., 2004). In this species, *cyp19a1a* and both *foxl2* transcripts were expressed specifically in the differentiating ovary, but their temporal expression patterns were differently. *Foxl2a* expression was correlated with the expression of *cyp19a*, whereas *foxl2b* was expressed later during development. Mutated or knockout *foxl2* gene caused premature ovarian failure in human and mice (Crisponi et al., 2001; Schmidt et al., 2004; Uda et al., 2004). In the Nile tilapia, ovarian expression of *foxl2* began in the early

stages of sexual differentiation and persisted until adulthood (Wang et al., 2004). *Foxl2* is also involved in the transcriptional regulation of *cyp19a1a* in Nile tilapia (Wang et al., 2004) and medaka (Nakamoto et al., 2006). The similar results were reported for the Japanese flounder *Paralichthys olivaceus*, displaying that expression of *cyp19a1a* and *foxl2* was down-regulated after exposure to high temperature water and *foxl2* activated *cyp19a1a* transcription *in vitro* (Yamaguchi et al., 2007). Exposure rainbow trout to estrogens resulted in the upregulation of *foxl2*, whereas androgen or aromatase inhibitor treatments induced *foxl2* downregulation (Baron et al., 2004). Hence, *foxl2* is considered as a potent stimulator of *cyp19a1a* expression, and therefore confirmed the important role of *foxl2* in fish ovarian differentiation. In female Nile tilapia, disruption of endogenous *foxl2* caused in variable degrees of male development, with some female-to-male inversion, further highlighting the critical role of *foxl2* in ovarian differentiation (Wang et al., 2007).

Obtaining basic knowledge on sex differentiation of a teleost like barramundi is vital to extend our view on the evolution and mechanism of the sex differentiation of vertebrate animals. From a practical aspect, elucidating these essential biological functions is critical to develop new tools or optimise existing methods for controlling the reproduction of farmed fish, supporting fisheries management as well as predicting possible consequences of anthropogenic impacts (Devlin and Nagahama, 2002).

1.4. Induction of sex change for aquaculture purposes

Unlike higher vertebrates, the course of gonadal development of fish has been proven to be easily modified by endocrine disrupting chemical (EDC) exposure, which can alter the phenotypic sex of individuals (Yamamoto, 1969; Piferrer, 2001; Devlin and Nagahama, 2002). A wide range of EDCs, including steroids and non-steroidal substances, which are

natural or synthetic origin, have been reported to have sex inversion potency to aquatic animals due to their steroid mimicking properties (Lee et al., 2006; Cheshenko et al., 2007).

Naturally, sex steroids are the sex hormones, mainly secreted by the gonads that are constantly detected at high level during the normal course of sex differentiation. Sex steroids include androgens, estrogens and progestogens, but only androgens and estrogens play an important role in gonadal differentiation of fish (Piferrer, 2011). Although both androgens and estrogens are present in both sexes at different levels, androgens are largely considered as male sex hormones, while estrogens are considered as female sex hormones. Endogenous androgens, mainly testosterone, are irreversibly converted into estrogen when catalysed by the enzyme aromatase (Guiguen et al., 2010). The ratio between testosterone and estradiol, not the quantities, is the determinant for the gonadal sex; thus, providing exogenous androgens or estrogens can interfere with this ratio and result in changes in phenotypic sex regardless of the genetically determined sex.

Many farmed fish, such as salmonids, sea basses and tilapias display sexually dimorphic differences in growth and maturation, leading to the desire to produce monosex population or sterile stocks (Piferrer, 2011). Generally, the purpose of sex control in farmed fish is to increase the profitability by having more high value commodities in the grow-out system, or by reducing the production cost for broodstock management in hatcheries. Illustration of the economic benefits of rearing sex reversed fish was reported for chinook salmon *O. tshawytscha* (Solar and Donaldson, 1991). Although, due to health concerns, direct use of sex hormones in aquaculture is restricted or completely banned (Budd et al., 2015).

The responsiveness of fish gonads to EDCs change during life stage. It is considered that there is a time window during early development, termed the labile period, where gonadal sex is relatively plastic and when gonadal differentiation can be strongly influenced with external

factors (Piferrer, 2001). Therefore, treatment with EDCs during this labile period of sex differentiation requires the least amount of exogenous chemicals and the shortest treatment duration to obtain a successful rate of production of the targeted gonadal sex. Studies on the labile period in multiple fishes showed that its timing is largely species-specific. For example, the labile period for estrogen treatment in the masu salmon *O. masou* is from 5 to 22 days post-hatch (dph) (Nakamura, 1984), while in coho salmon, it is between 8 days pre-hatch and 13 dph (Piferrer and Donaldson, 1989). Despite the importance of the labile period for sex control, it does not exclusively imply that fish cannot be successfully sex-reversed by hormonal treatment outside this period. For instance, in European seabass and chum salmon *O. keta*, the gonad is responsive to exogenous steroids outside the period of gonadal differentiation and well beyond the labile period (Nakamura, 1984; Blázquez et al., 1995). Nevertheless, hormonal treatments to differentiate fish require higher doses and longer duration than those that are administered during the labile period in order to successfully reverse gonadal sex.

Once gonadal differentiation has occurred, the effects are usually permanent in most reported fish species, although there are some cases where induced sex-reversed fish regress to their destined phenotype (Olito and Brock, 1991; Tan-Fermin, 1992; Lim and Wong, 1996). Noticeably, administration of sex steroids for inducing sex reversal was proven to be both effective for fish species possessing sex chromosomes and those where they are absent (Piferrer, 2001). This is because sex steroid administration affects the process of sexual differentiation, not sex determination.

For masculinisation, androgens and some non-steroidal aromatase inhibitors have been reported to be effective in producing male-skewed fish populations. Among androgens, the synthetic 17 α-methyl testosterone (MT) is the most widely used hormone. MT is a potent androgen, as its binding affinity to the androgen receptor is much stronger than natural

androgens (Fitzpatrick et al., 1994); however, MT is naturally converted to 17β-estradiol (E_2) via aromatase (Baroiller et al., 1999) and, as such, the non-aromatisable masculinising agents, including aromatase inhibitors, have their usage more recently rendered. While steroid hormones raise increasing public concern, alternatives for non-steroidal masculinising agents like aromatase inhibitors are attractive for future research. The non-steroidal aromatase inhibitors, such as fadrozole and letrozole, induce masculinisation by reducing estrogen biosynthesis in mammals, chicken, reptiles, and fish (Steele et al., 1987; Afonso et al., 1999; Afonso et al., 2001; Komatsu et al., 2006; Akbary et al., 2015). Aromatase inhibitors work by either irreversibly deactivating the aromatase enzyme, or through competitive exclusion of aromatase to receptors in estrogen producing cells. At the molecular level, both androgens and aromatase inhibitors have been reported to suppress the expression of the female-biased gene, *cyp19a1a*, in chicken (Elbrecht and Smith, 1992), zebrafish (Fenske and Segner, 2004), Japanese flounder (Kitano et al., 2000) and fathead minnow (Villeneuve et al., 2006).

Induced feminisation by means of sex steroids, mainly estrogens and other estrogenic agents, natural, or of synthetic origin, have been conducted successfully on various farmed fish (Piferrer, 2001). Although there is no strong correlation between the chemical structure and estrogenic function, synthetic estrogens generally have more pronounced sex-reversing effects than natural estrogens (Yamamoto, 1969; Piferrer and Donaldson, 1992; Kavumpurath and Pandian, 1993b; Van den Belt et al., 2004). Moreover, the elimination of synthetic estrogens is empirically slower than their natural types (Pandian and Sheela, 1995; Piferrer, 2001; Pandian and Kirankumar, 2003). Among estrogens, E_2, a natural estrogen, is the most widely used for the purpose of sex control in fish; while the synthetic estrogen 17α-ethinylestradiol (EE_2) and diethylstilbestrol (DES) have showed the most potency at feminising in a variety of teleost species (Piferrer, 2001).

Research on sex control of hermaphroditic fish also showed that exogenous steroids and non-steroidal aromatase inhibitors are effective in sex manipulation. Specifically, exposure of protogynous fish to androgens resulted in masculinisation in stoplight parrotfish *Sparisoma viride* (Cardwell and Liley, 1991), gag grouper *Mycteroperca microlepis* (Roberts Jr and Schlieder, 1983), dusky grouper *Epinephelus marginatus* (Sarter et al., 2006) and honeycomb grouper *E. merra*. Similarly, administration of aromatase inhibitors induced sex change in numerous protogynous females, such as red spotted grouper *E. akaara* (Li et al., 2005) and wrasse *Halichoeres trimaculatus* (Nozu et al., 2009). In protandrous fish, estrogen exposure induced feminisation of black seabream *Sparus microcephalus* (Ruan et al., 1996), sea bream *S. aurata* (Condeça and Canario, 1999) and common snook *Centropomus undecimalis* (Carvalho et al., 2014) before their normal time of sex reversal. In the case of protandrous black porgy, feeding with E_2 for 5 months at low doses (0.25 or 1.0 mg kg^{-1} feed) stimulated testicular development, while high concentration doses (4.0 mg kg^{-1}) induced complete feminisation in 1 and 2 year-old individuals (Chang et al., 1994; Chang et al., 1995a; Chang et al., 1995b). Two year-old black porgy fed with dietary E_2 at a concentration of 4.0 mg kg^{-1} food for 4.5 months at different timing in their reproductive season induced reversible sex reversal (Chang and Lin, 1998a). These results emphasise the importance of hormonal treatment variables such as dosage, duration and timing.

Administration of exogenous estrogen or androgen can cause abnormal development of gonads and lead to effects such as sterilisation, intersexuality and ‘paradoxical feminisation’ (Billard et al., 1981; Lessman and Habibi, 1987; Trudeau et al., 1993; Pandian and Sheela, 1995). Although excessive exposure to estrogens in some cases can result in sterilisation (Eckstein and Spira, 1965; Blázquez et al., 1998), estrogens are not intentionally applied for sterilising fish due to their deleterious effects if a particular threshold is surpassed. For example, significantly lower survival was observed in coho salmon and zebra cichlid

Cichlasoma nigrofasciatum after E_2 immersion with doses from 800 µg/L for 2 hr and dietary 200 mg E_2 kg^{-1} food for 20 days, respectively (Hunter et al., 1986; George and Pandian, 1996). Exogenous androgens or estrogen exposure is also reported to produce intersexuality, a phenomenon when both ovarian and testicular cells exist within the same gonadal tissues. Although not all observed intersexual fish were tested for fertility, most of the studied cases showed high ratios of low reproductive performance (Pandian and Sheela, 1995). 'Paradoxical feminisation' is a term used for cases when fish exposed to some aromatisable androgens at high concentration, or for extended duration, turned into females, which has been reported in several teleost species (Norris, 1987; Piferrer et al., 1993; Rinchard et al., 1999). Interestingly, Davis et al. (1990) reported an unclear case of channel catfish *Ictalurus punctatus* feminisation following exposure to either androgens or estrogens. Altogether, it has been repeatedly shown that determining hormonal variable treatments for sex manipulation, such as sex steroid types and dosages, duration and timing, are species-specific and should be empirically obtained.

Several methods for administration of EDCs to induce sex change in fish have been reported within the literature, including direct injection of the material into the muscle or body cavity of the fish, direct immersion into a bath containing the dissolved EDC for a certain amount of time, administration in the culture water or as a dietary supplement (Crim, 1985). The advantages and limitations of these methods have been discussed previously (Pandian and Sheela, 1995; Beardmore et al., 2001). Due to simplicity of administration and applicability at a commercial scale, dietary feeding of hormones is one of the most common techniques for sex reversal in aquaculture. The addition of hormones and chemicals to the feed successfully sex reversed Mozambique tilapia *O. mossambica* (Nakamura and Takahashi, 1973; Nakamura, 1975), European sea bass (Chatain et al., 1999), and rainbow trout (Sahafi et al., 2011). The oral route of hormone administration has some disadvantages, such as the

degradation of hormone in the digestive progress, variability in dosage among individuals due to non-uniformity and concentration of the hormone in the feed, and differences in the feeding rate of individuals leading to differences in how much of the hormone the fish may actually ingest. For those species in which the gonadal labile period occurs before first feed (such as during embryogenesis or in yolk sac larvae like most of the egg-laying salmonids) immersion in water containing sex steroids offers an effective alternative. Immersion of coho salmon larvae with 400 μg/L of E_2 for a period of 2 hr results in clutches comprising 97% females (Hunter et al., 1986). Generally, methods of hormone delivery should be chosen based on the size/age/life stage of fish at the time of treatment, as well as considering other factors such as environmental and human health concerns.

1.5. Barramundi farming

Barramundi, *Lates calcarifer*, also known as Asian seabass, are widely distributed in coastal and brackish waters throughout the Indo-West Pacific region from the Arabian Gulf to southern China, the Philippines, Indonesia, Papua New Guinea and northern Australia (Nelson et al., 2016). Barramundi is one of the most important commercial aquaculture species of many tropical countries, including Australia, Malaysia, Indonesia, Thailand and Vietnam. They are amenable for farming, and well known for their high growth rate, good quality flesh and easy husbandry. Global production in 2014 was approximately 71,000 tonnes (FAO, 2018a). Most of the seed supply for farming of barramundi is derived from hatchery production.

In Australia, barramundi is farmed in all states except Tasmania. The industry produces around 7,000 tonnes of product. It has an estimated value of production at around $70 million at farm gate. There is every indication the industry will continue to expand, with growth

coming from existing farms and new entrants to the industry (Australian Barramundi Farmers Association, http://www.abfa.org.au/barramundi.html).

The future growth and increase in productivity of Australian barramundi aquaculture requires efficient selective breeding programs that target genetic gains for growth and disease tolerance. One of the biggest problems that significantly affects the efficiency of selective breeding is the fact that barramundi is a protandrous hermaphrodite, where fish first mature as males and then a few years later sex change into females (Moore, 1979). In Australia, under natural conditions, barramundi reach sexual maturity as males at 2 - 4 years, and then undergo the sex change process between 6 and 8 years to become mature females (Moore, 1979; Davis, 1982). Although a sexually precocious population in the Gulf of Carpentaria, Australia, has been surmised to change their sex after 4-5 years of age (Davis, 1984). Under culture conditions, barramundi mature as male at around 2 years post-hatching and then naturally sex change into female at around 3-4 years. This natural sex change challenges the broodstock management for barramundi hatcheries, as male broodfish of high quality constantly change sex into female requiring recruitment of new male fish for broodstock. In addition, in selective breeding, sequential sex change of barramundi most often results in one-generation age difference between sexes, with females older than the males in spawning groups. Inter-generation breeding thereby halves the annualised rate of genetic progress that could otherwise be made through a single-generation selection program (Robinson and Jerry, 2009). Therefore, sex control of sequential hermaphrodites, as in the case of barramundi, through inducing earlier sex reversal of males into females or preventing the natural sex change to maintain males in the breeding population, is of great importance.

Synchronising generations between male and female fish could be feasible if it was possible to promote early maturation of females. Literature shows that sex reversal in other fish species can be achieved by manipulating certain environmental conditions. For instance,

altering water temperature or administrating exogenous steroidal hormones and aromatase inhibitors are among the most common practices to induce sex change (Pandian and Sheela, 1995; Baroiller et al., 1999; Piferrer, 2001; Baroiller et al., 2009); however, the effectiveness of these methods is not always stable and is significantly affected by many factors. Regarding the hormone addition method, successful sex reversal ratios of a species are dependent on the timing and duration of treatment, along with the types and dosage of hormone. Basically, a sufficient amount of exogenous hormone should be administered to fish larvae starting at the sexually undifferentiated stage and lasting through the stage of gonadal sex differentiation (Yamamoto, 1969). At an optimal dosage, the duration period of hormone treatment should be limited, without compromising the efficiency of the sex reversal induction, to reduce the side effects of hormones on fishes, as well as minimise other economic issues such as cost of labour, time and hormone, etc. Time of onset of treatment, ideally, is the “critical” period when developing gonads are most sensitive to differentiation. Therefore, determining the precise window of the gonadal differentiation process plays an important role in establishing the protocol for sex reversal induction in barramundi.

Barramundi is found to reach their sex reversal period earlier in captive conditions than in the wild where male barramundi have mature testis as early as 9 months post-hatching (Szentes et al., 2012) and a portion of fish cultured in sea cages have been observed to sex change at 3 years of age (Guiguen et al., 1994). This suggests that sex differentiation of this species could be dependent on environmental factors. In fact, a study on barramundi by Athauda et al. (2012) showed that plasma, brain and gonadal levels of key sex steroidal hormone levels and gonadal stages in 14 month-old barramundi were strongly effected by rearing water temperature. This labile characteristic of gonad development leads to the hypothesis of speeding up the process of sex maturity by exogenous components, such as manipulating environmental conditions or administering sex hormones.

The sex determining mechanism of barramundi has not been firmly elucidated, as sex chromosomes have not been detected. Not all barramundi individuals undergo the sex change process because there is existence of primary females that do not go through the male phase and males that do not reverse sex (Moore, 1979; Davis, 1982). Most smaller fish are males, dictating genetic involvement in determining sex of this species; however, the role of environmental factors has not been fully elucidated.

1.6. Thesis aims and structure

Control of sex change in barramundi is one of the biggest impediments when conducting selective breeding programs for the species. The research reported in this thesis therefore aimed to understand initial male sex development and then control the important process of sex differentiation in barramundi. It documents, for the first time, the development of the male testis from hatching to spermatogenesis, along with some of the underlying gene expression changes associated with early male sex development. Based on the timing of testes differentiation (indicating first phase of sexual development), trials then examined if the administration of exogenous estrogen fed to larvae in pelleted feeds induces precocious females. Further trials were then conducted examining if 6 month to 2-year-old males could be sex changed into females using estrogen implants. This was trialled because successful induction of sex differentiation may allow males harvested for selective breeding programs to be sex changed into females and bred together with non-sex changed males of the same age. Finally, the use of an aromatase inhibitor, fadrozole, was examined to determine if sex change from male to female could be inhibited to keep broodstock as males if desired.

The thesis has been prepared to report on a series of trials each representing an article for publication.

The first data chapter (*Chapter 2*) focused on investigating the biological basis of the sex differentiation of farmed barramundi, including the morphological changes and expression

levels of putative male-related genes in the gonadal tissues. This study used histology to examine the morphological changes in the gonad tissues of barramundi from newly hatched undifferentiated tissue to completed testicular development. Description of changes at cellular levels in the gonad were reported with a focus on early signs of male differentiation that have been detected for other teleost species. Also, RT-qPCR was used to study the expression of the two key male-biased genes, *dmrt1* and c*yp11b*, during the critical timing of sex differentiation, as molecular changes generally precede visual morphological signs. The method, *in situ* hybridisation with a mRNA probe, was used to detect the location of *dmrt1* on the gonad sections to determine which gonadal cells are responsible for the initiation of barramundi sex differentiation. Combined information on morphological changes and key male gene expression are used to determine the labile period, believed to be the most responsive time of the gonad to external influences. This study was published in the peer-reviewed journal *Aquaculture* (Banh et al., 2017) with minor editing in this thesis chapter for readability and links with other parts of the thesis as a whole.

Chapter 3 examined the potential of the two estrogens, E_2 and EE_2, on the feminisation of barramundi, by dietary administration to barramundi juveniles during their pre-determined labile period (as determined in *Chapter 2*). As the hormonal sex change for fish is species-specific in term of steroid type, dosages, treatment timing and duration, the information in this chapter is useful for future sex control of barramundi. The effect of these estrogens on the gonad development and liver morphology of barramundi juvenile was examined by histology. The former analysis included description on the gonadal phenotypes and appearances of any abnormal cells. The latter analysis was to check if there were any detrimental effects of the hormones on fish health. Additionally, RT-qPCR was used to study the effect of exogenous estrogen on the expression of the key sex genes, *dmrt1* and *cyp19a1a*. These gene expression patterns were used to correlate with the morphological changes in the

hormone-induced gonads. This study was submitted to the peer-reviewed journal *Aquaculture* and is under review.

Chapter 4 investigated an alternative method to deliver E_2 to the sexually differentiated barramundi for sex reversal. As the feminised barramundi are for selective breeding purposes, only a certain number of males are required to be sex changed to female. The E_2 hormone implantation 'dosage-range-finding' trial was initially conducted on mature male barramundi (6-8 kg body weight (BW)). The differentiated fish were implanted with cholesterol-based pellets containing different dosages of E_2 into the peritoneal cavity. This preliminary trial was successful in providing an efficient range of E_2 for feminising male barramundi and suggested not to implant into the fish cavity due to inflammation risk. Based on the effective E_2 doses determined from the preliminary experiment, pellets of the required E_2 dose were inserted into the left dorsal musculature of each fish in the primary trial. Histology analyses on morphology of gonads and livers were used to evaluate the feminising effect and health condition of the treatments. Gene expression patterns of five key sex-related genes (*dmrt1*, *cyp11b, esr1, cyp19a1a* and *foxl2*) in the E_2 treated fish were examined by RT-qPCR. The information obtained from this chapter provides an effective and suitable method for barramundi breeders to use when selectively changing sex of targeted individuals.

Chapter 5 assessed the effects of a non-steroidal aromatase inhibitor, Fadrozole (FAD), administered solely, or with E_2 cholesterol pellet implants, on the gonadal development of mature male barramundi. This study assessed if FAD can block the natural sex change of barramundi and keep fish as males. Identifying large numbers of natural sex changing fish is difficult due to timing and unreliability of identifying gonads in transition without destructive sampling; thus, in this trial, the optimised E_2 dosage determined previously for inducing feminisation was used to drive a sufficient number of fish for experimental purposes towards the female gonadal state. In this trial, gonad development, fish health and gene expression of

the key sex-related genes of male barramundi implanted with FAD, E_2 and a mixture of FAD and E_2 were examined. Information from this last trial is important for better understanding mechanism of sex change in barramundi, as well as providing barramundi breeders a way to maintain broodfish as male.

Collectively, this thesis provides new information on the biology of male sex differentiation in barramundi, along with establishing methods useful for industry to sex-change males into precocious female broodstock, or keep them male, using exogenous endocrine disruptors.

Chapter 2. Morphological changes and regulation of the genes *dmrt1* and *cyp11b* during the sex differentiation of barramundi

2.1. Introduction

The study of teleost gonadal development offers unique insights for the broader understanding of vertebrate sex differentiation, as well as facilitating strategies to control reproductive processes in farmed aquaculture species. This is because, unlike most vertebrates, sex determination and differentiation in fishes are often flexible processes and may be subject to modification by extrinsic factors, especially during early gonadal development when animals are most sensitive to environmental stimuli (widely referred to as the labile period) (Piferrer, 2001; Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002). Manipulation of environmental factors (e.g. addition of sex steroids, changing water temperature or pH) during early gonad development may override genetically pre-programmed sex determination mechanisms, thus altering the resultant phenotypic sex of the fish (Baroiller and D'Cotta, 2001; Budd et al., 2015). It has been shown in many fish that this critical labile period occurs early in embryonic/juvenile development, primarily before the germ and ancillary cells destined to become the gonad differentiate (Nakamura and Takahashi, 1973; Hackmann and Reinboth, 1974). By the time the gonad is fully differentiated, the lability of the sex determination process dramatically decreases in most species (Piferrer, 2001).The determination of when the fish gonad actually commences sexual differentiation, especially testicular differentiation, is often complicated by sexual differences in timing of development, as well as how differentiation can be morphologically detected. For instance, testicular differentiation studies of several fish species reveal that germ cells remain quiescent for a prolonged time after formation of the zygote compared to ovarian tissue. In the two tilapia species, *O. mossambicus* and *O. niloticus*, ovarian differentiation becomes histologically recognizable around 20 and 35 dph, respectively; however, testicular

differentiation only commences around 50 and 70 dph (Nakamura and Takahashi, 1973; Nakamura and Nagahama, 1985; 1989). Similarly, in the African catfish *Clarias gariepinus,* signs of gonadal differentiation are observed at 25-28 dph in females and later at 42-45 dph in males (Van Den Hurk et al., 1989; Santi et al., 2016). As PGCs of male fish remain morphologically unchanged for longer periods than in females, it is often hard to determine based on histological observation of the germ cells alone if gonadal differentiation has actually commenced. Accordingly, other types of early histological indicators for testicular differentiation are often used. These include the appearance of a slit-like lumen in stromal tissue, which subsequently becomes the efferent duct, appearance of blood vessels, invagination of the epithelial tissues into lobular structures, and differentiation of the primitive somatic cells into functional cells (Takahashi, 1977; Patiño et al., 1996; Aberle et al., 1997; Nakamura et al., 1998; Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002). In addition to histology, molecular cues have also been used to narrow the timing of male sex differentiation in fishes. The *dmrt1* gene, for instance, is known to be a key determinant in testicular development of vertebrates (Raymond et al., 1998; Smith et al., 1999a; Guan et al., 2000; Kettlewell et al., 2000; Shibata et al., 2002; Koopman and Loffler, 2003; Matsuda, 2003; Kobayashi et al., 2004). *In situ* hybridisation and immune-histochemical studies in Nile tilapia, orange-spotted grouper, zebrafish and medaka have shown *dmrt1* to be particularly expressed in testicular Sertoli cells as they differentiate; thus, observation of the expression of this gene in these cells provides an indication of testes development (Devlin and Nagahama, 2002; Kobayashi et al., 2004; Guo et al., 2005; Xia et al., 2007). Similarly, increased activity of 11 β-hydroxylase, a steroidogenic enzyme, which catalyses the biosynthesis of the potent androgen 11-ketotestosterone in male fish (Kime, 1993), is detected during the early testicular differentiation of Nile tilapia (Ijiri et al., 2008), rainbow trout (Govoroun et al., 2001; Baron and Guiguen, 2003; Vizziano et al., 2007),

anemonefish *Amphiprion clarkia* (Miura et al., 2008) and Japanese eel *Anguilla japonica* (Jianga et al., 1996).

Barramundi is an important commercial species farmed throughout tropical waters of the Indo-Pacific due to their high growth rate, good flesh quality and easy husbandry (Jerry, 2013). In recent times, there has been interest in improving the productivity of the species and several selective breeding programs have been investigated. However, one of the major impediments in implementing selective breeding in barramundi is that this fish is a protandrous hermaphrodite (Moore, 1979; Davis, 1982; Robinson and Jerry, 2009). That is, fish mature first as males, before subsequently changing sex to the female gonadal state some years later. As a result, it is not possible to mate male and female broodfish of the same age, with males currently mated in breeding programs to females that originate from prior generations. This inability to mate fish of the same age reduces potential genetic gains through selection by half, as the annualised rate of genetic gain in a breeding program is limited by the generation interval (Falconer, 1975). If precocious females could be produced through early manipulation of sex differentiation processes, barramundi breeding programs would benefit from being able to mate fish of the same age, substantially lowering the generation interval. Accordingly, the ability to control and/or manipulate sex is considered as a critical goal for future efficient selective breeding programs. To achieve this goal though, information on the early gonad differentiation process is required, along with an estimate on when the application of extrinsic environmental stimuli for sex control would most likely be effective for the species.

Traditionally, studies determining sex differentiation in fish have been based on histological examination of gonadal morphology throughout ontology. In barramundi, histology indicates that individuals are functional males with full formation of the testes by nine months of age (Guiguen et al., 1994; Szentes et al., 2012). However, there is no information on testicular

development before this time and, in particular, when and how initial timing of testicular differentiation commences in the species. An understanding of the timing of initial gonadal differentiation into the male testis state in barramundi is important as this may give indications of the labile period whereby their sex could be manipulated through the addition of exogenous factors like hormones to produce primary females.

The aim of this study was to document for the first time in the protandrous barramundi, formation and early testicular development of the male gonad, with special attention to identifying early indicators and developmental patterns of sex differentiation. In addition, the expression pattern of two key male genes, namely *dmrt1* and *cyp11b*, within developing gonadal tissue was studied by RT-qPCR. Lastly, to better understand the role *dmrt1* plays during this process, *dmrt1* expression was also localised at the cytological level in the gonad tissue by mRNA *in situ* hybridisation. Together, the profiles of the male genes and the histological changes in gonadal morphology were used to describe the period of testicular differentiation of barramundi and indicate the likely labile period where sex manipulation strategies would be likely to be effective for this economically important aquaculture species.

2.2. Material and methods

All the experiments conducted for this study were approved by the Animal Ethics Committee of James Cook University (Approval A2014).

2.2.1. Experimental and rearing conditions and sampling schedule

Fertilised eggs (~20,000) obtained from a commercial barramundi hatchery were distributed among five 600 L tanks (5 replicates). Fish were cultured following common hatchery practices in Australia (Schipp et al., 2007), whereby they were kept in seawater at 28-31^{o}C and 12 h light and 12 h dark photoperiod. Fish were weaned at 18 dph and fed *ad libitum* with formulated pellets (Ridley Corporation, Australia). From 30 dph, fish were periodically

graded and extreme size classes removed to reduce the amount of fish per tank to ~ 500 by 61 dph. Juvenile barramundi samples were collected for histological analysis starting from 1 dph for a period of 8 months to cover the presumed entire development period of the male barramundi gonad according to the following regime: 1-60 dph samples collected every second day (3 individuals/tank), 60-100 dph every 5 days (3 individuals/tank), 110-160 dph every 10 days (3 individuals/tank), 6-8 months every 30 days (5 fish/age group). For gene expression analyses, fish (n = 5) were also sampled at 70, 90, 100, 120, 140 and 160 dph. For *in situ* hybridisation, fish gonads (n = 5) were sampled and analysed at 120, 140 and 160 dph.

2.2.2. Histology analysis

Sampled fish were humanely euthanized with AQUI-S® anaesthetic (Aqui-S New Zealand Ltd, New Zealand) followed by cervical dislocation. For newly hatched larvae up to 18 dph, whole larvae were fixed for histological analyses, while for fish aged 20-60 dph only trunks were fixed (through removal of the head at the dorsal fin and the tail at the anal fin). Finally for those fish 65 dph and older the gonads were individually dissected. Fifteen individuals were collected for each time point. All samples regardless of type were fixed in 10% neutral buffered formalin containing 4% formalin for 24 h, and then subjected to standard histology procedures. The preparations were sectioned serially at a five µm thickness according to the established paraffin method and stained with haematoxylin – eosin. Serial histological preparations covered the whole length of the abdominal cavity. Approximately 30-50 slides were obtained from each sample. For the large fish, which had their entire gonads removed, three pieces (anterior, middle and posterior) from the left and right gonad were sampled from each fish. These pieces were then serially sectioned and 10-15 slides were obtained for each gonad region. The slides were examined using an Olympus CelSens Microscope Digital Camera System (Olympus, Japan).

2.2.3. *In situ* hybridisation with *dmrt1* cRNA probe

Sense and antisense digoxigenin (DIG)-labeled cRNA probes were synthesised according to (Leonard, 1995) with some modification. The antisense RNA probe was designed to bind to the mRNA of the target gene, *dmrt1*, while the sense probe was used as a negative control to eliminate background staining. Firstly, a cDNA template was obtained from the gonadal RNA of a 12-month-old barramundi individual (see the cDNA synthesis section).

Amplification of *dmrt1* cDNA was then conducted by PCR using sense and antisense primers (Table 2.1.) with T7 promoter. The thermal cycling conditions consisted of an initial step of 5 min at 95 ^{o}C followed by 10 cycles of 95 ^{o}C (30 s), 60 ^{o}C (90 s), 72 ^{o}C (40 s), and 20 cycles of 95 ^{o}C (30 s), 57 ^{o}C (90 s), 72 ^{o}C (40 s), and final extension at 60 ^{o}C (30 min). The obtained PCR products (575 bp) were confirmed to correspond with the *dmrt1* sequence of barramundi by Sanger sequencing (Australian Genome Research Facility (AGRF), Melbourne, Australia). The digoxigenin (DIG)-labelled RNA probes were then generated in both antisense and sense direction by *in vitro* transcription with a DIG RNA labelling Kit (Roche Molecular Biochemicals, Switzerland), according to manufacturer directions using T7 RNA polymerase (Promega, USA). Probe quality was checked by electrophoresis on a 1.5% 1.5x TBE gel for 20 min at 90 V.

Sampled tissues were rapidly rinsed in diethylpyrocarbonate (DEPC)-treated PBS and fixed in 4% paraformaldehyde for 24 h at 4 ^{o}C. The fixed tissues were then cryoprotected by incubation in 20% sucrose-PBS at 4 ^{o}C for 6 h, followed by embedding in Tissue-Tek® Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA Inc., USA). Tissues were sectioned (8 µm thickness) with a cryotome (Leica Biosystems Inc., Germany) and mounted on superfrost silane treated slides (Menzel-Glaser, Germany).

In preparation for *in situ* hybridisation transverse sections were rehydrated in a sodium phosphate buffer with saline (PBS) and permeabilised with 1 µg/mL proteinase K7 in PBS containing 1% Tween 20 for 20 min. Slides were incubated in either sense or antisense digoxigenin-labelled RNA probes with hybridisation buffer (50% formamide, 5x saline-sodium citrate, 500 µg/mL of tRNA, 50 µg/mL of heparin, and 0.1 % Tween-20 [pH 6.0]) at 58 ^{o}C overnight. Slides then were cooled to room temperature and subjected to stringency washes to remove excess probe. Alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Molecular Biochemicals, Switzerland) at 5000-fold dilution was used to detect the *dmrt1* mRNA binding reaction. Colour reaction was performed with fresh staining solution containing NBT/BCIP in a dark humid chamber for 12 h, followed by incubating slides in stop buffer [10 mM Tris-HCl (pH 8.1), 1 mM EDTA]. After briefly washing in distilled water, slides were mounted using aqueous mounting solution (Mountant Immu-Mount, Thermo Fisher Scientific) then imaged.

Table 2.1. Primer sequences used for synthesis of *in situ* hybridisation probes (*dmrt1*) and RT-qPCR (for the genes *dmrt1*, *cyp11b* and *ubq*)

Primer		Nucleotide sequences (5'-3')	Functions	References
ISH-dmrt1	Forward Reverse	TGTCTTTTTACTCTCCCTGC TGGTATTGCTGGTAGTTGTAG	Synthesis of cRNA probe	Ravi et al. (2014) with modification
RT-dmrt1	Forward Reverse	GTGACTCTGACTGGCCCAGAG CAGCAGGTCGGACGTTCC	RT-qPCR	Ravi et al. (2014)
ubq	Forward Reverse	ACGCACACTGTCTGACTAC TGTCGCAGTTGTATTTCTGG	Reference gene for RT-qPCR	De Santis et al. (2011)
cyp11b	Forward Reverse	ACACCGGGGTTCTGGGCCAG CACCGCTGTCGTGTCGACCC	RT-qPCR	Ravi et al. (2014)

2.2.4. Quantification of *dmrt1* transcripts in gonadal tissue by RT-qPCR

Quantitative reverse transcription PCR (RT-qPCR) was conducted to obtain a relative gene expression analysis of the *dmrt1* and *cyp11b* genes in barramundi gonads at 70, 90, 100, 120, 140 and 160 dph. As the size of the gonad from a single barramundi did not suffice for RNA extraction according to the Trizol® RNA Isolation (Thermo Fisher Scientific, USA) protocol

(less than 50 mg in weight), pools of gonads of 20 fish (70 dph), 10 fish (90 dph), 5 fish (120 dph and 140 dph) and 3 fish (160 dph) were processed as a single biological replicate with a total of eight replicates for each age group.

2.2.4.1. RNA extraction, DNAse treatment and cDNA synthesis

Total RNA was extracted from barramundi gonad using Trizol® RNA Isolation Reagents (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The obtained RNA was quantified spectrophotometrically (NanoDrop 1800, Nanodrop Technologies, Wilmington USA) and stored at -80°C until DNAse treatment. DNAse treatment was conducted on the RNA extracts (2-4 µg RNA) to eliminate any residual traces of genomic DNA with TURBO DNA-free™ kit (Invitrogen™, USA) according to manufacturer's instructions. DNAse treated RNA was then cleaned with an ammonium acetate precipitation protocol (Osterburg et al., 1975). Quality of RNA was checked by electrophoresis on 1.5% agarose gel (in 1x TBE made with DEPC treated water) with GelGreen™ (Biotium Inc., USA) staining, as well as by OD260/280 and OD260/230 absorbance ratios measured on a NanoDrop spectrophotometer (NanoDrop Technologies, USA).

Synthesis of cDNA was conducted using a Tetro cDNA synthesis kit (Bioline, USA). Briefly, 3 µg of DNAse-treated RNA was placed in a RNAse free 200 µL PCR strip tubes (Astral Scientific, Australia) with 0.5 µL Oligo $(dT)_{18}$, 0.5 µL Random Hexamer, 1 µL of 10 mM dNTP mix, 4 µL of 5x RT buffer, 1 µL of RiboSafe RNAse Inhibitor, 1 µL Tetro Reverse Transcriptase (200u/µL) and DEPC treated water to a total 20 µL. To ensure absence of residual DNA within DNAse treated RNA samples, no amplification control (NAC) reactions were performed by using an aliquot of some of the samples DNAse-treated RNA diluted to the same concentration as the RNA used in the real cDNA syntheses without reverse transcriptase. All tubes (including the real cDNA syntheses and NAC) were then placed in a

C1000 Thermal Cycler (Bio-Rad, USA) using the following cycling conditions: 45 ^{o}C for 30 min, 25 ^{o}C for 10 min followed by 45 ^{o}C for 30 min then terminated reaction by incubating at 85 ^{o}C for 5 min, before chilling on ice. The cDNA then was stored at -20 ^{o}C.

2.2.4.2. Quantitative reverse transcription PCR (RT-qPCR)

Primer pairs for *dmrt1* and *cyp11b* were derived from Ravi et al. (2014), whereas primers for the previously validated endogenous control, or reference gene ubiquitin (*ubq*) were derived from De Santis et al. (2011) (Table 2.1.). Reaction efficiencies were validated (in triplicate) from standard curves prepared from 3-fold serially diluted cDNA. Reaction efficiency ranged from 95% to 103%, and reproducibility of the assay (R^2) was always greater than 0.99. RT-qPCR reactions were performed in a total volume of 15 µL and consisted of 7.5 µL of SsoFast^{TM}Evagreen® master mix (Bio-Rad, USA), 0.6 µL of 0.2 µM forward and reverse primers, 5 µL of 1:500 diluted cDNA templates and 1.3 µL of water, previously prepared by an automatic pipetting system (Corbett Robotics, Qiagen, Germany). The RT-qPCR cycling conditions were 95 ^{o}C for 30 s followed by 40 cycles consisting of 5 s at 95 ^{o}C and 10 s of 58 ^{o}C for *dmrt1* and *cyp11b,* or 61^{o}C for *ubq,* and melting step ramping from 60 ^{o}C to 95 ^{o}C with 0.5 ^{o}C increments each step (Roto-Gene Q, Qiagen, Germany).

For each gene, all 30 samples from all six age groups were run in triplicate and assayed in the same 100-well ring with two standard dilutions of standard curve used to enable the importation of the baseline determined by the standard curve through the Rotor-gene 6000 Series software (Roto-Gene Q, Qiagen, Germany). Melt curve analysis was included for all genes to check the assay specificity, whereby only single amplicons were detected for each of the three gene products. Extraction negatives and no amplification controls were included in each ring and produced no signal, confirming no interference by genomic DNA. *Dmrt1* and

cyp11b were normalized to *ubq* using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The mean and standard deviation were determined from the triplicate samples.

2.2.5. Statistical analysis

Statistical analyses were performed using the SPSS software package (IBM SPSS Statistics 23, USA). Homogeneity of variance was tested using Levene test and data that did not conform was log-transformed with any outliners removed. One-way analysis of variance (ANOVA) was used to compare expression values of the target genes at different time points. Post-hoc multiple comparisons were conducted using Tukey test. Differences of *p* values less than 0.05 were considered significant.

2.3. Results

2.3.1. Early gonadal development and sex differentiation

The primordial germ cells (PGCs) of barramundi were first observed at 4 dph situated at the dorsal wall of the coelomic cavity, close to the distal end of the swim bladder (Fig. 2.1.A). PGCs were clearly characterized by their relatively large size (about 4-5 µm in diameter), low nucleocytoplasmic ratio, distinct round outline, weakly stained cytoplasm and a single, clear nucleus. Barramundi PGCs comprised of single, prominent eccentrically located nucleolus. In serial cross sections, PGCs were not detected in the posterior part of the gonad. The pear-like germinal ridge (or primordial gonad), which was composed of a few somatic cells, was first detected at 14 dph (6.4 ± 0.6 mm in total length (TL)) without germ cell attachment (Fig. 2.1.B). At 28 dph, when larvae were approximately 12.6 ± 0.7 mm TL, the paired gonads were observed to be suspended on both sides from the swim-bladder wall by a thin sheet of somatic cells of the mesenteries (Fig. 2.1.C). The paired gonads ran caudalwards (i.e. head to tail) from the proximal part of the pectoral fins, alongside the swim-bladder and ended in the peritoneum near the genital pore. All the gonads observed 36 dph showed a relatively simple pattern of cellular association, and no signs of somatic or germ cell sex differentiation could

be recognised, at least morphologically (Fig. 2.1.D). However, by 44 dph, the cross section of the gonad showed evidence for formation of a slit-like lumen, which was represented as a split in the stroma tissue when the fish reached 25.8 ± 3.3 mm TL (Fig. 2.1.E). During subsequent development, this space could be identified as the efferent duct. This split in the stroma tissue was the first discernible morphological evidence of differentiation of gonadal tissue in barramundi. Thus, testes differentiation appeared to commence ~ 44 dph in this species. The germ cells with multiple nucleoli (granular chromatin), were observed with a relative smaller size (3-5 µm in diameter), along with others comprising a single nucleolus. In cross section, the germ cells were distributed at the periphery close to the epithelial cells of the developing gonad. At 60 dph (50.1 ± 6.8 mm TL), blood vessels were observed at the distal part of the gonad (Fig. 2.1.F). Invagination was observed along the epithelial cells at the ventral side of the gonad resulting in latero-ventral lobes. PGCs were often found in mitosis (Fig. 2.1.F'). Later, at 70 dph (69.8 ± 5.2 mm TL), blood vessels were found to be substantially larger in size (Fig. 2.1.G). Aggregation of somatic cells in the gonad at this age resulted in increased size of the whole gonad. By 90 dph (102.7 ± 24.6 mm TL), the slit-like lumen was extended and became recognisable as the efferent duct at the dorsal side, which was distinguished from the smaller testicular lobules (Fig. 2.1.I). A noticeable feature at this age was evidence of cysts of PGCs nested in the latero-ventral lobes (Fig. 2.1.H), while the stroma tissues were distributed at the dorsal side.

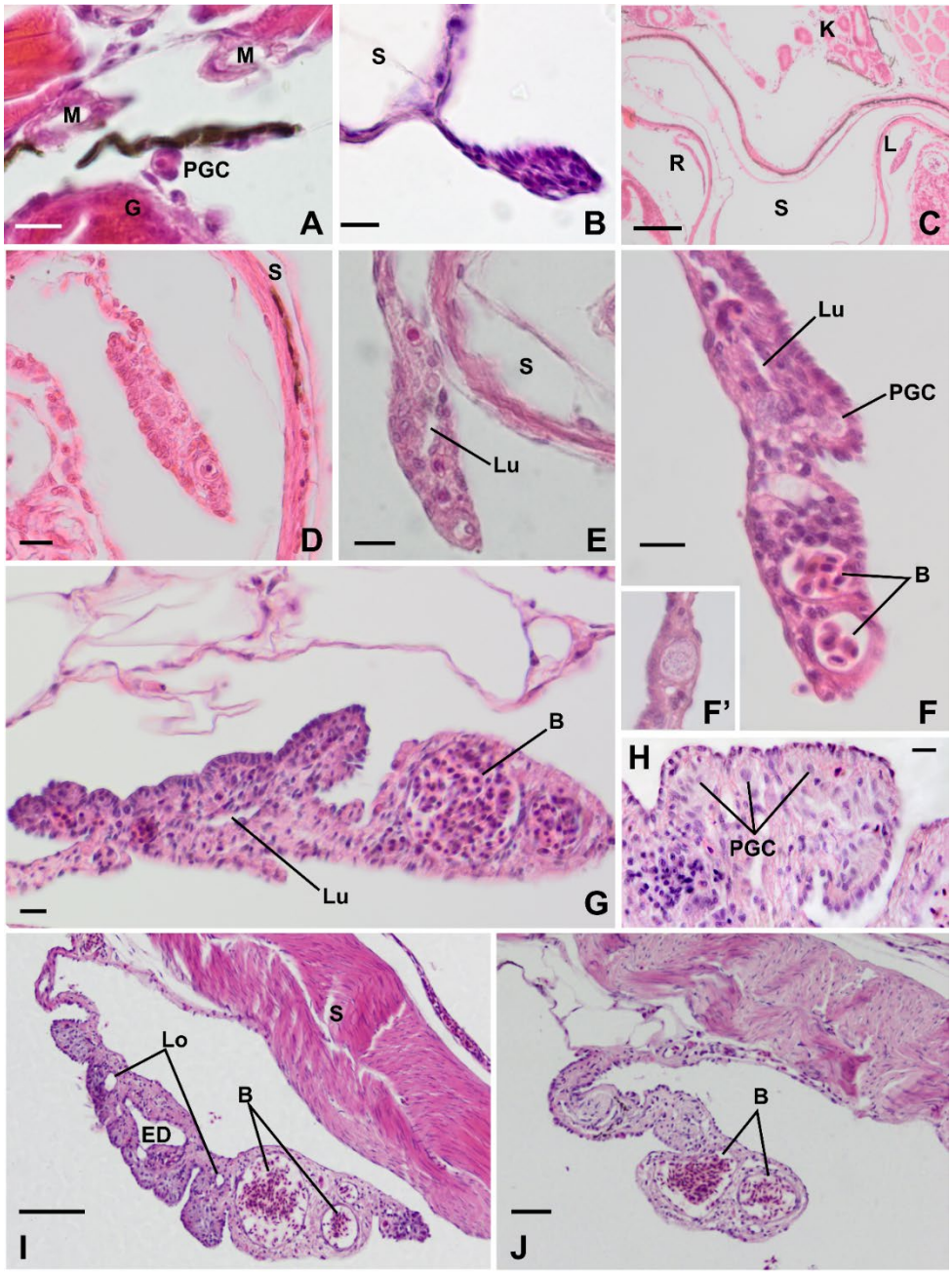

Figure 2.1. Early gonadal development and differentiation of farmed barramundi in seawater

A) PGC solitarily located between the dorsal peritoneal wall and the gut (G) in the cross section. **B)** Indifferent gonad at 14 dph. **C)** Cross section of barramundi at 28 dph showing the indifferent pair of gonads suspended from swim-bladder wall by a thin sheet of somatic cells of the mesenteries. **D**) Undifferentiated gonad at 36 dph. **E)** Slit-like lumen in the gonad at 44 dph. **F**) Gonad at 60 dph with blood vessels at the peripheral region and; **F'**) the PGCs in mitosis. **G**) Gonad at 70 dph. **H)** High magnification of left gonad at 90 dph showing the strips of PGCs. **I)** Left gonad at 90 dph. **J)** Right gonad at 90 dph. Abbreviation: PGC, primordial germ cell; M, mesonephric duct; S, swim bladder; K, kidney; L, left gonad; R, right gonad; Lu, lumen; B, blood vessel; Lo, testicular lobule; ED, efferent duct. Scale bar indicates 10 μm (A); 5 μm (B, D, E, F, F', G, H); 20 μm (C, I, J).

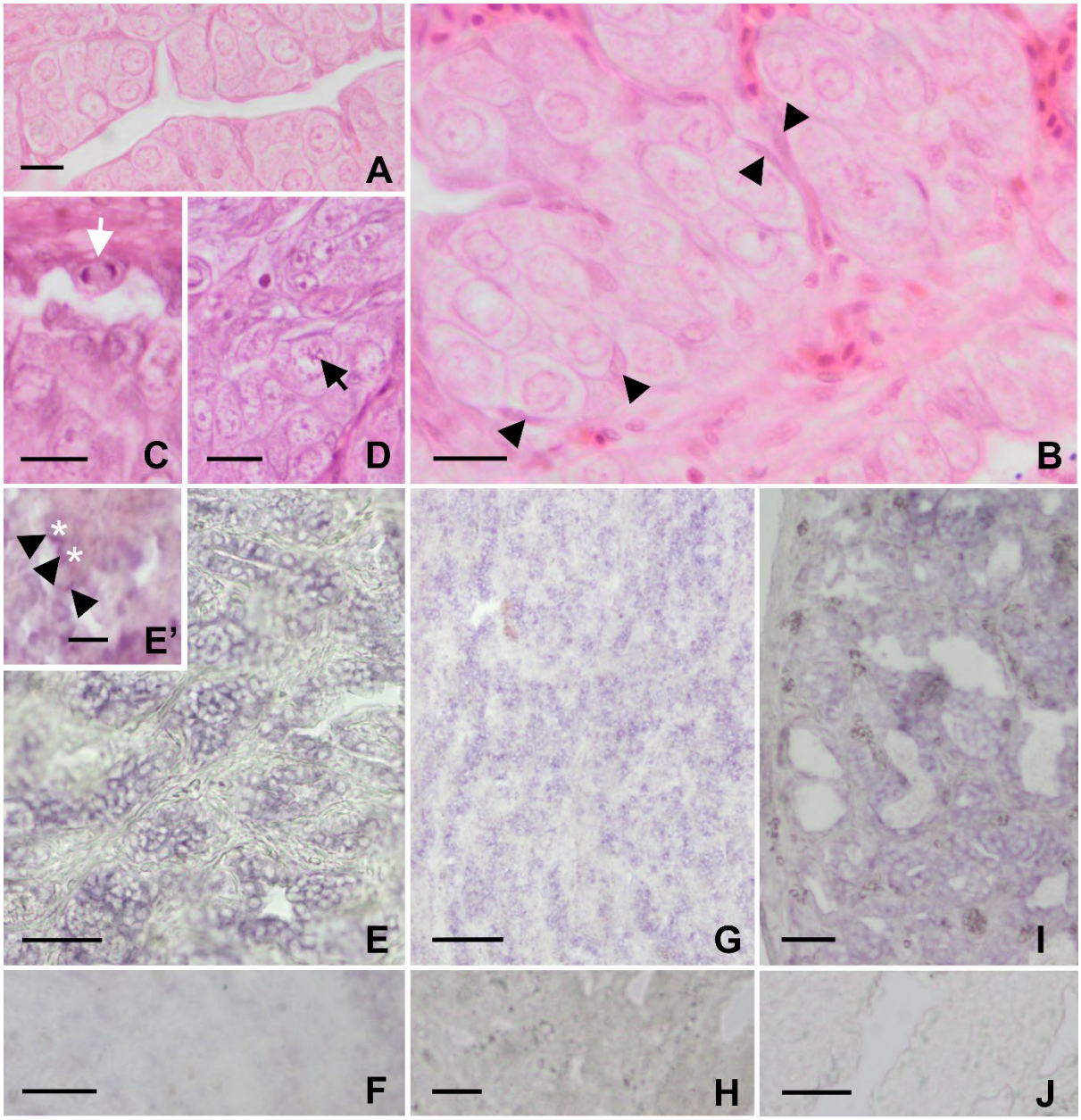

Figure 2.2. Histological images of the differentiated gonad of barramundi at 120 dph and 130 dph; and *in situ* hybridisation with antisense and sense probes of *dmrt1* in gonad at 120, 140 and 160 dph

A) Cysts of germ cells and lobular lumen in the cross section of gonad at 120 dph. **B)** Sertoli cells (black arrow heads) envelop the cysts of germ cells at 120 dph. **C)** A germ cell in mitosis (white arrow) in the gonad at 120 dph. **D)** Germ cell in meiosis (black arrow) in the gonad at 130 dph. **E, G** and **I)** *dmrt1* positive staining (purple) in the gonad at 120 dph, 140 dph and 160 dph. **E')** *dmrt1* was positively stained in Sertoli cells (black arrowheads) while no positive stain was seen in the spermatogonia (white asterisks). **F, H** and **J)** No positive staining in sense probe for negative control. Scale bar indicates 5 µm (A, B, C, D and E'); 50 µm (E, F, G, H, I and J).

As fish aged the gonadal structures became more obvious and by 120 dph (141.2 ± 22.3 mm TL) the testes were nearly fully formed. At this stage of development, the germ cells were

observed to be noticeably clustered in seminal lobules, which were separated by a defined layer of fibrous connective tissue. Blood vessels were regularly found at the interstitial areas among cysts of germ cells (Fig. 2.2.A, 2.2.B and 2.2.C). The somatic cells surrounding the germ cells were also observed to have differentiated into Sertoli cells. These Sertoli cells had an elongated triangular shape with a strongly basophilic appearance. Sertoli cells enveloped the cysts of germ cells, which had matured into spermatogonia. By 130 dph, gonads contained germ cells in the 'bouquet' stage of meiosis, with the chromatin condensed at one end of the nucleus and a single nucleus laying over the chromatin, a feature typical of zygotene (Fig. 2.2.D). Finally, by 140 dph, most of the primordial germ cells had become differentiated into primary spermatocytes (Fig. 2.3.A) when the fish reached 169.4 ± 40.3 mm TL. Efferent ducts were located on the dorsal side of the testes, while ventrally invagination of the gonad formed latero-dorsal lobes. In cross sections, spermatocytes were distributed in the lobular shape close to the ventral side, while stromal tissues composed of connective fibroblast cells were situated in the gonad dorsally. Active spermatogenesis was observed in the testes of most sampled fish by 160 dph (Fig. 2.3.I) (197.5 ± 35.7 mm TL). No sampled fish were found to have germ cells differentiated into oocytes. The key morphological events during early gonadal development and differentiation of barramundi are summarised in Table 2.2.

2.3.2. Gradient in barramundi testes development

During early stages, differentiation of cells was uniform in the proto-gonad. However, once differentiation had begun histology revealed that development occurred in an anterior to posterior manner, with the first signs of tissue differentiation observed in the anterior portion of the developing testes (e.g. slit-like lumen, blood vessels, differentiation of germ cells). This observation was consistent for both developing left and right gonads from the same cross section. The gradient of gonadal development along the length of the gonad was

particularly noticeable during spermatogenesis when the fish were 140 to 160 dph. As well as the gonad developing anterior to posteriorly, there was some evidence that the left gonad matured faster than the right gonad. Here cross sections of the left gonad were observed to be larger than the right gonad (by 90 dph), although the cellular organisation was indistinguishable (Fig. 2.1.I and 2.1.J). By 140 dph, though, cross sections of the anterior left testes revealed large numbers of spermatocytes, the posterior gonad was observed to contain a few spermatocytes and large numbers of spermatogonia. However, in the right gonad at the same time of development only approximately half of the germ cell lobules in the anterior testes had spermatocytes, while germ cells remained undifferentiated in the posterior region of the testes (Fig. 2.3.A, 2.3.B, 2.2.C and 2.3.D). Similarly, in the 160 dph samples, spermatozoa were detected within the lumen of the seminiferous tubules of the anterior part of the left and right gonads; in the posterior left testes, mostly spermatocytes and spermatids were evident, while in the right posterior testes only a few lobules of spermatozoa were observed (Fig. 2.3.E, 2.3.F, 2.3.G and 2.3.H). Therefore, spermatogenesis and gonadal development in barramundi is not a uniform process between the testes, as differences in morphology were seen in tissues sampled from the left or right gonad, or from the anterior, middle or posterior sections. By 8 months, however, tissues were fully formed and production of spermatozoa was relatively uniform in both gonads (Fig. 2.3.J).

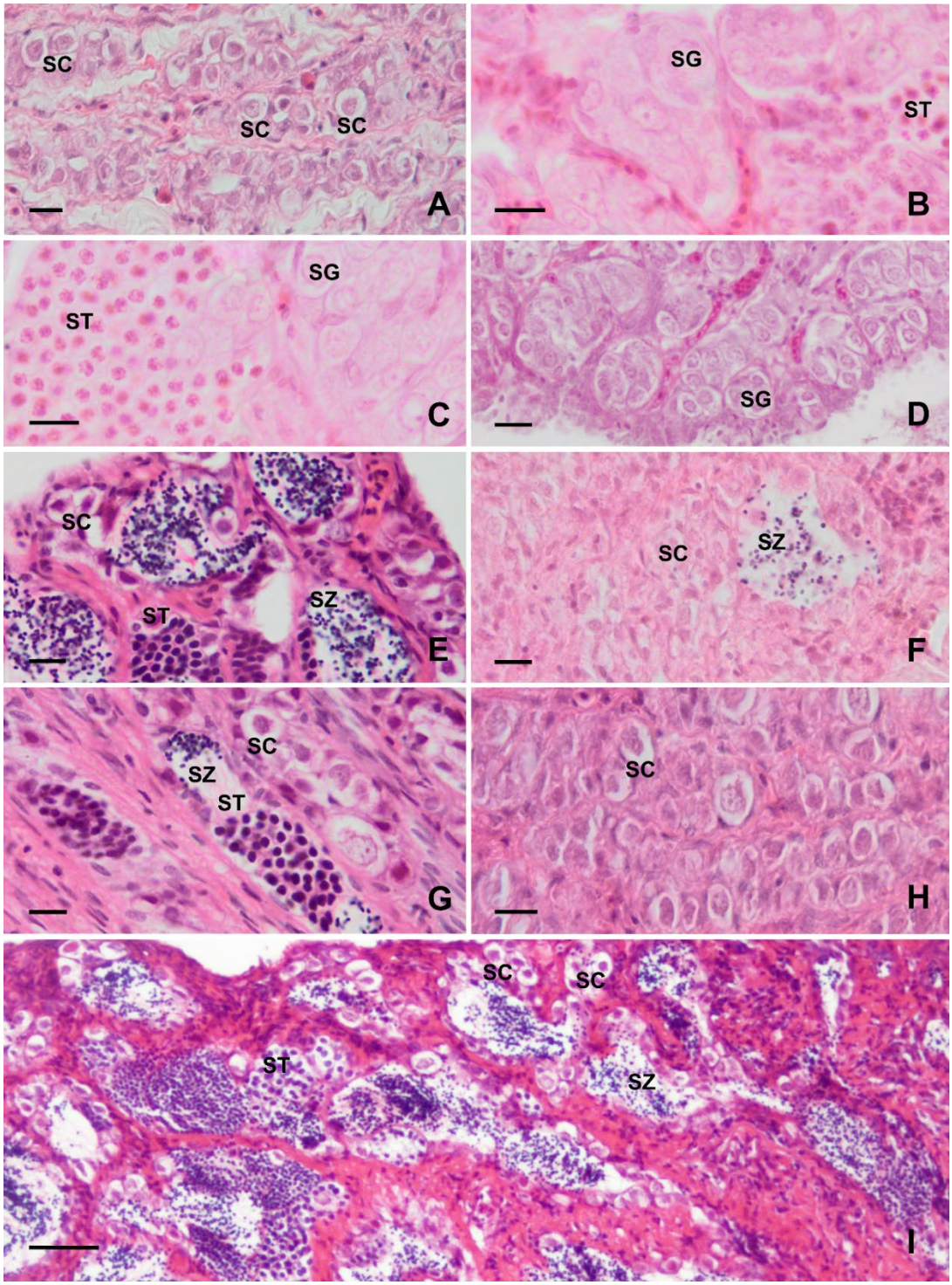

Figure 2.3. Gradient in gonad development of barramundi at 140 and 160 dph

A) Cross section of the anterior part of left gonad at 140 dph. **B)** Cross section of the posterior part of left gonad at 140 dph. **C)** Cross section of the anterior part of right gonad at 140 dph. **D)** Cross section of the posterior part of right gonad at 140 dph. **E)** Cross section of the anterior part of left gonad at 160 dph. **F)** Cross section of the posterior part of left gonad at 160 dph. **G)** Cross section of the anterior part of right gonad at 160 dph. **H)** Cross section of the posterior part of right gonad at 160 dph. **I)** Gonad of 8 month post-hatching barramundi. Abbreviation: SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa. Scale bar indicates 10 μm (A-H); 20 μm (I).

Table 2.2. Summary of morphological events and features of gonadal development and sex differentiation of seawater farmed barramundi at 28 ºC

DPH	Morphological events, gonad description	Observed individuals/Total samples	Gonadal classification
14	Pear-like germinal ridge composed of a few epithelial cells without germ cell attachment.	12/12	Undifferentiated
28	Gonad increased size by increasing in number of somatic cells. Germ cells were detected.	9/9	Undifferentiated
44	First sign of testicular differentiation whereby the slit-like lumen is evident.	9/12	Differentiated
60	Blood vessels were observed at the distal part of the gonad. Invagination was observed along the gonad resulting in formation of lobular shape tissues. Epithelial cells developed and lined the lobular organisation. Primary germ cells were often found in mitosis.	10/12	Differentiated
70	Blood vessels were found larger in size. Aggregation of somatic cells in the gonad at this age resulting in increased size of the whole gonad.	9/12	Differentiated
90	The efferent duct was expanded and distinguished from the unrestricted lobular organisations or testicular lobules. PGCs were presented as in the strips nested in the lobes distributed along the epithelial layer.	11/12	Differentiated
120	Germ cells were clustered in the seminal lobules which were separated by a layer of fibrous connective tissue. The somatic cells surrounding the germ cells were differentiated to functional cells such as Sertoli cells.	7/9	Differentiated
130	Meiosis of germ cells were detected	7/9	Spermatogenesis
140	Most of the primordial germ cells had become differentiated into primary spermatocytes	8/9	Spermatogenesis
160	Active spermatogenesis was observed in the testes.	9/9	Spermatogenesis
210	Gonads were relatively uniform and composed of large area of spermatozoa and spermatids with some spermatocytes.	7/9	Spermatogenesis

2.3.3. Expression of *dmrt1* and *cyp11b* in the differentiating gonadal tissue

The expression of the genes *dmrt1* and *cyp11b* in the barramundi gonad was assessed by RT-qPCR from 70 to 160 dph (Fig. 2.4.A and 2.4.B). This coincided with the most active differentiating phase of the gonad as revealed via histology. Results showed that by 70 dph, when RT-qPCR was first able to be assessed, *dmrt1* and *cyp11b* expression could already be detected at low levels. Over this timeframe *dmrt1* expression was observed to peak at 120 dph, with an almost 30-fold increase in expression compared to 70 dph samples ($P < 0.05$). Thus, expression levels of *dmrt1* increased as a factor of time from 70 to 120 dph. This expression pattern coincided with the evidence of large blood vessels at 70 dph, enlargement of the lumen at 90 dph and differentiation of the somatic cells into Sertoli and Leydig cells by 120 dph. After 120 dph, *dmrt1* expression appeared to gradually reduce over time in the discernibly differentiated gonad, although expression was not significantly different ($P = 0.065$) (Fig. 2.4.A). Similarly, c*yp11b* expression in the barramundi gonad was observed to increase dramatically, almost eight folds, from 70 to 90 dph. From 90 dph, as the gonad continued to develop this gene was seen to gradually lower in expression and was reduced by 50% from 120 dph to160 dph ($P < 0.05$).

Distribution of *dmrt1* transcripts in the gonad at 120, 140 and 160 dph were also investigated by *in situ* hybridisation. This revealed that *dmrt1* was expressed in the somatic cells surrounding germ cells at 120 dph, but was not detectable in the germ cells. As shown in Fig. 2.2.E and 2.2.E', *dmrt1* expression was clearly localised around the germ cells. As spermatogenesis proceeded, more intensive *dmrt1* signals were detected in the gonad of barramundi at 140 and 160 dph.

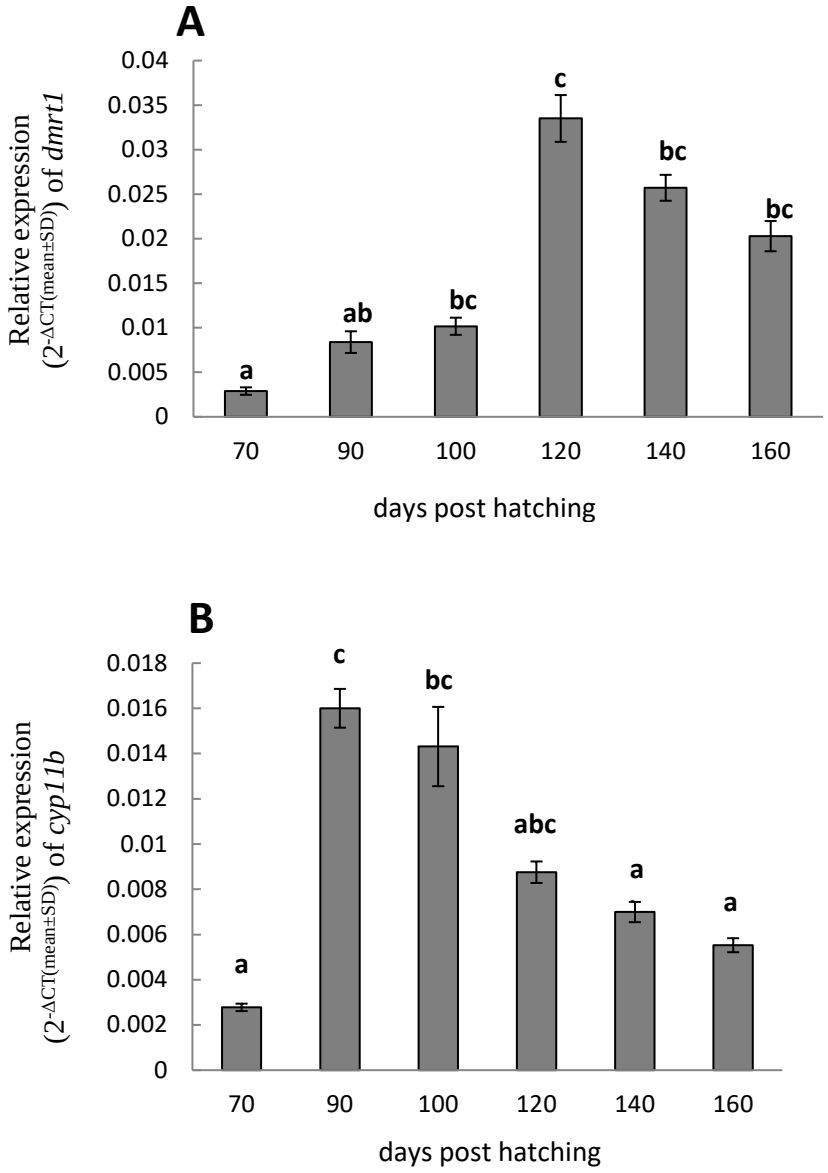

Figure 2.4. Relative *dmrt1* (A) and *cyp11b* (B) expression measured by RT-qPCR in gonad tissue at 70, 90, 100, 120, 140 and 160 dph.

The values were calibrated with the reference gene *ubq* according to Livak and Schmittgen (2001). Different letters represent statistical differences ($P < 0.05$) between age groups ($n = 8$ for each value).

The expression of *cyp11b* and *dmrt1* was detectable as early as possible (70 dph), their peak were observed at 90 and 120 dph, respectively. Also, the final signs of testicular differentiation occurred at 140 dph when the total fish length were 169.4 ± 40.3 mm. This

data suggests that the period for testing external treatments for sex control would likely cover the window time from 44 to 140 dph for captive cultured barramundi in seawater (from 25.8 ± 3.3 mm to 169.4 ± 40.3 mm TL, respectively).

2.4. Discussion

This research describes for the first time the early gonadal development and testicular differentiation of barramundi. In barramundi, the gonad was shown to originate from an undifferentiated mass of cells situated at the dorsal wall of the coelomic cavity, which then developed to show initial signs of testicular differentiation by 44 dph, where a slit-like invagination in the stromal tissue first appears; this slit-like opening later formed the efferent duct. The gonad continued to develop whereby blood vessels formed, ventral invagination of the epithelial surface resulted in latero-dorsal lobes with cysts of PGCS that become lobules (90 dph). By 120 dph, the testes had appeared to have formed as germ cells became clustered into seminal lobules and Sertoli supporting cells were obvious. The described sequence of testicular differentiation in barramundi is in close agreement to that seen in other teleost species (Mozambique tilapia (Nakamura and Takahashi, 1973); Nile tilapia (Nakamura and Nagahama, 1989), pejerrey (Strüssmann et al., 1996), chum salmon (Nakamura, 1984), and white-spotted charr *Salvelinus leucomaenis* (Nakamura, 1982)). Similarly, in these other species, the testes developed from cells originating in the dorsal coelomic cavity and the first clear sign of testicular differentiation was the appearance of the anlages of the efferent duct.

Detecting testes morphological differentiation based on germ cell activity is relatively difficult, as germinal cells destined to develop into spermatocytes commonly stay quiescent until quite late in the development process (Nakamura and Nagahama, 1989; Chiba et al., 1997; Nakamura et al., 1998; Devlin and Nagahama, 2002; Matsuda, 2005; Nakamura, 2013). In barramundi, germ cells were not observed to differentiate until around day 120, where

Sertoli cells were first detected, and not to differentiate into spermatocytes until around day 140. However, early indicators for male differentiation can be based on the behaviour of somatic cells in the gonads, including formation of the presumptive efferent duct, occurrence of blood capillaries near the efferent duct (Hatakeyama et al., 2005; Rasmussen et al., 2006; Haugen et al., 2012) and appearance of distinct aggregation of stromal cells at the gonad hilus (Itahashi and Kawase, 1973; Van Den Hurk et al., 1989; Patiño et al., 1996; Strüssmann and Nakamura, 2002). The observation of these other morphological features in testes has been used to pinpoint the time where the testes begins to differentiate. In barramundi, therefore, testicular differentiation is predicted to commence prior to 44 dph (~25.8 ± 3.3 mm TL).

The expression of the two male genes *cyp11b* and *dmrt1* significantly increased at 90 dph and 120 dph, respectively. The highest expression of *cyp11b* was at 90 dph, when the lumen was enlarged. It is possible that secretion of 11-ketotestosterone at this time in the differentiating gonad is associated with triggering the differentiation of somatic cells. For instance, levels of 11KT increased during the testicular differentiation of European sea bass (Papadaki et al., 2005). This expression of *cyp11b* shows a sexually dimorphic pattern, whereby more mRNA is detected in the male in several fish species, including barramundi (Liu et al., 2000; Socorro et al., 2007; Liu et al., 2008; Ravi et al., 2014). As the physiological differentiation precedes histological differentiation, findings suggest that in barramundi *cyp11b,* or endogenous androgen, plays a crucial role in testicular differentiation. Meanwhile, the peak expression of *dmrt1* was observed at 120 dph, followed by the differentiation of primordial germ cells into spermatogonia. Moreover, *dmrt1* transcripts were exclusively found in supporting cells such as the Sertoli cells, supporting evidence from other studies that suggest *dmrt1* plays an important role in testicular development and spermatogenesis. According to Svingen and Koopman (2013), Sertoli cells serve as master regulators driving the differentiation of the androgen-producing Leydig cells and the spermatogonia of the mammalian testis. In Nile

tilapia, *dmrt1* was also observed in somatic cells and parenchyma/medullary cell mass cells, which differentiated into Sertoli cells and the efferent duct during testicular differentiation, respectively (Kobayashi et al., 2008). Sexual dimorphic expression of *dmrt1* has been reported in various vertebrates (Marchand et al., 2000; Brunner et al., 2001; Devlin and Nagahama, 2002; Berbejillo et al., 2012). This gene is upregulated considerably in developing testes, but not in developing ovaries. The timing of this upregulation varies among species, but it generally occurs in late sex determination, or the early testicular differentiation period. In the gonad of protandrous gilt seabream, *dmrt1* mRNA levels increased during spermatogenesis and decreased just prior to sex change (Liarte et al., 2007). *Dmrt1* is shown to be conserved in the sex determination process through evolution and gonad-specific expression during the period of sex determination. Matson et al. (2011) showed that *dmrt1* was vital for maintenance of testicular function. Loss of the *dmrt1* transcription factor in adult mice can cause trans-differentiation of testicular Sertoli cells to ovarian granulosa cells and ectopic *dmrt1* can reprogram granulosa cells to Sertoli-like cells in the ovary (Lindeman et al., 2015). In the protandrous black porgy, *dmrt1* plays a crucial role in testis differentiation and spermatogenesis (Wu et al., 2012). These characteristics of *dmrt1* expression in different species suggest that *dmrt1* is specifically involved in early testicular development.

Observations within the current study identify that the barramundi gonad develops from an undifferentiated gonad into testicular tissues during their second to sixth month of development, as testis containing spermatozoa released in the ducts could be found as early as 160 dph. Development of the proto-gonad directly into the testes implies that differentiation as a male is a mandatory initial phase of barramundi sexual development. Prior to the present study, the earliest report on barramundi gonadal development was after 9 months of growth, with (functional) testes identified in barramundi reared in an intensive freshwater

recirculation system (Szentes et al., 2012). In this study, only males were also observed. Although there has been evidence of primary barramundi females in the wild (Moore, 1979; Davis, 1982), our study likewise found no evidence for an ovary-like gonad in the individuals examined, nor intersex gonads that contained testicular and ovarian tissues. This confirms that barramundi is a protandrous hermaphrodite where all fish mature initially as male.

Barramundi gonadal sex differentiation and development showed a developmental gradient, which proceeds from anterior to posterior in the gonad (head to tail), and was more advanced in the left compared to the right gonad. The same pattern of gonadal development were observed in pejerrey, where differentiation of the testes proceeded caudally and only commenced in the right testes when 10-30% of the left testes had differentiated (Strüssmann and Ito, 2005). Similar to the pejerrey, where sex differentiation in the testes starts anteriorly, regions of barramundi gonad, which were more developed, were always accompanied with the presence of larger blood vessels.

2.5. Conclusions

Based on our observations, in barramundi the first signs of sex differentiation, specifically the formation of the efferent duct primordium, start at 44 dph when the fish reached 25.8 ± 3.3 mm in total length. Other indicators such as the invagination of the epithelium resulting in latero-dorsal lobes, appearance of blood vessels and later increases in size, and differentiation of supporting cells into Sertoli and Leydig cells, were evident from 60 to 120 dph. The gene *cyp11b* was highly expressed at 90 dph implying its important role in testicular differentiation. The expression of *dmrt1* sharply increased on 120 dph, just prior to spermatogenesis and exclusively in surrounding Sertoli cells. Based on the findings, it is suggested that the sex differentiation process is completed for most fish at 140 dph corresponding to a TL of 169.4 ± 35.7 mm.

Chapter 3. Dietary 17 β-estradiol and 17 *α*-ethinylestradiol alter gonadal morphology and gene expression of the two sex-related genes, *dmrt1* and *cyp19a1a*, in juvenile barramundi

3.1. Introduction

Barramundi are protandrous hermaphroditic fish that mature initially as male at around 2 years of age before naturally undergoing permanent sex change to female at 4-5 years, depending on cues from the environment and population (Davis, 1982). Despite being an important aquaculture species, efficient commercial selective breeding programs for barramundi have largely been impeded by this protandrous sexual development; hatcheries in Australia must keep and sustain superior males for an additional 2-3 years past male maturity in order for the fish to sex change and breed as females (Robinson and Jerry, 2009). Mature females are 90-120 cm in length (10-20 kg) (Davis, 1982; Robinson and Jerry, 2009) requiring large-volume tank facilities. Moreover, as the rate of genetic progress in breeding programs is factored by generation interval (Falconer and Mackay, 1996), only half the potential genetic gain can be made as males and females of the same age (generation) cannot be mated (Robinson and Jerry, 2009). Developing methods to precociously feminise male barramundi and, therefore, produce both sexes simultaneously would be beneficial for selective breeding of barramundi.

Induced feminisation has been widely and successfully applied in numerous teleost species. This is because gonadal development in fish is particularly flexible and susceptible to the influence of external factors (Devlin and Nagahama, 2002). Manipulation of physical parameters (such as temperature and pH) during the time window when the undifferentiated gonads are responsive, also known as the labile period has been shown to override genetically determined sex mechanisms, thus altering the phenotypic sex of the fish (Baroiller et al., 1999; D'Cotta et al., 2001; Piferrer, 2011). Application of exogenous estrogenic sex steroids

has also been successful in changing phenotypic sex in fish (Piferrer, 2001; Strüssmann and Nakamura, 2002; Budd et al., 2015). One of the most common and effective estrogenic hormones for inducing feminisation is the natural estrogen compound E_2 (Nakamura et al., 2003). E_2 is known to play a key role in growth, promotion of gonadal development and hepatic vitellogenin production (Ng and Idler, 1983; Christiansen et al., 1998; Klinge, 2000; Nilsson et al., 2001). The synthetic EE_2 is also used for hormone treatment in aquaculture breeding programs due to its two- to -three-fold potency in feminising fish when compared to E_2 (Yamamoto, 1969; Blázquez et al., 1998; Piferrer, 2001). Both E_2 and EE_2 have been documented as effective hormone treatments for feminising teleosts such as coho salmon (Goetz et al., 1979; Piferrer et al., 1994a), common snook (Carvalho et al., 2014; Passini et al., 2016), black porgy (Chang et al., 1995a; Chang and Lin, 1998b) and sablefish *Anoplopoma fimbria* (Luckenbach et al., 2017).

Masculinisation or feminisation of the undifferentiated gonad, as influenced by hormones, is determined by the balance between a masculinising or feminising molecular pathways (Todd et al., 2016; Capel, 2017). One of the key male-supporting genes, *dmrt1*, plays an important role in the testicular development and differentiation of many fish species (Smith et al., 1999b; Guan et al., 2000; Kettlewell et al., 2000; Guo et al., 2005; Johnsen et al., 2010; Masuyama et al., 2012; Shen and Wang, 2014). *Dmrt1* expression is significantly higher in male pejerrey (Fernandino et al., 2008), European sea bass (Deloffre et al., 2009), and Atlantic cod *Gadus morhua* (Johnsen et al., 2010), compared to females of the same species. In barramundi undergoing gonadal differentiation, *dmrt1* is upregulated and peaks at 140 dph, coinciding with the differentiation of the supporting somatic cells into Sertoli cells (Banh et al., 2017). In mature adult barramundi, *dmrt1* expression is downregulated in ovarian tissue (Ravi et al., 2014; Domingos et al., 2018). In an antagonistic fashion to *dmrt1*, the gene *cyp19a1a* promotes production of P450 aromatase, a key enzyme responsible for the

irreversible conversion of androgens to estrogens (Kazeto et al., 2004; Luckenbach et al., 2009). *Cyp19a1a* is responsible for the provision of all endogenous estrogens by controlling steroidogenic conversion of androgens and, as a result, is regarded as a key determinate for ovarian differentiation and development (Kitano et al., 1999; Kobayashi et al., 2003; Piferrer and Guiguen, 2008; Smith et al., 2013). *Cyp19a1a* is upregulated in ovarian tissues of various teleost species (Kwon et al., 2001; Blázquez et al., 2008; Ijiri et al., 2008; Wu et al., 2008; Wen et al., 2014; Caruso et al., 2016) and is also considered an early marker of ovarian development (Piferrer, 2011). However, Ravi et al. (2014) reported *cyp19a1a* gene expression was 5.5-fold higher in testes than in ovaries of barramundi. The role of *cyp19a1a* in barramundi sex differentiation is thus still unclear.

The effect of two exogenous estrogen hormones on the differentiation of barramundi gonads was studied in context of their application to induce feminisation. The hormones, E_2 and EE_2, were delivered orally through the feed at two dosages from 30 to 160 dph. Morphological and histological effects of hormone treatment on fish gonads were assessed throughout treatment, after treatment and at 12 months post hatch (mph) to understand functional changes during sex differentiation. Histopathological analysis of liver of the treated fish were performed to determine the impacts on fish health. Expression patterns of *dmrt1* and *cyp19a1a* in the gonad were investigated through RT-qPCR at 160 dph and 12 mph.

3.2. Materials and methods

3.2.1. Experimental design

Rearing and sampling methods were approved by the Animal Ethics Committee of James Cook University (Approval A2014). Juvenile barramundi (22 dph) that were fully weaned onto commercial diets were obtained from a commercial supplier and transferred into the experimental system. Prior to commencing hormone treatment, fish (27 dph) underwent a 3-day acclimation period and were fed *ad libitum* with the control diet. Hormone-treated and

control diets were administered from 30 to 160 dph covering the period of initial gonadal differentiation, which has been previously determined to occur from 44 to 140 dph in barramundi (Banh et al., 2017) as in the *Chapter* 2 in this thesis.

Four hormone treatments were administered to juvenile barramundi; each treatment was replicated three times and included a no-hormone control group. In brief, at day one of treatment, 6,000 fish of average 9.1 ± 0.4 mm TL were distributed into fifteen 40 L tanks (i.e. 400 individuals per unit). From 80 to 160 dph, fish from each unit were transitioned into fifteen 500 L tanks to accommodate for density and size effects due to growth. Control fish were fed a commercial pelleted diet without the addition of hormones (Ridley Corporation, Australia). Treated fish were fed the same commercial diet with the addition of either E_2 (Sigma-Aldrich, E8875) at 10 mg ($10E_2$) or 20 mg per kg ($20E_2$), or EE_2 (Sigma-Aldrich, E4876) at 5 mg ($5EE_2$) or 10 mg per kg ($10EE_2$). This first effort in orally delivered estrogen to barramundi for a duration of 130 days (from 30 to 160 dph) was based on the relationship between dose and duration of estrogen treatment generalised by Piferrer (2001). The hormone dosage for feminisation is highly species-specific. As this is the first attempt to induce sex change in barramundi by oral route, the chosen doses were based on the results of other species with the safety for fish health in mind. Example of duration and dose treatment of E_2 and EE_2 of several species were plotted in a diagram in Piferrer (2001). The duration treatment was 130 days so the doses of estrogen were generally chosen less than 25 mg per kg feed. EE_2 is more potent than E_2, hence, EE_2 dosages were as half as the E_2.

Hormones were added to the commercial feed using the ethanol evaporation method (Shved et al., 2007). Fish were fed the manipulated and control diets until day 160, where after they were individually PIT tagged, pooled into a single 2,500 L tank, and fed with a commercial floating pellet (Ridley Corporation, Australia) until final sampling at 12 mph.

Fish rearing and water quality monitoring were conducted as demonstrated in *Chapter 2*. The experimental systems were equipped with two cartridges of activated carbon (8-10 kg activated carbon /cartridge) to absorb any hormone leaching into the water from feeds. Activated carbon (Acticarb GC1200, Activated Carbon Technologies Pty Ltd., Australia) in filters was replaced every 2 weeks. Finally, water supplied to experimental tanks was sampled every week using E_2 (quantitative analysis ranges from 0.05 μg/L to1 μg/L) and EE_2 (quantitative analysis ranges from 0.05 μg/L to 3 μg/L) ELISA kits (Ecologiena for Environmental Pollutants, Tokiwa Chemical Industries Co., Ltd., Japan) to detect residuals of E_2 or EE_2. No E_2 or EE_2 was detected in the water supplied to experimental units.

3.2.2. Samplings

Fish were sampled 1 day prior to commencement of the experiment (30 dph), throughout the hormone feeding trial (50, 100 and 160 dph) and at 12 mph. On the day of sampling, fish in each tank were counted to establish survival rate and any individuals removed for destructive histological and molecular analyses were euthanised with an over dose of AQUI-S® (Aqui-S New Zealand Ltd, New Zealand). Thirty-six fish per treatment (n = 12 per tank) were sampled at 30, 50, 100 and 160 dph; and 12 fish per treatment at 12 mph. Sampled fish were measured to the nearest millimetre and weighed to the nearest gram. For the fish sampled at 30 and 50 dph, the head and tail were trimmed from the body and the whole trunk preserved for histology. For the fish sampled at 100 dph, 160 dph and 12 mph, individual gonads and livers were sampled and preserved for histological as described in 2.3. Gonad tissues were sampled for gene expression analyses (as described in *2.2*) at 160 dph (at the cessation of hormone treatment when untreated barramundi are completely sexually differentiated (Banh et al., 2017)) and at 12 mph to examine if the hormone effect lasted after 6 months withdrawing the hormone treatment.

3.2.3. Histological analysis

Trunks of barramundi juveniles (at 30 and 50 dph), or individual tissues (gonad and liver) of older fish (100 dph, 160 dph and 12 mph) were placed in 10% neutral buffer formalin for 24 h before being processed for histology. Specimens were dehydrated and histological sections conducted using standard paraffin embedding techniques (*see* section *2.2.3*). Gonad surface areas were measured using Thresholding in the ImageJ software (Schneider et al., 2012).

Testicular development during spermatogenesis was categorised according to Guiguen et al. (1994). Specifically, gonads at stage M0 were immature with no visible differentiated germ cells; M1 gonads exhibited predominance of spermatogonia; M2 gonads were filled with mostly spermatocytes and spermatids, while gonads containing predominantly spermatozoa were classified as stage M3.

3.2.4. Gene expression analyses by RT-qPCR

The expression of two genes, *dmrt1* and *cyp19a1a*, was examined at 160 dph (at the cessation of hormone treatment) and 12 mph (approximately 6 months post hormone treatment). For gene expression analyses, sampled tissues were cut into small pieces (less than 2 mm), preserved in RNAlater^{TM} Stabilization Solution (Thermo Fisher Scientific), and incubated at 4 ^{o}C overnight prior to storage at -20 ^{o}C for 3 to 4 days before RNA isolation.

RNA extraction, DNAse treatment, cDNA synthesis and RT-qPCR reactions were conducted as described previously (*see Section 2.2.3*). RT-qPCR was routinely optimised prior to mRNA gene expression analysis. Gene expression level was compared in the gonads of 12 fish from each treatment at two time points (160 dph and 12 mph). Primers for *dmrt1*, *cyp19a1a*, and the internal reference gene *ubq* (ubiquitin) were derived from previous studies (De Santis et al., 2011; Ravi et al., 2014; Domingos et al., 2018) (Table 3.1). RT-qPCR

efficiencies (E) for each gene were validated using standard curves prepared from five points of 3-fold serially diluted cDNA (Table 3.1).

Table 3.1. Primer sequences used for reverse transcription (RT)-qPCR to study the expression of the genes *dmrt1* and *cyp19a1a* in the gonads of barramundi fed with different dosages of E_2 and EE_2

Primer		Nucleotide sequences (5'-3')	References
dmrt1	Forward	GTGACTCTGACTGGCCCAGAG	Ravi et al. (2014)
	Reverse	CAGCAGGTCGGACGTTCC	
cyp19a1a	Forward	CACTGTTGTAGGTGAGAGACA	Domingos et al. (2017)
	Reverse	CTGTAGCCGTCTATGATGTCA	
ubq	Forward	ACGCACACTGTCTGACTAC	De Santis et al. (2011)
	Reverse	TGTCGCAGTTGTATTTCTGG	

At each time point, two 100-well rings were used for each gene. Each ring contained six samples (run in triplicate) from each treatment with two standard dilutions of the standard curve to normalise possible variability between runs. RT-qPCR product specificity for each gene was confirmed by analysis of melting curves, visualisation of the size of products on a 1.5% agarose gel, and Sanger sequencing (AGRF).

The relative abundance of the target genes (*dmrt1* and *cyp19a1a*) were normalised using the reference gene *ubq* according to the $2^{-\Delta Ct}$ method of Livak and Schmittgen (2001). The stability of the reference gene for normalisation was examined using the GeNorm (Primer Design, Ltd., UK).

3.2.5. Statistical analysis

Statistical analyses were performed using the SPSS software package (IBM SPSS Statistics 23, USA). A mean C_T sample value was obtained for each RT-qPCR sample triplicate. Normality and homogeneity of variance were tested using the Kolmogorov-Smirnov and Levene tests, respectively. C_T values that did not meet criteria of either of these tests were log-transformed with outliners removed. One-way analysis of variance (ANOVA) and Post-

hoc Tukey's test were used to determine differences among hormone treatments on fish growth (BW and TL). Data that could not be normalised, or that presented heterogeneous variances after transformation were analysed using a non-parametric Kruskal-Wallis test, followed by the Mann-Whitney U test for pairwise comparisons. Within the same treatment, an independent Mann Whitney U test was used to compare gene expression at 160 dph and 12 mph. Differences were regarded as statistically significant when $P < 0.05$.

3.3. Results

3.3.1. Growth performance and survival

The effects of oral administration of E_2 and EE_2 on the growth performance (BW and TL) and survival of barramundi are shown in Table. 3.2. Treatment commenced at 30 dph at BW 12.8 ± 2.2 g and length 9.8 ± 1.2 mm. Over the course of 130 days, barramundi in the control group grew significantly faster than in either of the hormone treatments ($P < 0.05$). Exposure to estrogenic compounds retarded growth, even after the termination of hormone administration at 12 mph. Feeding with E_2 and EE_2 in this trial did not affect survival of barramundi, as no statistically significant difference was found among the treatments of fish fed with E_2 and EE_2, or the control group ($P > 0.05$).

Table 3.2. Effects of E_2 and EE_2 at various dosages on growth and survival of barramundi during the hormonal treatment (from 30 to 160 dph) and after termination of hormone supplement (at 12 mph)

Hormonal dose (mg kg^{-1} feed)		Control	10 mg E_2 kg^{-1}	20 mg E_2 kg^{-1}	5 mg EE_2 kg^{-1}	10 mg EE_2 kg^{-1}
BW (g)	50 dph	20.0 ± 4.5^{a}	16.5 ± 4.5^{ab}	14.8 ± 2.5^{b}	18.4 ± 4.6^{ab}	14.0 ± 4.7^{b}
	100 dph	122.9 ± 14.5^{a}	91.0 ± 10.5^{b}	93.5 ± 7.4^{b}	110.1 ± 12.8^{ab}	99.0 ± 11.1^{b}
	160 dph	324.6 ± 32.1^{a}	207.2 ± 20.0^{b}	185.4 ± 22.8^{c}	218.3 ± 18.2^{b}	203.5 ± 26.6^{b}
	12 mph	1094.8 ± 147.0^{a}	850.0 ± 175.3^{b}	823.0 ± 162.6^{b}	878.8 ± 226.7^{b}	825.0 ± 185.2^{b}
TL (mm)	50 dph	11.6 ± 0.9	11.0 ± 1.1	12.2 ± 3.6	11.2 ± 1.3	10.4 ± 1.3
	100 dph	16.1 ± 1.5^{a}	14.6 ± 1.9^{b}	14.0 ± 1.5^{b}	14.11 ± 1.33^{b}	14.4 ± 1.6^{b}
	160 dph	21.4 ± 2.3^{a}	19.7 ± 3.6^{b}	19.1 ± 2.6^{b}	19.8 ± 2.7^{b}	18.8 ± 2.6^{b}
	12 mph	29.2 ± 3.4^{a}	28.2 ± 3.0^{b}	28.3 ± 3.1^{b}	28.1 ± 2.3^{b}	28.2 ± 2.5^{b}
Survival (%)	50 dph	82.1 ± 4.3	83.8 ± 9.6	78.7 ± 2.5	76.2 ± 2.8	79.2 ± 5.7
	100 dph	79.0 ± 7.4	75.2 ± 4.1	78.0 ± 6.8	80.5 ± 9.9	78.9 ± 4.8
	160 dph	92.4 ± 3.1	94.0 ± 9.4	88.9 ± 4.1	93.2 ± 9.2	87.1 ± 6.1

12 mph	92.2	87.3	89.3	88.4	90.6

Different letters represent statistical differences ($P < 0.05$) of the value (mean ± standard deviation) in the same row. No superscripts means non-significant difference ($P > 0.05$). BW: body weight; TL: total body length. At 12 mph, survival of each group was only recorded from one common tank.

3.3.2. Morphological changes in the gonads of barramundi fed with E_2 and EE_2 at different dosages

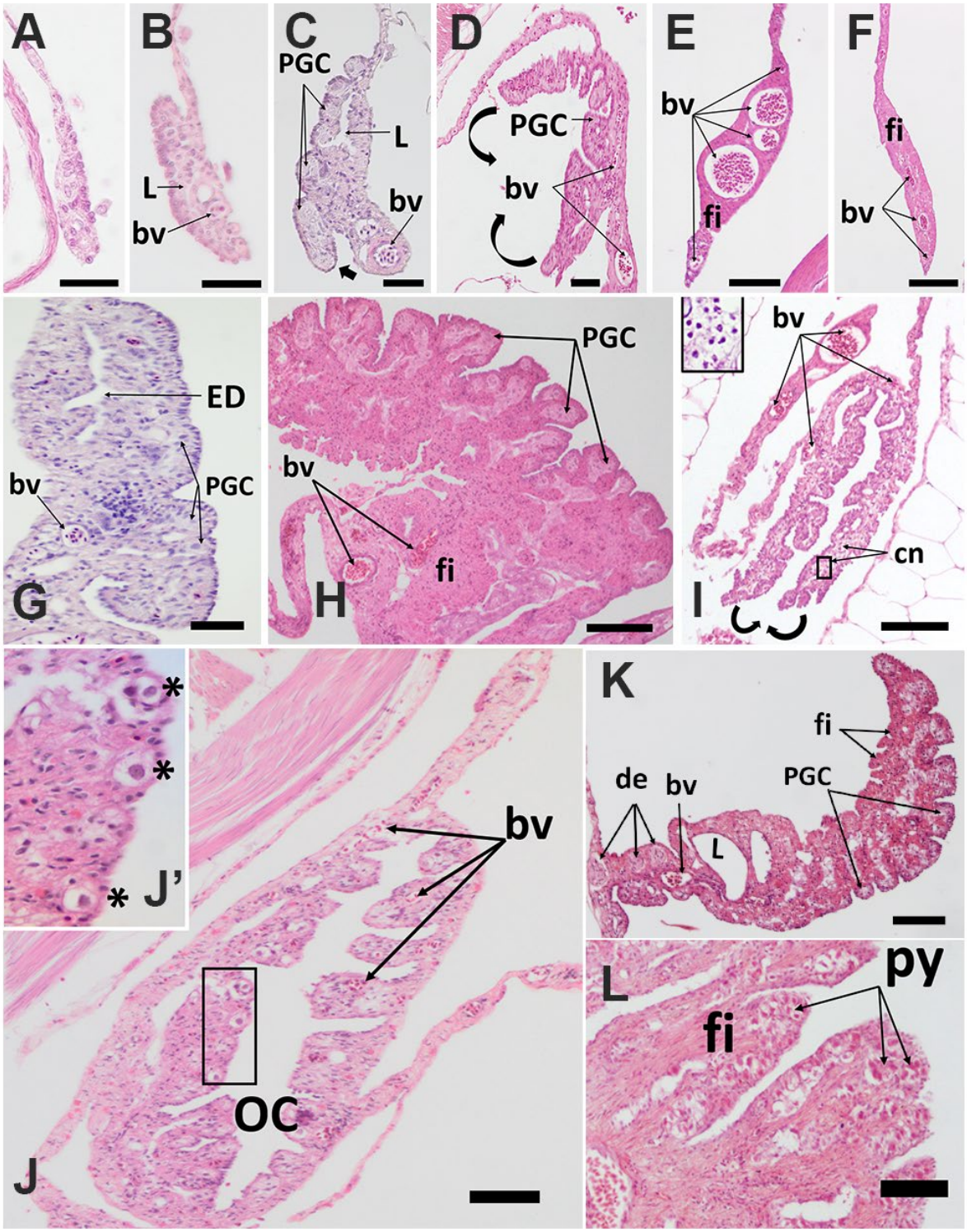

Figure 3.1. Transverse sections of barramundi gonads received different treatments of dietary E_2 and EE_2 at 50 and 100 dph

A) Undifferentiated gonad of barramundi larvae at 30 dph. Scale bar 20 µm. **B)** Control barramundi at 50 dph showing a narrow slit-like lumen as a presumptive efferent duct. Scale bar 20 µm. **C)** Barramundi gonad fed with 10 mg E_2 kg^{-1} diet at 50 dph. Scale bar 20 µm. **D)** Barramundi gonad fed with 20 mg E_2 kg^{-1} diet at 50 dph. The arrows indicate how the two lobes would be closed to form ovarian cavity. Scale bar 20 µm. **E)** Gonad of fish fed with treatment 5 mg EE_2 kg^{-1} diet at 50 dph. Scale bar 10 µm. **F)** Gonad of fish fed with treatment 10 mg EE_2 kg^{-1} diet at 50 dph. Scale bar 10 µm. **G)** Gonad of the control barramundi at 100 dph. Scale bar 20 µm. **H)** Barramundi gonad at 100 dph in the treatment 10 mg E_2 kg^{-1} diet. Scale bar 20 µm. **I** and **J)** Barramundi gonad at 100 dph in the treatment 20 mg E_2 kg^{-1} diet: **I)** In some fish, the dual lobes did not close. Note the presence of oocytes in the chromatin-nucleolus stage (cn) enlarged in the inset. Scale bar 50 µm. **J)** The ovarian cavity formed. Note the presence of oogonia (*) which are enlarged in the inset **(J')**; **K)** Barramundi gonad at 100 dph in the treatment 5 mg EE_2 kg^{-1} diet. Scale bar 20 µm. **L)** Barramundi gonad at 100 dph in the treatment 10 mg EE_2 kg^{-1} diet. Scale bar 20 µm. Abbreviations: bv, blood vessel; cn, chromatin-nucleolus stage oocytes; de, degenerative cells; ED, efferent duct; fi, fibrosis; L, lumen; OC, ovarian cavity; PGC, primordial germ cell; py, pyknosis.

Morphological changes in barramundi gonads induced by dietary E_2 and EE_2 are summarised in Table 3.3. At 30 dph, prior to the beginning of the experiment, there were no histological signs of gonadal differentiation in any individual and the primordial gonads appeared as thin filaments suspended on both sides from the swim-bladder wall by a somatic cell sheet (Fig. 3.1.A). The transverse section of these primordial gonads was 410.86 ± 27.15 μm^2. At 50 dph, control fish gonads showed a narrow, slit-like lumen as the first sign of testicular differentiation (Fig. 3.1.B), while gonads of fish fed with E_2 and EE_2 diets had distinct morphological characteristics. Gonads of the fish in the $20E_2$ treatment showed paired elongated aggregations of somatic cells in the proximal and distal portions, with a thin sheet of somatic cells at the dorsal side connecting to the mesentery. Subsequently, at 100 dph, the outgrowths of the aggregations fused together to form the ovarian cavity (Fig. 3.1.D). The $10E_2$ treated individuals had a clear and large lumen (Y shape) in the gonad tissue on the

dorsal side, whilst at the ventral side there was an outgrowth of tissue (Fig. 3.1.C). The surface area of gonads belonging to fish exposed to $10E_2$ and $20E_2$ treatments (2022.3 ± 337.1 μm^2 and 1823.6 ± 198.7 μm^2, respectively) were significantly larger than in the control fish (1049.5 ± 213.3 μm^2). The $5EE_2$ treatment (Fig. 3.1.E) did not significantly affect the size of the gonad surface (997.2 ± 112.4 μm^2) compared to the control. The high concentration of EE_2 ($10EE_2$) resulted in thin and narrow gonads (Fig. 3.1.F), which were significantly smaller than all other treatments (724.9 ± 102.5 μm^2). Exposure to EE_2 also resulted in the development of abnormal gonads, observed as fibrosis surrounding the blood vessels of gonadal tissue. Fibrosis is abnormal connective tissue, which is described as an increased proliferation of fibroblasts followed by increased deposition of collagen leading to an appearance of thickened connective tissue (Gray et al., 1999; Dietrich and Krieger, 2009).

At 100 dph, lobular structures containing strips of PGCs were observed in control fish (Fig. 3.1.G). Anatomically, gonads of control fish were filiform and attached to the side of the swim bladder. In the $10E_2$ treatment, gonads of all fish were more complex in organisation, consisting of multiple lobular structures containing large cysts of undifferentiated germ cells surrounded by dense connective tissues (Fig. 3.1.H). Ten fish of the $20E_2$ treatment (83%) had a developed ovarian cavity in the middle of the gonad, representing feminisation of gonads (Fig. 3.1.J). The remaining 17% (2/12) fish had gonads with dual lobes, indicating that the enclosing process to form the ovarian cavity was not complete (Fig. 3.1.I). Early stages of oocyte development (as chromatin nucleus) were detected in all $20E_2$ fish, including the two fish that did not exhibit the ovarian lumen (Fig.3.1.I and 3.1.J). In the $5EE_2$ treatment, there was a large lumen, which was different from the efferent duct in the control fish and ovarian cavity in the $20E_2$ treatment. Mild fibrosis and degenerative material of the somatic cells were obvious (Fig. 3.1.K). At 100 dph, morphological features of apoptotic germ cells / pyknosis (the irreversible condensation of chromatin in the nucleus of a cell undergoing

necrosis or apoptosis) were observed in gonads of 10E_2 treated fish, in addition to cell shrinkage and condensation of the nucleus (Fig. 3.1.L). Some normal germ cells also existed and were surrounded by fibrotic connective tissue.

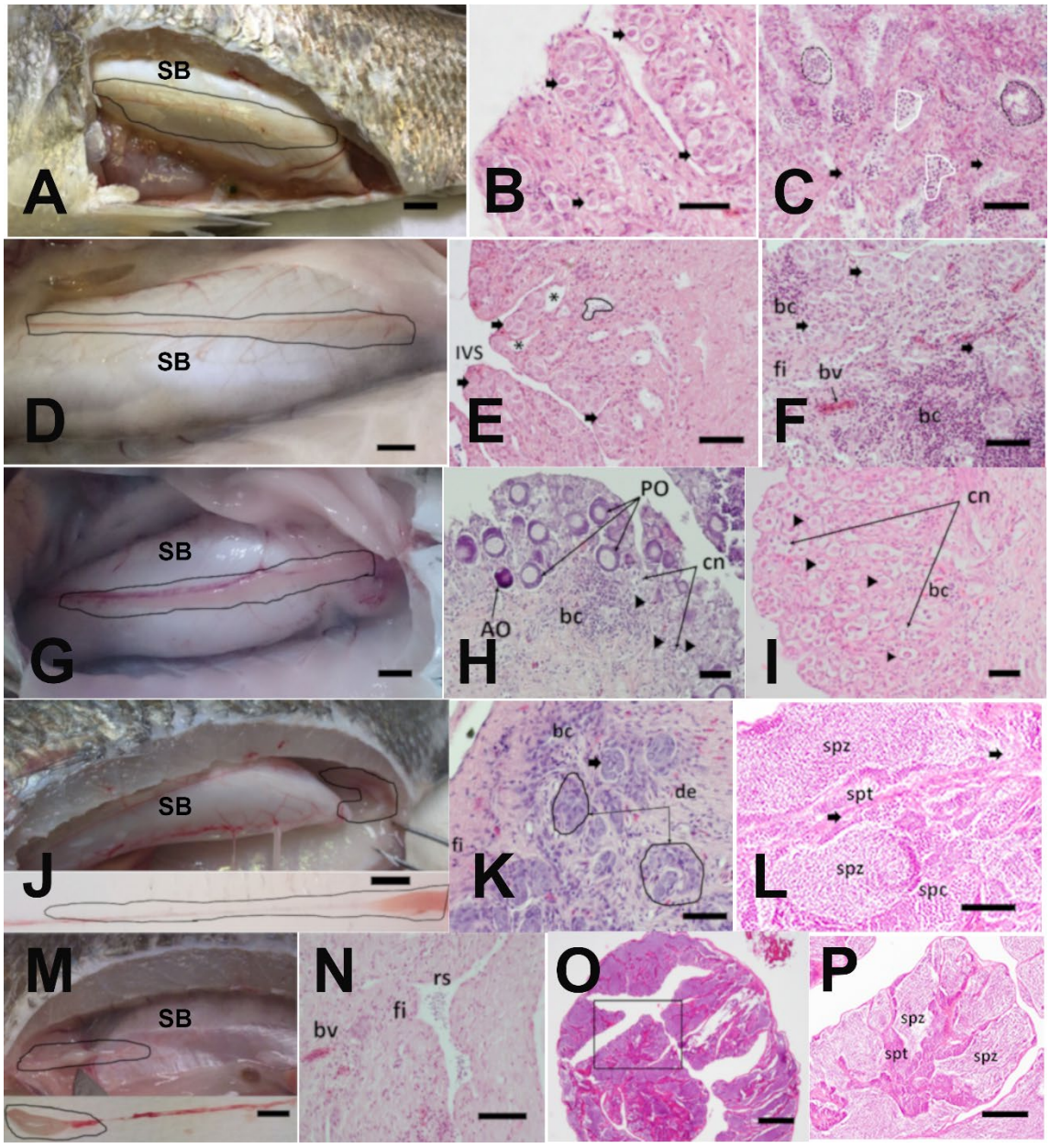

Figure 3.2. Gonadal histology of 160 dph barramundi exposed to different treatments of dietary E_2 and EE_2

Fig. A, B, and C: Control barramundi gonads. **A)** Anatomically, gonads of control fish were thin and attached to the side of the swim bladder. Scale bar 1 cm. **B)** Testis of the control fish at M1 stage. Black arrow indicates spermatogonia. Scale bar 20 µm. **C)** Testis of the control fish at M2 stage.

Different stages of testicular cells were observed: spermatogonia (black arrows), spermatocytes (in white line), spermatids (in black dash line). Scale bar 20 µm. **Fig. D, E and F**: Barramundi gonad of the treatment 10 mg E_2 kg^{-1} diet. **D)** Gonads of fish fed with 10 mg E_2 kg^{-1} diet did not show visible differences compared to the control when observed with naked eye. Scale bar 1 cm. **E)** Black arrows indicate spermatogonia distributed along the periphery at the ventral side of the gonad. Some acellular areas were present, indicated by the asterisks. Scale bar 50 µm. **F)** Basophilic cells observed. Black arrows indicate spermatogonia. Scale bar 20 µm. **Fig. G, H and I**: Barramundi gonad of the treatment 20 mg E_2 kg^{-1} diet: **G)** Gonads of fish fed with 20 mg E_2 kg^{-1} diet were large and had light pink colour with clear blood vein. Scale bar 1 cm. **H)** Black arrow heads indicate oogonia. Scale bar 20 µm. **I)** Black arrow heads indicate oogonia. Scale bar 20 µm. **Fig. J, K and L:** Gonad of barramundi in the treatment 5 mg EE_2 per kg diet. **J)** Gonad of barramundi in the treatment 5 mg EE_2 per kg diet had enlarged tubular form in the posterior part, enclosed by the black line. Scale bar 1 cm. **K)** Degraded cells are in the black line. Anterior filiform part of the gonad. Scale bar 20 µm. **L)** Black arrows indicate spermatogonia. Posterior part of the gonad. Scale bar 50 µm. **Fig. M, N, O and P**: Barramundi gonad of the treatment 20 mg E_2 per kg diet: **M)** Gonad of barramundi in the treatment 10 mg EE_2 per kg diet had enlarged tubular form at the posterior part, enclosed by the black line. Scale bar 1 cm. **N)** Anterior filiform part of the gonad. Scale bar 50 µm. **O)** Posterior part of the gonad. Scale bar 200 µm. **P)** Posterior part of the gonad at higher magnification. Scale bar 50 µm. Abbreviations: AO, atretic oocyte; bc, basophilic cells; bv, blood vessel; cn, chromatin-nucleolus stage oocytes; de, degenerative cells; fi, fibrosis; IVS, invagination of the ventral side of the gonad; PO, perinucleolar oocytes; rs, residual spermatozoa; SB, swim bladder; spg, spermatogonia; spc, spermatocytes; spt, spermatids; spz, spermatozoa.

At 160 dph, the testicular stage of maturation in control fish was classified as M1 (75%, 9/12 fish) or M2 (25%, 3/12 fish) (Fig. 3.2.A). Exposure to E_2, at 160 dph, caused an inhibitory effect on barramundi testicular development. In the $10E_2$ group, all gonads contained spermatogonia, separately or in cysts, distributed predominately along the peripheries of the gonad (Fig. 3.2.B). In 50% (6/12) of fish, a few spermatozoa were detected (Fig. 3.2.B). Spermatogonia have a spherical nucleus containing peripherally condensed chromatin and lightly basophilic cytoplasm. Some acellular areas were observed in the tissues next to cysts of testicular germ cells (either spermatogonia or spermatozoa). Mild fibrosis and basophilic cells (immune related cells) were also noticed in the gonads of fish from the $10E_2$ treatment.

In the $20E_2$ treatment, spermatogenesis was strongly suppressed and there were no residual testicular germ cells. Early stage of oocytes as oogonia were observed along the periphery at the ventral side of the gonad, while the remaining tissues were dense connective tissues (Fig. 3.2.I). Oogonia are typically recognised as spherical cells possessing a single large spherical nucleus containing fibrillary chromatin. Some oogonia were undergoing the process of meiosis (chromatin nucleus stage). Aggregations of basophilic cells were also observed in E_2 treated gonads. Four fish in the $20E_2$ treatment group (33%) had a few oocytes at early stages of development (perinucleolar stage) (Fig. 3.2.H). Perinucleolar oocytes (~20 µm) were distributed at the periphery of the ventral side of the gonad, while the remaining gonad tissue was composed of dense connective tissues, clusters of basophilic cells and blood vessels. A few oocytes were found in atresia (Fig. 3.2.H).

In the EE_2 treatments, at 160 dph, 100% (12/12) of $5EE_2$ fish and 67% (8/12) of $10EE_2$ fish exhibited abnormalities in gonad development. Approximately 70% of the anterior gonad was distinctly filiform and the remaining posterior portion had an enlarged tubular form (Fig. 3.2.J and 3.2.M). The filiform parts of these gonads were highly fibrous. In the $5EE_2$ treatment, some cysts of PGCs, basophilic cells and degenerative material were observed among the fibrotic tissue. The filiform of $10EE_2$ gonads were severely fibrotic with some free spermatozoa present, but not distributed in the organised lumen (Fig. 3.2.N). In the enlarged tubular posterior region, spermatids and spermatozoa were present within enlarged lobules (Fig. 3.2.L and 3.2.P). Gonads of the remaining fish (33%) of the $10EE_2$ treatment displayed complete fibrosis along the gonad without any presence of germinal cells.

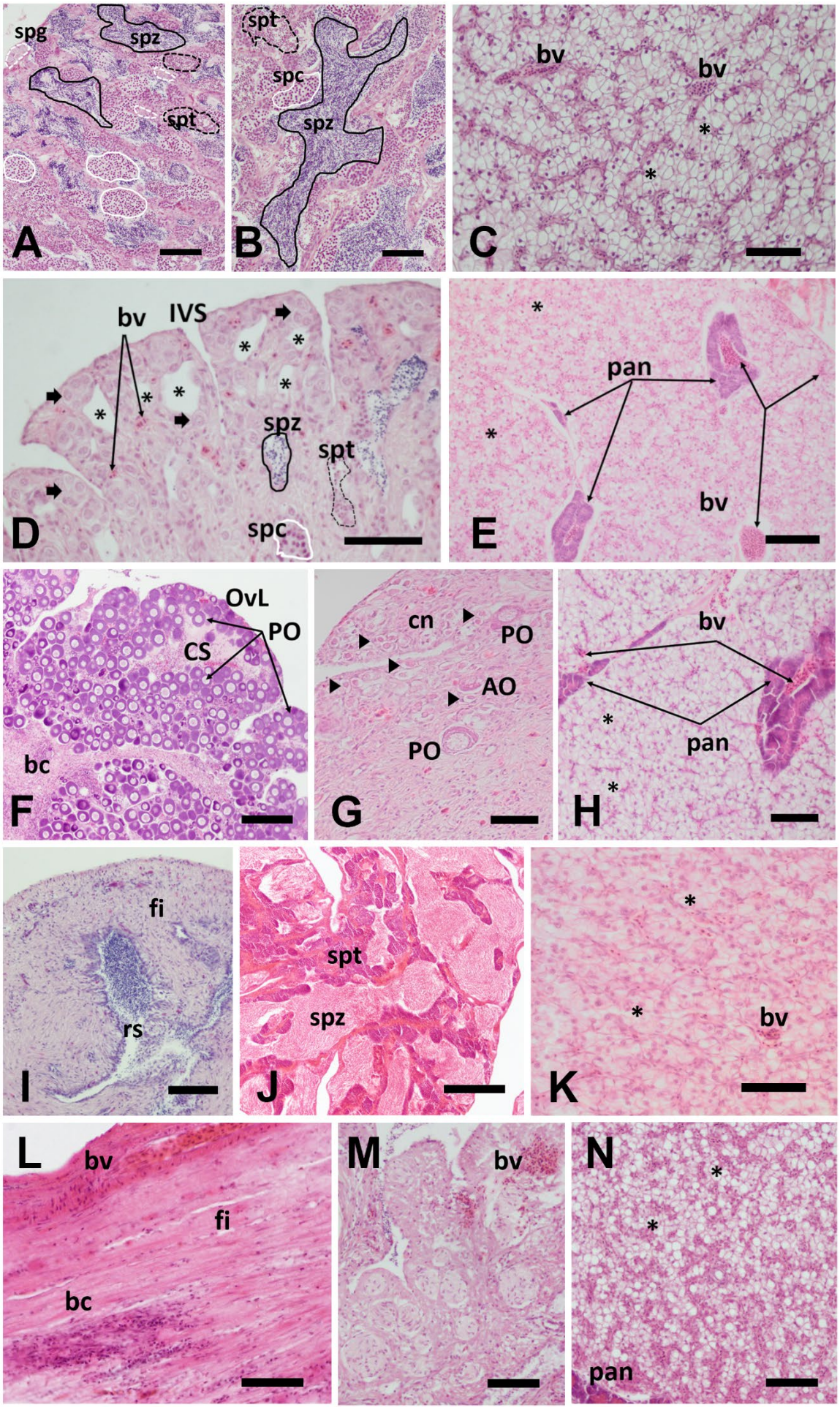
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Figure 3.3. Histological images of gonads and livers of barramundi exposed to different treatments of dietary E_2 (10 mg kg^{-1} and 20 mg kg^{-1}) and EE_2 (5 mg $kg^{-1}$and 10 mg kg^{-1}) at 12 mph

Fig. A, B, and C: Control barramundi: **A)** Testis of control fish at M2 stage. Different stages of testicular cells were observed: spermatogonia (white dash line), spermatocytes (in white line), spermatids (in black dash line) and spermatozoa (black line). Scale bar 20 µm. **B)** Testis of control fish at M3 stage. Different stages of testicular cells were observed: spermatogonia (white dash line), spermatocytes (in white line), spermatids (in black dash line) and spermatozoa (black line).Scale bar 20 µm. **C)** Liver of control fish. Asterisks indicate vacuoles. Scale bar 20 µm. **Fig. D** and **E**: Treatment 10 mg E_2 kg^{-1} ($10E_2$) diet **D)** Gonad of barramundi in the 10E2 treatment showing mostly spermatogonia distributed along the periphery at the ventral side of the gonad (indicated by black arrows). Some acellular areas present, indicated by the asterisks. Scale bar 50 µm. **E)** Liver of $10E_2$ fish. Asterisks indicate vacuoles. Scale bar 50 µm. **Fig. F, G**, and **H:** Treatment 20 mg E_2 kg^{-1} ($20E_2$) diet: **F)** Gonad of a feminised barramundi in the $20E_2$ treatment showing POs. Scale bar 50 µm. **G)** Gonad of a $20E_2$ treated showing some POs, oogonia chromatin-nucleolus stage oocytes. Arrow heads indicate oogonia. Scale bar 20 µm. **H)** Liver of fish in the treatment 20 mg E_2 kg^{-1} fish. Asterisks indicate vacuoles. Scale bar 50 µm. **Fig. I, J** and **K:** Treatment 5 mg EE_2 kg^{-1} diet ($5EE_2$). **I)** Anterior part of the gonad in the $5EE_2$ showing mostly fibrous connective tissues and an area of residual spermatozoa (rs). Scale bar 50 µm. **J)** Posterior part of the gonad in the $5EE_2$ were enlarged and filled with spermatids and spermatozoa. Scale bar 20 µm. **K)** Liver of in the $5EE_2$ fish. Asterisks indicate vacuoles. Scale bar 20 µm. **Fig. L, M and N:** Treatment 10 mg EE_2 kg^{-1} diet ($10EE_2$): **L)** Most fish (70%) in treatment 10 EE_2 had gonadal tissues containing severe fibrosis in tissue as represented. Scale bar 20 µm. **M)** Gonads of some fish in the treatment $10EE_2$ had blood vessels and degenerative cells (*). Scale bar 20 µm. **N)** Liver of in the $10EE_2$ fish. Asterisks indicate vacuoles. Scale bar 20 µm. Abbreviations: AO, atretic oocyte; bc, basophilic cells; bv, blood vessel; cn, chromatin-nucleolus stage oocytes; CS, connective stroma; de, degenerative cells; fi, fibrosis; IVS, invagination of the ventral side of the gonad; OvL, ovarian lamellae; pan, pancreatic cells; PO, perinucleolar oocytes; rs, residual spermatozoa; spg, spermatogonia; spc, spermatocytes; spt, spermatids; spz, spermatozoa.

At 12 mph, histological observations of testis from control fish revealed the presence of large lobules containing a complete series of male germ cells (spermatocytes, spermatids, and spermatozoa) and a larger number of released spermatozoa in the lumen as M2 (83%) and M3 (17%) stages (Fig. 3.3.A and 3.3.B). While hormonal exposure ceased 6 months earlier, fish from E_2 treatments exhibited suppressed spermatogenesis; fish in the $10E_2$ treatment did

not show noticeable difference in gonad morphology compared to the gonads of the same treatment sampled at 160 dph. Specifically, cysts of spermatogonia were still observed distributed along the periphery of gonads with some acellular areas (Fig. 3.3.D). A few spermatocytes, spermatid and spermatozoa were also observed in the gonad. Fifty percent (six out of 12 fish) in the $20E_2$ treatment had ovaries mainly filled with perinucleolar oocytes arranged to form ovarian lamellae (Fig. 3.3.F). These perinucleolar oocytes were relatively small with diameter approximately ~20-30 µm. The remaining 50% of the $20E_2$ treatment fish had gonads mostly containing some perinucleolar oocytes and atretic oogonia (Fig. 3.3.G). Basophilic cells were detected in the dense connective tissues in all $20E_2$ fish.

At 12 mph, all $10EE_2$ fish showed some degree of fibrosis of gonad tissue, with extreme fibrosis present in 75% of fish (9/12) (Fig. 3.3.L). There were some blood vessels, basophilic cells and degenerative materials distributed among the fibrotic gonad tissues. In the remaining three males and 100% of fish in the $5EE_2$ treatment, gonads were found similar to the EE_2 treated fish at 160 dph. Specifically, each gonad is composed of the major filiform fibrosis and a short enlarged tubular part containing only spermatids and spermatozoa (Fig. 3.3.I and 3.3.J).

Liver histological analysis of the experimental barramundi were used to examine the potential side effects of exogenous E_2 and EE_2 administration. No differences were observed between the untreated control and the treated E_2 and EE_2 treatments at all samplings. For all sampled liver of different groups, liver sections showed uniform hepatocytes with distinct nuclei and nucleoli, abundance of cytoplasmic lipid and vacuolisation. These signs are typical of healthy farmed fish. The histological images of the livers of the control and the treated fish in the $10E_2$, $20E_2$, $5EE_2$ and $10EE_2$ treatment at 12 mph are shown in the Fig. 3.3.C, 3.3.E, 3.3.H, 3.3.K and 3.3.N, respectively.

Table 3.3. Morphological changes in gonad development of barramundi due to oral administration of various dosages of E_2 and EE_2 during treatment (from 30 to 160 dph) and after treatment termination (at 12 mph)

30 dph	
Prior to hormone treatment, there were no histological signs of gonadal differentiation and gonads appeared as thin filaments suspended on both sides from the swim-bladder wall by a thin sheet of somatic cells (Fig. 3.1.A) (100%, 45/45 fish). Average gonad surface was 410.86 ± 27.15 μm^2.	
50 dph	
Control Fig. 3.1.B	Gonads appeared as slit-like lumen (100%, 12/12 fish). Average gonad surface was 1049.5^a ± 213.3 μm^2.
10 mg E_2 Fig. 3.1.C	A clear, large, tri-lobular lumen on the dorsal side and an outgrowth of tissue on the ventral side (83%, 10/12 fish). Average gonad surface was 2022.3^b ± 337.1 μm^2.
20 mg E_2 Fig. 3.1.D	Gonads showed paired, elongated aggregations of somatic cells in the proximal and distal portions and a thin sheet of somatic cells at the dorsal side connecting to the mesentery (75%, 9/12 fish). Average gonad surface was 1823.6^b ± 198.7 μm^2.
5 mg EE_2 Fig. 3.1.E	Abnormal gonads with fibrosis surrounding the blood vessels (100%, 12/12 fish). Average gonad surface was 997.2^a ± 112.4 μm^2.
10 mg EE_2 Fig. 3.1.F	Abnormal gonads with fibrosis and small blood vessels (100%, 12/12 fish). Average gonad surface was 724.9^c ± 102.5 μm^2.
* Different letters represent statistical differences ($P < 0.05$) of the value (mean ± standard deviation).	
100 dph	
Control Fig. 3.1.G	Efferent duct expanded and clearly visible. Lobular structures contained strips of primary germ cells distributed along the epithelial layer (100%, 12/12 fish).
10 mg E_2 Fig. 3.1.H	Gonads larger and more complicated in organisation with multiple lobes containing large cysts of undifferentiated germ cells surrounded by dense connective tissues (100%, 12/12 fish).
20 mg E_2 Fig. 3.1.I, 3.1.J and 3.1.J	Ovarian lumen in the middle of gonad tissues (83%, 10/12 fish). Remaining fish (17%, 2/12) gonads with dual lobes, indicating that the enclosing to form ovarian cavity was not complete. Chromatin nucleus (early stage oocytes) detected in all fish.
5 mg EE_2 Fig. 3.1.K	Large lumen, mild fibrosis and degenerative material of the somatic cells (100%, 12/12 fish).
10 mg EE_2 Fig. 3.1.L	Apoptotic germ cells/pyknosis, cell shrinkage, and condensation of the nucleus. Some normal germ cells also existed and surrounding by fibrotic connective tissue (100%, 12/12 fish).
160 dph	
Control Fig. 3.2A, 3.2.B and 3.3.C	Active spermatogenesis observed in the testes. Testis stage M1 and M2 observed in 9 of 12 (75%) and 6 of 12 (25%) fish, respectively.

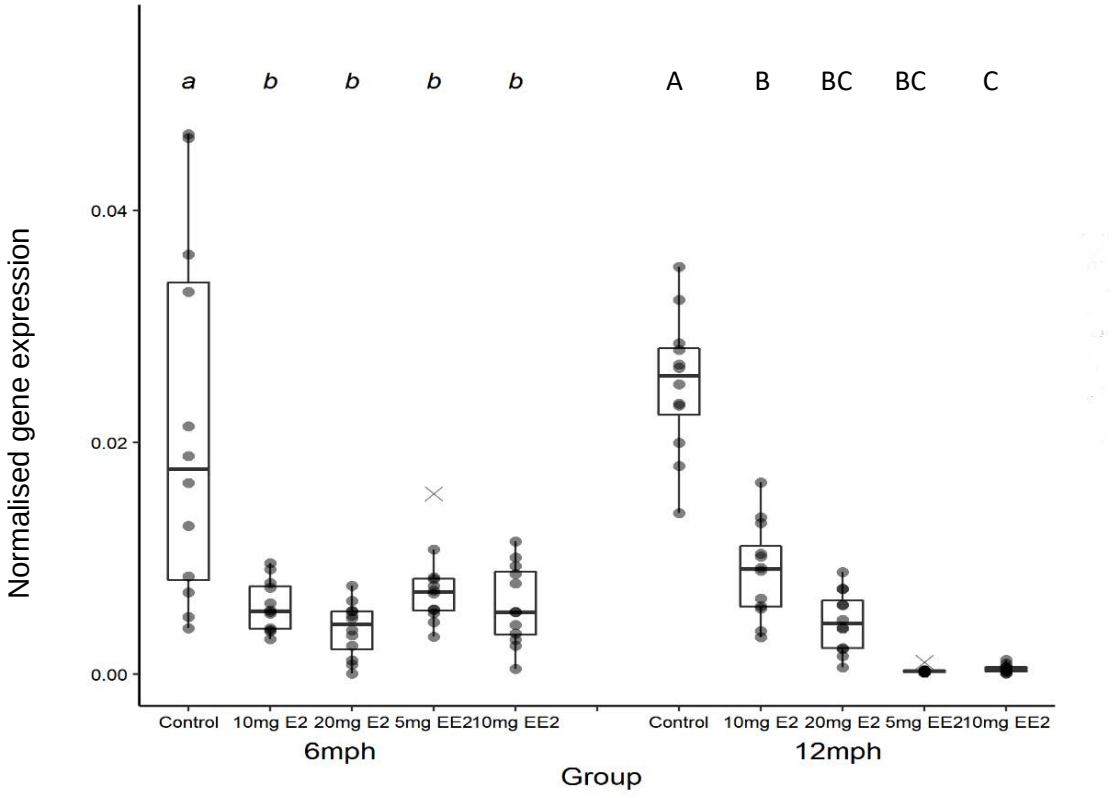
10 mg E_2 Fig. 3.2D, 3.2.E and 3.2.F	Cysts of spermatogonia distributed mostly along tissue peripheries in all fish. In 50% (6/12) of fish, a few spermatozoa were detected. Some acellular areas were observed in the tissues next to cysts of testicular germ cells. Mild fibrosis and basophilic cells were also noticed in gonads.
20 mg E_2 Fig. 3.2G, 3.2.H and 3.2.I	Oogonia distributed along the periphery at the ventral side of gonads and the remaining tissue were dense connective tissues (67%, 8/12 fish). Aggregations of basophilic cells also observed. Few oocytes at early stages (perinucleolar oocytes, ~20 µm) distributed at the periphery of the ventral side; remaining gonad tissue composed of dense connective tissues, clusters of basophilic cells and blood vessels (33%, 4/12 fish). Few perinucleolar oocytes were found in atresia.
5 mg EE_2 Fig. 3.2J, 3.2.K and 3.2.L	Anterior gonad filiform (approximately 70% of whole gonad length) (75%, 9/12 fish). Cysts of PGCs, basophilic cells and degenerative material observed among fibrotic tissue. In the enlarged tubular posterior gonads, spermatozoa and spermatids were present only in enlarged lobules.
10 mg EE_2 Fig. 3.2.M, 3.2.N, 3.2.O and 3.2.P	Anterior filiform section severely fibrotic with some free spermatozoa present, but not in the organised lumen (67%, 8/12 fish). In the enlarged, tubular posterior gonads, only spermatozoa and spermatids were present only in enlarged lobules. 33% (4/12) fish had complete fibrosis of the gonad.
12 mph	
Control Fig. 3.3.A and 3.3.B	Large lobules containing a complete series of male germ cells (spermatocytes, spermatids, and spermatozoa) and a larger number of released spermatozoa in the lumen of the gonads as M2 (83%. 10/12 fish) and M3 (17%, 2/12 fish) stages.
10 mg E_2 Fig. 3.3.D	Spermatogonia were present at the periphery of the gonads. Some acellular areas were present as empty lobules. A few spermatocytes, spermatid and spermatozoa also found. Blood vessels and basophilic cells were distributed among fibrosis at the dorsal side of the gonads.
20 mg E_2 Fig. 3.3.F and 3.3.G	Ovaries mainly filled with perinucleolar oocytes arranged to form ovarian lamellae (50%, 6/12 fish). Alternatively, gonads mainly contained oogonia, some perinucleolar and atretic oogonia (50%, 6/12 fish). Basophilic cells were detected in the dense connective tissues of all fish.
5 mg EE_2 Fig. 3.3.I and 3.3.J	Gonads of all fish composed of major filiform fibrosis with some free spermatozoa, and a short enlarged tubular part containing only spermatids and spermatozoa.
10 mg EE_2 Fig. 3.3.L and 3.3.M	Testicular fibrosis observed in the whole gonad (75%, 9/12 fish). Some blood vessels, basophilic cells and degenerative materials distributed among the fibrotic gonad tissues. Alternatively, in 25% (3/12) fish, gonads were similar to those of treatment 5EE_2 at 12 mph.

3.3.3. Altered expression of *dmrt1* and *cyp19a1a* genes in the gonad

Gene expression patterns of two important sex genes, *dmrt1* and *cyp19a1a*, were investigated by RT-qPCR (Fig. 3.4). The results of Sanger sequencing on the RT-qPCR products of *dmrt1*

and *cyp19a1a* were aligned and confirmed the correct mapping to the reported gene sequences of barramundi. Based on the expression levels of control fish, provision of both dietary E_2 and EE_2 resulted in downregulated expression of *dmrt1* in barramundi gonads at 160 dph and 12 mph. The largest difference in expression, relative to control, was observed in 12 mph $5EE_2$ and $10EE_2$ fish ($P < 0.05$). At 12 mph, *dmrt1* expression was significantly downregulated in the E_2 fed groups in a dose-dependent manner. Conversely, the expression of *cyp19a1a* was upregulated in the $20E_2$ treatment at 160 dph and 12 mph ($P < 0.05$). At both time points, 160 dph and 12 mph, the expression of *cyp19a1a* of the $10E_2$ fish was detected at significantly lower levels than the $20E_2$ fish ($P < 0.05$); and was not significantly different from the control. Similarly, at 160 dph, expression of *cyp19a1a* of both EE_2 treatment did not statistically differ from the control fish. However, *cyp19a1a* was found to be significantly downregulated in the $5EE_2$ and $10EE_2$ fish at 12 mph ($P < 0.05$).

A. *dmrt1*



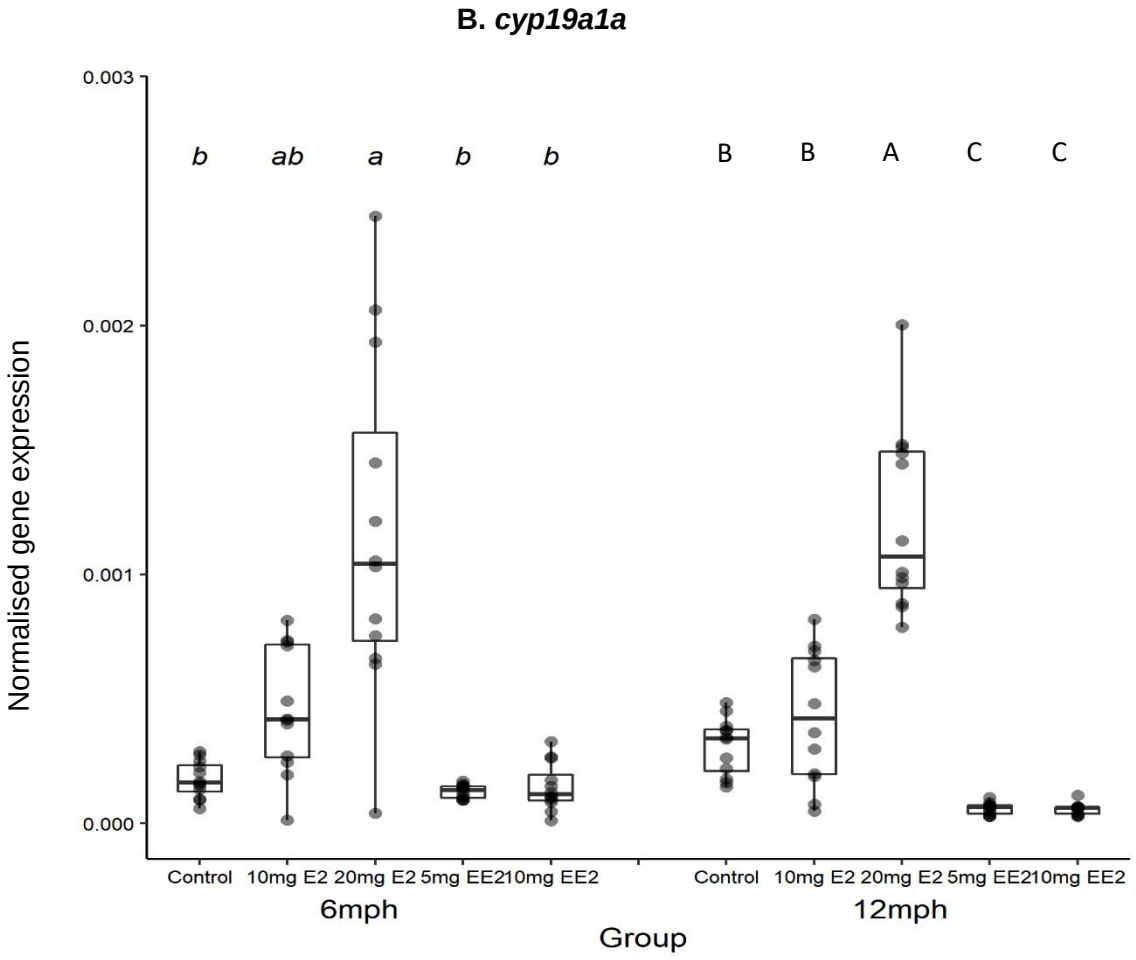


Figure 3.4. Relative *dmrt1* (A) and *cyp19a1a* (B) expression measured by RT-qPCR in gonads of barramundi fed with E_2 and EE_2 at different dosages sampled at 160 dph and 12 mph

The values were calibrated with the reference gene *ubq* according to Livak and Schmittgen (2001). Different lowercase and uppercase letters represent statistical differences ($P < 0.05$) between treatments at the same time-point and different time-points of the same treatment, respectively ($n = 12$ for each value).

3.4. Discussion

Selective breeding programs for barramundi have been challenged by the protandrous sexual development of the species. Investigating methods that influence the sex differentiation process, and in particular, precocious sex change of males to females is therefore of

commercial aquaculture interest. This is because the ability to produce uniformly-aged females and males will not only increase potential genetic gains through selection, but also substantially lower infrastructure requirements for the management of breeding programs (Robinson and Jerry, 2009). Sex control for aquaculture species, particularly the success of feminising fish through the application of exogenous estrogens, shows diverse results depending on fish species, hormone used, treatment timing and duration (Piferrer, 2001; Budd et al., 2015). These treatment variables generally need to be determined empirically for each species as different responses to hormonal manipulation are often observed, even for closely related species. In the current study, trials evaluating the effect of feeding estrogenic sex steroids on the sexual development of juvenile barramundi were performed. Feeding barramundi with either E_2 or EE_2 between 30 to 160 dph altered gonadal morphology and development, and changed expression levels of the key sex-related genes *dmrt1* and c*yp19a1a*.

Exposure to exogenous estrogen for feminising purposes has been tested for numerous aquaculture species. According to Yamamoto (1969), induction of sex reversal is most successful when the effective treatment (hormone type and dose) is given during the labile period when the gonads are most responsive to exogenous hormones. Using the previously determined labile period of barramundi from 44 to 140 dph as a guide (Banh et al., 2017), feeding fish 20 mg E_2 per kg feed produced individuals with distinguished female gonadal features, including ovarian-like lumen and perinucleolar oocytes. This is in accordance with other studies where estrogen exposure during early life induced feminised effects such as the presence of an ovarian cavity and/or oocytes (Nakamura and Takahashi, 1973; Piferrer and Donaldson, 1989; Blázquez et al., 1998; Brion et al., 2004; Filby et al., 2007). Obtaining sex-reversed fish in the $20E_2$ treatment, but not in the $10E_2$ treatment, confirms the important part

of E_2 in the sex change process of barramundi, as well as the key role of sex steroid quantity in triggering this process to occur.

Exposure to E_2 and EE_2 induced different responses in barramundi gonads reflecting the divergence in estrogenic potencies of these two steroids. In the studies testing both E_2 and EE_2 on feminisation of fish, EE_2 has generally been shown to be more efficient. For example, in chinook salmon EE_2 produced feminising effects when fish were exposed to shorter treatment durations than E_2 (Piferrer and Donaldson, 1992), or at lower hormone concentrations in the guppy *Poecilia reticulate* (Kavumpurath and Pandian, 1993a). In European seabass, EE_2 resulted in a higher feminising rate than E_2 when using the same concentration and duration treatment (Blázquez et al., 1998). Nevertheless, in the present study, feminised fish were only evident in the fish fed 20 mg E_2 per kg of feed; fish in the control and other treatments, including the fish fed 10 mg E_2 and both EE_2 treatments, did not exhibit significant evidence of feminisation. Compared to those reported *in vitro* and *in vivo* studies, EE_2 is generally two to three times more potent than E_2 (Piferrer, 2001; Van den Belt et al., 2004). In the present study, results that the relative estrogenic potencies of EE_2 compared to E_2 on barramundi are also possibly different, possibly related to their influence the expression of certain sex related genes.

Undesirable side effects resulting from the application of exogenous estrogen, including E_2 and EE_2, during gonadal differentiation observed in this experiment have been reported in other species. These include sterility, pyknosis, apoptotic germ cells and acellularity (absence of cells). Germ cell apoptosis in estrogen exposed fish was reported in medaka (Weber et al., 2004) and fathead minnows (Länge et al., 2001). Previous studies have also noted increased acellularity or reduced amount of released spermatozoa in the testes of E_2 and EE_2 treated fish (Gimeno et al., 1998; Weber et al., 2003; Oropesa et al., 2014). The acellularity observed in this study appeared to have occurred through complete or partial elimination of

spermatocytes without changing the overall testicular tissue structure. Similar observations were reported in fish exposed to other estrogenic compounds, including E_2 and EE_2 (Gimeno et al., 1998; Weber et al., 2003; Panter et al., 2006). Administration of EE_2 to the fish during sexual differentiation may affect the responsiveness of the developing germinal and/or somatic cells, by disturbing the gonadal differentiation pathway and steering them away from what has been genetically programmed.

Increased testicular fibrosis in response to estrogen administration, similar to that observed in this experiment, has been previously reported in European sea bass (Blázquez et al., 1998; Gorshkov et al., 2004), medaka (Kang et al., 2002; Seki et al., 2002), and zebrafish (Weber et al., 2003; Schäfers et al., 2007). According to Dietrich and Krieger (2009), fibrosis tissue in steroid-treated gonads is likely to be a wound-healing reaction to retain the overall critical structures of the organ (i.e. lumen and ducts). As exogenous estrogens have been well-characterised as broad endocrine-disrupting compounds, it is likely that gonads treated with estrogen may result in an altered physiological process, such as an immune response (Lai et al., 2002). The infiltration of basophilic cells (immune-related cells) possibly appeared because of damaged tissues undergoing necrotic cell removal by phagocytosis. According to Guiguen et al. (1994), formation of basophilic cells, which sometimes contain dark pigment, was characteristic of all natural sex reversal barramundi gonads. In protandrous gilthead seabream, E_2 and EE_2 promoted an inflammatory process as indicated by the appearance of immune cells in the exposed gonads (Chaves-Pozo et al., 2007; Cabas et al., 2011). Interestingly, substantial leukocyte infiltration was observed before naturally occurring sex change in gilthead seabream, suggesting an immunological surveillance, physiological regulation and tissue modelling response is required to maintain the gonadal structure while the testicular degenerative process occurs (Liarte et al., 2007).

Estradiol treatments did not significantly increase mortality, but negatively affected barramundi growth during and after treatment. Compromised growth and/or appetite of fish fed with E_2 food have been previously reported as a deleterious effect of estrogen hormone exposure (Johnstone et al., 1979; Condeça and Canario, 1999; Hendry et al., 2003; Davis and Ludwig, 2004; Kojima et al., 2008). Interestingly, feeding sea bream (~ 40 g BW) with a diet containing 15 EE_2 mg per kg resulted in poor appetite and growth; however, replacing the treatment with E_2 at the same dosage did not reduce appetite and growth (Condeça and Canario, 1999). Investigation of the molecular effects of estrogen exposure in rainbow trout determined that an EE_2 treatment suppressed the expression of key genes in the growth hormone and insulin-like growth factor systems, leading to reduced growth (Hanson et al., 2012). Conversely, chinook salmon treated with estradiol resulted in increased growth (Piferrer and Donaldson, 1992). In yellow perch, *Perca flavescens*, E_2 was apparently anabolic in fish larger than ~10 g and was thought to mediate sexually-related dimorphic growth in which females grow faster than males (Malison et al., 1985). While other studies have found both reduced survival and growth rate (Donaldson and Hunter, 1982; Piferrer and Donaldson, 1992; Sun et al., 2010), it is possible that the hormone concentrations used in the present study were not high enough to cause lethal toxicity in the treated groups.

Exposure barramundi to E_2 in this study suppressed the expression of the male-associated gene, *dmrt1*, and upregulated the female-associated gene *cyp19a1a*. The opposite result in which *dmrt1* was not suppressed by EE_2 was reported in rainbow trout fed with 20 mg EE_2 kg^{-1} of food for 2 months (Vizziano-Cantonnet et al., 2008). Different from barramundi, dietary estrogen down-regulated male genes relating to 11-oxygenated androgens in Leydig cells instead of Sertoli cell marker like *dmrt1*. Contrarily, the *dmrt1* expression pattern in this study is in agreement with most studies on induction of sex reversal for other species. For example, administration of exogenous estrogen to pejerrey during early development down-

and up-regulated the expression of *dmrt1* and *cyp19a1a*, respectively (Fernandino et al., 2008; Pérez et al., 2012). Exposure of sexually undifferentiated juvenile African catfish to the exogenous MT repressed *dmrt1* and up-regulated *cyp19a1* expression (Raghuveer et al., 2005). Dietary E_2 induced 100% male Nile tilapia and upregulated the gene *cyp19a1* and downregulated *dmrt1* (Kobayashi et al., 2003; Kobayashi et al., 2008; Kobayashi and Nagahama, 2009). Likewise, estrogen treatment induced the down-regulation of *dmrt1* of zebrafish (Schulz et al., 2007; Reyhanian Caspillo et al., 2014), fathead minnow (Filby et al., 2007) and rainbow trout (Marchand et al., 2000). The findings in the present study indicates that *dmrt1* is also estrogen-sensitive in barramundi and estrogen treatment has prolonged suppressive effects on *dmrt1* up to seven months post-hormone treatment. Although there were enlarged regions of testis developed in the EE_2-treated fish, *dmrt1* expression of these fish were significantly lower than the untreated fish. The gonad of these fish subjected to RT-qPCR included both fibrotic filiform tissue and an enlarged region containing only mature spermatids and spermatozoa. This result may suggest that not only the filiform gonad, but also the enlarged region, do not have full function despite the presence of advanced testicular germ cells.

The mRNA for aromatase (*cyp19a1a*) has higher expression in the ovary than in the testis in numerous teleosts (Blázquez et al., 2008; Fernandino et al., 2008; Johnsen et al., 2013). Exposing male medaka and sexually maturing fathead minnows of mixed sex to EE_2 at 10 ng per litter resulted in upregulated expression of gonadal aromatase (Scholz and Gutzeit, 2000; Filby et al., 2007). However, *cyp19a1a* was suggested to be expressed higher in the testes than in ovaries of barramundi (Ravi et al., 2014). The present study showed though that the relative expression of the female-related gene *cyp19a1a* in barramundi exposed to $20E_2$ was elevated compared to other treatment groups and may have contributed to the observable feminisation effects compared to other treatments. This may be because the 5’ flanking

region of *cyp19a1a* was found to contain estrogen responsive elements (Yoshiura et al., 2003), explaining the up-regulation of *cyp19a1a* by administration of exogenous estrogen.

Exposure of zebrafish juveniles to EE_2 downregulated the transcription of c*yp19a1a* (Kazeto et al., 2004), and significantly suppressed the early gonadal development in both sexes of zebrafish (Hill Jr and Janz, 2003; Weber et al., 2003). Aromatase was detectable in testes of XY males zebrafish exposed to 10 ng per L EE_2 (Scholz and Gutzeit, 2000). Due to the strong estrogenic potential of EE_2, exposure to EE_2 during early stages of the barramundi lifecycle may cause irreversible damage to the gonadal tissues, preventing the normal sex differentiation into testis and not supporting the sex inversion into ovary. This may explain the near complete suppression of both *cyp19a1a* and *dmrt1* in gonadal tissues of 12 mph barramundi. This possibility should be explored in further studies.

3.5. Conclusions

In this study E_2 treatment induced some feminising effects when fed to juveniles at a higher dose (20 mg kg^{-1} food), and inhibited spermatogenesis at a lower dose (10 mg kg^{-1} food). Higher dosage and/or longer treatment duration should be tested to optimise the sex manipulation protocol for barramundi with particular caution given to liver damage and overall animal health. Both doses of EE_2 (5 and 10 mg kg^{-1} food) suppressed the expression of important feminising gene *cyp19a1a* at 12 mph, which was 205 days after the termination of the treatment (ceased at 160 dph). The EE_2 treatment also led to phenotypically detrimental effects, such as severe fibrosis and abnormally developed gonads. This suggests that E_2 is a better steroidal candidate to produce precocious females in barramundi.

Chapter 4. Induction of precocious females in the protandrous barramundi through 17β-estradiol implants

4.1. Introduction

Barramundi are protandrous sequential hermaphrodites, which dictates that male fish require long-term housing for several years after harvest until they undergo sex change to female (often by this time the females are 10+ kg in size), and females are usually one generation older than the males in spawning groups. Sequential sex change thereby results in substantial maintenance and infrastructure costs for hatcheries to hold males and inter-generation breeding halves the annualised rate of genetic progress that could otherwise be made through a single-generation selection program (Robinson and Jerry, 2009). As such, the development of methods to obtain precocious females, which can be bred with males of the same generation, would not only reduce broodstock holding costs, but also increase genetic gains in selective breeding programs for the species.

Controlled feminisation has been successfully achieved in a diverse range of teleost species by taking advantage of the significant plasticity of phenotypic sex (Devlin and Nagahama, 2002; Budd et al., 2015). The most commonly used approach is to administer exogenous steroids to override the sex of the fish species, which would otherwise be determined by genetic, social or environmental factors (Piferrer, 2001). Exposure to E_2 has been shown to be highly effective in inducing feminisation in fishes of a number of taxonomic families, namely, Cyprinidae, Anabantidae, Poecilidae, Ictaluridae, Salmonidae and Cichlidae (Pandian and Sheela, 1995; Piferrer, 2001). Sex manipulation is most efficient when fish are exposed to hormone during the labile period, a highly-sensitive sex development period when fish gonads are undifferentiated; however, this does not preclude manipulation of sex outside this period as sex plasticity of some teleost species remains after sexual differentiation (Piferrer, 2001; Takatsu et al., 2013). For instance, feminisation of Chum salmon, for which

sex is distinguishable by 25 dph, was observed in testes by exposing individuals to 1 µL E_2/L from 34-100 dph (Nakamura, 1984). For the protandrous common snook, induced feminisation was achieved in 90% of juveniles using feed supplemented with 100 $mg.kg^{-1}$ E_2 for 45 days (Carvalho et al., 2014) and in 100% of 3 year old male fish implanted with exogenous E_2 (Passini et al., 2016).

In spite of genetically determined sex, phenotypic sex in teleost species is the result of an antagonistic interaction between feminising and masculinising gene networks, where the prevailing network acts to continuingly suppress the opposing network. Genes central to the underlying feminising network are *cyp19a1a* and *foxl2*, and those key to the masculinising network are *dmrt1*, *cyp11b* and *esr1* (Liu et al., 2015; Todd et al., 2016). Specifically, the key female gene *cyp19a1a* is activated by the transcription factor encoded by the gene *foxl2; cyp19a1a* encodes the enzyme aromatase, which catalyses the conversion of testosterone to estradiol (Kazeto et al., 2004; Guiguen et al., 2010). For males, the transcription factor *dmrt1* regulates the expression of male promoting genes (e.g. *cyp11b*, *sox9, amh*) and downregulates the female pathway by suppressing *foxl2* and, subsequently, reducing *cyp19a1a* expression and the presence of endogenous estrogen (Wang et al., 2010; Herpin and Schartl, 2011; Kobayashi et al., 2013). Experimentally induced downregulation or upregulation of either *foxl2* or *dmrt1* has been shown to result in the reprogramming of pluripotent sex cells and induced sex change in both teleost fishes and mammals (Uhlenhaut et al., 2009; Matson et al., 2011; Li et al., 2013; Lindeman et al., 2015). Similarly, sexually dimorphic patterns of *cyp19a1a* gene expression have also been reported for many species (i.e. higher levels in females than in males) (Guiguen et al., 1999; Sudhakumari et al., 2005; Blázquez et al., 2008).

The effect of estrogen across specific tissues is mediated by positive and negative feedback interactions with estrogen receptors, *esr1* and *esr2* (Cheskis et al., 2007). In the protandrous

gilt-head seabream, expression of *esr1* is highly specific to the testis, while *esr2* is present in most tissues, but more abundantly in ovary, testis, liver, intestine and kidney (Socorro et al., 2000). Similar male-specific expression of *esr1* has been reported in medaka (Chakraborty et al., 2011), zebrafish (Menuet et al., 2002), Atlantic croaker *Micropogonias undulates* (Hawkins et al., 2000), seabream (Socorro et al., 2000) and barramundi (Ravi et al., 2014). Conversely, *esr1* expression is less in testicular tissue than in ovarian tissue of the protandrous black porgy (Lee et al., 2001; Chakraborty et al., 2011) and the protogynous orange-spotted grouper (Chen et al., 2011), and differs throughout gonadal development and gametogenesis. For instance, expression of *esr1* is low during early ovarian development (pre-vitellogenic stage), but increases in the matured ovary of goldfish *Carassius auratus* (Choi and Habibi, 2003), rainbow trout (Nagler et al., 2000) and eel (*Anguilla* sp.) (Lafont et al., 2016). As the expression of *esr1* has been shown to be species-specific, examining its regulation in barramundi induced to sex change may help to clarify its role in the sex changing process of this particular species.

This research aimed to develop protocols to effectively produce precocious barramundi females from 1-year-old males using exogenous E_2 implants. In addition to investigating the effects of E_2 treatment on gonadal morphology, gene expression of known masculinising (*dmrt1*, *cyp11b*, *esr1*) and feminising (*cyp19a1a* and *foxl2*) genes were also examined by RT-qPCR to further understand the underlying genetic pathways of sex determination in this species.

4.2. Material and methods

4.2.1. Experimental design

4.2.1.1. E_2 'dosage-range-finding' trial

An E_2 hormone implantation 'dosage-range-finding' trial was initially conducted on 18 male barramundi (6.5 ± 0.8 kg BW) individually tagged with passive integrated responder (PIT)

tags. The fish were stocked in three 2500 L tanks (n = 6 fish/tank) connected to a 13,500 L closed freshwater recirculation system. All experimental systems were equipped with two cartridges of activated carbon (8-10 kg activated carbon /cartridge) to absorb potential hormones in the water. Activated carbon (Acticarb GC1200, Activated Carbon Technologies Pty Ltd., Australia) was replaced every 2 weeks. Two fish in each of the three tanks received cholesterol pellets, containing either 0 mg E_2 kg^{-1} BW (n = 6), 10 mg E_2 kg^{-1} BW (n = 6) or 20 mg E_2 kg^{-1} BW (n = 6), implanted into the peritoneal cavity. Control fish were implanted a 'dummy' cholesterol pellet that did not contain hormone. Preparation of E_2 hormonal pellets and the implantation procedure is described in *Section 4.2.3*.

Prior to implantation, three fish from the same cohort of fish as those subsequently implanted were randomly chosen and sacrificed to sample gonad tissue for histological analysis and confirm that fish used at the start of the trial were all males. All fish contained testis at stage M3 (i.e. containing mostly spermatids and spermatozoa, as defined in Guiguen et al. (1994)). At days 15 to 20 post-implantation, mortality was observed in four fish treated with 20 mg E_2 kg^{-1} BW and three fish treated with 10 mg E_2 kg^{-1} BW, suggesting both E_2 dosage rates were too high. Gonads and livers from deceased animals were collected and subjected to histological examination. After consideration for the health of remaining fish, they were sacrificed 20 days post-implantation and the gonads and livers collected for histology. The gonads of E_2 treated fish, including those observed as mortalities, contained early stage oocytes (previtellogenic oocytes). The livers of the treated fish were detrimentally compromised, showing necrosis and thickened arteriole walls containing deposits (i.e. arterial hyalnosis) (Fig. 4.3.C). These pathological findings have been previously reported in studies on the effects of estrogenic compounds in fish (Herman and Kincaid, 1988; Weber et al., 2004; Zha et al., 2007). As results of the preliminary trial indicated that E_2 overdose had occurred, the primary experiment dosages were lowered (described below). This trial also

proved that exogenous E_2 could induce sex change of barramundi in a freshwater environment.

4.2.1.2. The primary E_2 implantation experiment

In the primary experiment, male barramundi (405 ± 50 g BW, 317.1 ± 13.3 mm TL) were obtained from a commercial supplier, where they were cultured in freshwater before being transferred into the acclimation system. Fish were acclimated for one week in a 13,500 L recirculating freshwater system (previously described), subsequently anesthetised with AQUI-S (Aqui-S New Zealand Ltd, New Zealand), individually PIT tagged, BW (g) and TL (mm) recorded, and then stocked into the experimental system. The experimental system consisted of nine 600 L fiberglass conical tanks receiving recirculated water at 5 L min^{-1} from a 5,000 L sump. Fish were fed a commercial barramundi diet (Ridley Corporation) twice daily at 3% BW per day. Fish rearing and water quality monitoring were conducted as demonstrated in *Chapter 2*. The rearing system was also equipped with two cartridges of activated carbon and hormone residual test was conducted as presented in *Chapter 3*. No E_2 was detected in the water supplied to experimental units.

The experiment to induce feminisation of barramundi consisted of two hormonal dosage rates, 4 mg E_2 kg^{-1} BW (‘low dose’) and 8 mg E_2 kg^{-1} BW (‘high dose’), and a control group (‘untreated control’: 0 mg E_2 kg^{-1} BW). Eighteen individuals were randomly assigned to each group. Estrogen-exposed fish in the ‘low dose’ treatment group received cholesterol pellet implants containing 1.6 mg E_2 that achieved an average effective dose of 4 mg E_2 kg^{-1} BW. Likewise, fish in the ‘high dose’ treatment group received a cholesterol pellet with 4 mg E_2 that achieved an average effective dose of 8 mg kg^{-1} BW. Controls also received a cholesterol implant that contained no E_2 hormone. Following implantation (procedure described below),

two fish from each treatment group were placed in each of the nine tanks to account for any unexpected potential tank effects on gonadal development.

4.2.2. Samplings

Initial tissue sampling was conducted one-day prior to the beginning of the experiment (n = 5; called pre-implant), and was repeated at 4 weeks (n = 9 per treatment group) and 9 weeks (n = 9 per treatment group) post-implantation. As the release of exogenous hormone from the implant to the fish blood stream can be from several days up to 28 days post-implantation (Crim et al., 1988; Sherwood et al., 1988; Yamada et al., 1997; Piferrer, 2001; Wang et al., 2005), exogenous E_2 in the implants in this study were possibly completely metabolised by the time of sampling at 4 weeks post-implantation.

Fish were euthanised by immersion in AQUI-S solution until reaching stage IV anaesthesia (Coyle et al., 2004), followed by subsequent cervical dislocation. Gonad and liver tissues were collected for histological examination (morphology and condition) at all samplings. At 9 weeks post-implantation, gonad tissues were sampled for both histology and gene expression analyses as indicated in Fig. 4.1. Tissue preservation for histological and gene expression analyses are mentioned in detail in the *Sections 2.4* and *2.5*.

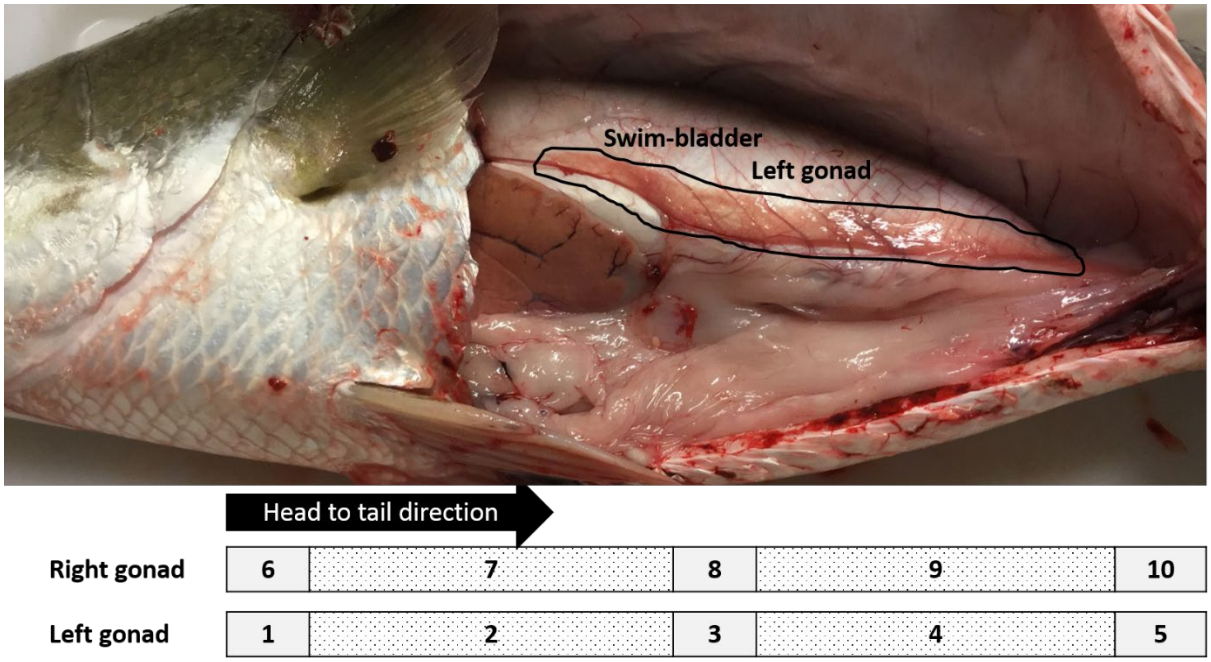


Figure 4.1. Gonad sampling designation for histological and genetic analyses of barramundi implanted with different dosages of E_2

The left gonad is outlined in black. The left and right gonads were collected and sectioned into five fragments each. The below schematic indicates how gonads were subsampled and described in the text for histological analysis (1, 3, 5, 6, 8 and 10), Anterior left gonad (1), Middle left gonad (3), Posterior left gonad (5), Anterior right gonad (6), Middle right gonad (8), Posterior left gonad (10); and pooling four pieces (2, 4, 7 and 9) for gene expression analysis.

4.2.3. Hormone pellet preparation and implantation procedures

Hormone pellet implants of the same size (2.3 mm diameter and 15 mm long) and weight (20 mg), containing either 0, 1.6 and 3.2 mg E_2, were prepared according to Lee et al. (1986), with some modification. Briefly, E_2 powder was dissolved in 80% ethanol and thoroughly mixed with cholesterol (C8867, Sigma-Aldrich) and coconut oil (5% w/w). The E_2-cholesterol-coconut oil mixture was dried in a fume hood at room temperature until a paste-like consistency was achieved. Holes (2.3 mm in diameter) drilled into a sheet of 15 mm plastic acted as a mould for pellet size and shape, while a similar flat plastic sheet acted as a base. The mixture was compressed into the mould and compacted by hand with the flat end of a 2.3 mm drill bit. Once the mould was full, a sharp strike of the drill bit with a hammer

expelled a compacted cylindrical pellet. Hormonal pellets were stored at 4 ^{o}C until implantation.

For implantation, fish were individually transferred from the tanks and anaesthetised. According to the experimental treatment allocations, pellets of the required dose were inserted into the left dorsal musculature of each fish with a RalGun pellet injector (Syndel Laboratories Ltd.). All fish successfully recovered from implantation and survived until completion of the experiment.

4.2.4. Histological analysis

Tissues (gonad and liver) sampled for histological analyses were kept in 10% neutral buffered formalin for 24 h before processing. Formalin-fixed tissues were dehydrated and sectioned using standard paraffin embedding techniques (*see* Section 2.2.2). Approximately 20-40 slides were obtained from each sample and specifically for gonads to ensure at least three parts (anterior, middle and posterior) of both left and right gonads were assessed. The slides were examined using an Olympus CelSens Microscope Digital Camera System (Olympus, Japan).

Testicular and ovarian development was categorised according to Guiguen et al. (1994). Specifically, gonads classed as stage M0 were immature with no visible differentiated germ cells; M1 gonads exhibited predominance of spermatogonia; M2 gonads were filled with mostly spermatocytes and spermatids; M3 gonads contained predominantly spermatozoa; M4, also known as post-spawning, testicular lobules are devoid of spermatozoa. Transitional gonads that are classed as stages T1 and T2 corresponds to the degeneration of testicular tissue without and with ovarian tissue, respectively. T3 and T4 stages are identified by the presence of ovarian tissue that is distributed less or more than 50% in the histological section, respectively. Female ovarian development stages were classed as: stage F1 when ovaries

contained oogonia and previtellogenic oocytes; stage F2 and F3 when less or more than 50% of the cross-section contained vitellogenic oocytes, respectively; stage F4 when oocytes were atretic. Histopathology of liver tissues was also conducted to assess the health condition of hormone-treated fish.

4.2.5. Gene expression analyses by RT-qPCR

The gonadal expression of five genes, *dmrt1*, *cyp11b*, *esr1*, *cyp19a1a* and *foxl2*, was studied in barramundi on completion of the 9-week treatment period. For gene expression analyses, sampled tissues were cut into small pieces (less than 2 mm), preserved in RNAlater™ Stabilization Solution (Thermo Fisher Scientific), and incubated at 4 ^{o}C overnight prior to storage at -20 ^{o}C for 3 to 4 days before RNA isolation.

RNA extraction, DNAase treatment, cDNA synthesis and quality control procedures were conducted as described in *Chapter 2* (*see Section 2.2.3*). RT-qPCR was optimised and performed to compare the level of target gene mRNA expression in the gonads of nine fish from each treatment. Primers from *dmrt1*, *cyp11b*, *esr1*, *cyp19a1a* and *foxl2* and the validated reference gene *ubq* (ubiquitin) were derived from previous studies (De Santis et al., 2011; Ravi et al., 2014; Domingos et al., 2018) (Table 4.1). Reaction efficiencies (E) for each gene were validated using standard curves prepared from serially diluted *c*DNA (E = 0.98-1.03, $R^2 \geq 0.99$).

For each target gene, 100-well rings contained nine samples from each of the three treatments run in triplicate and included a non-template control and two standard dilutions of the standard curve. RT-qPCR product specificity for each gene was confirmed by analysis of melting curves and Sanger sequencing (AGRF).

Table 4.1. Primer sequences used for RT-qPCR to study the expression of the genes *dmrt1, cyp19a1a, cyp11b, esr1* and *foxl2* in the gonads of barramundi implanted with different dosages of E_2

Target gene	Accession	Nucleotide sequences (5'-3')	References
dmrt1	KR232516.1	FO-GTGACTCTGACTGGCCCAGAG RO-CAGCAGGTCGGACGTTCC	Ravi et al. (2014)
cyp11b	KF444447	FO-ACACCGGGGTTCTGGGCCAG RO-CACCGCTGTCGTGTCGACCC	Ravi et al. (2014)
esr1	KF444452	FO-CTGCTCCAGGGTGCTGAGCC RO-TGGCCCAGGCATCATGTGG	Ravi et al. (2014)
cyp19a1a	KR492506.1	FO-CACTGTTGTAGGTGAGAGACA RO-CTGTAGCCGTCTATGATGTCA	Domingos et al. (2018)
foxl2	KF444454	FO-CAACCGCCCACCCCGATGTC RO-CTGGGGAGCGCCATGCTCTG	Ravi et al. (2014)
ubq	XM_018704769	FO-ACGCACACTGTCTGACTAC RO-TGTCGCAGTTGTATTTCTGG	De Santis et al. (2011)

The relative abundance of the target genes (*dmrt1*, *cyp11b*, *esr1*, *cyp19a1a* and *foxl2*) were normalised using the reference gene *ubq* according to the $2^{-\Delta Ct}$ method of Livak and Schmittgen (2001).

4.2.6. Statistical analysis

Statistical analyses were performed using the SPSS software package (IBM SPSS Statistics 23). All samples for RT-qPCR were run in triplicate and a mean C_T sample value obtained. Normality and homogeneity of variance were tested using the Kolmogorov-Smirnov and Levene tests, respectively. Normalised C_T values that did not meet criteria of either of these tests were log-transformed with outliers removed. One-way analysis of variance (ANOVA) and Post-hoc Tukey's test were used to determine differences among hormonal treatments on gene expression of *dmrt1*, *cyp19a1a* and *foxl2*. Normalised C_T values of the gene *cyp11b* and *esr1* that did not present heterogeneous variances after transformation were analysed using a non-parametric Kruskal-Wallis test, followed by the Mann-Whitney U test for pairwise comparisons. Differences were regarded as statistically different at $P < 0.05$.

4.3. Results

4.3.1. Morphological changes of barramundi gonads induced by exogenous 17β-estradiol

Sampling of gonads for histological analysis was conducted one-day prior to implantation of the hormone pellets (initial sampling), 4 weeks post-implantation and 9 weeks post-implantation (final sampling). Gonadal phenotype percentages of barramundi at these three time points are shown in Figure 4.2.

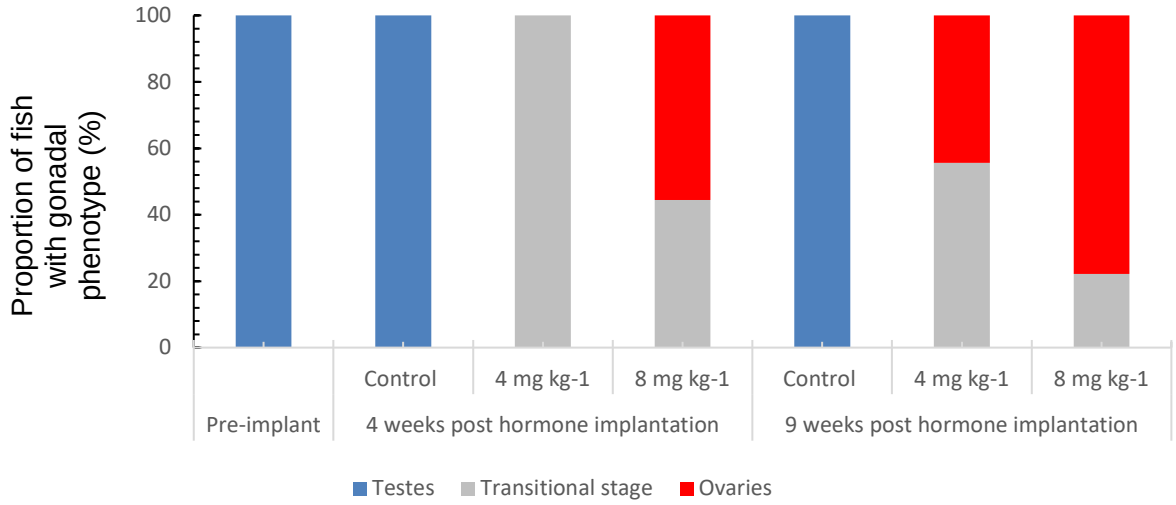


Figure 4.2. Proportion of barramundi that exhibited various gonadal phenotypes (testis, transitional stage and feminised gonad with previtellogenic oocytes) before implant and after 4 and 9 weeks of implantation with E_2 at concentrations of 4 mg kg^{-1} BW or 8 mg kg^{-1} BW

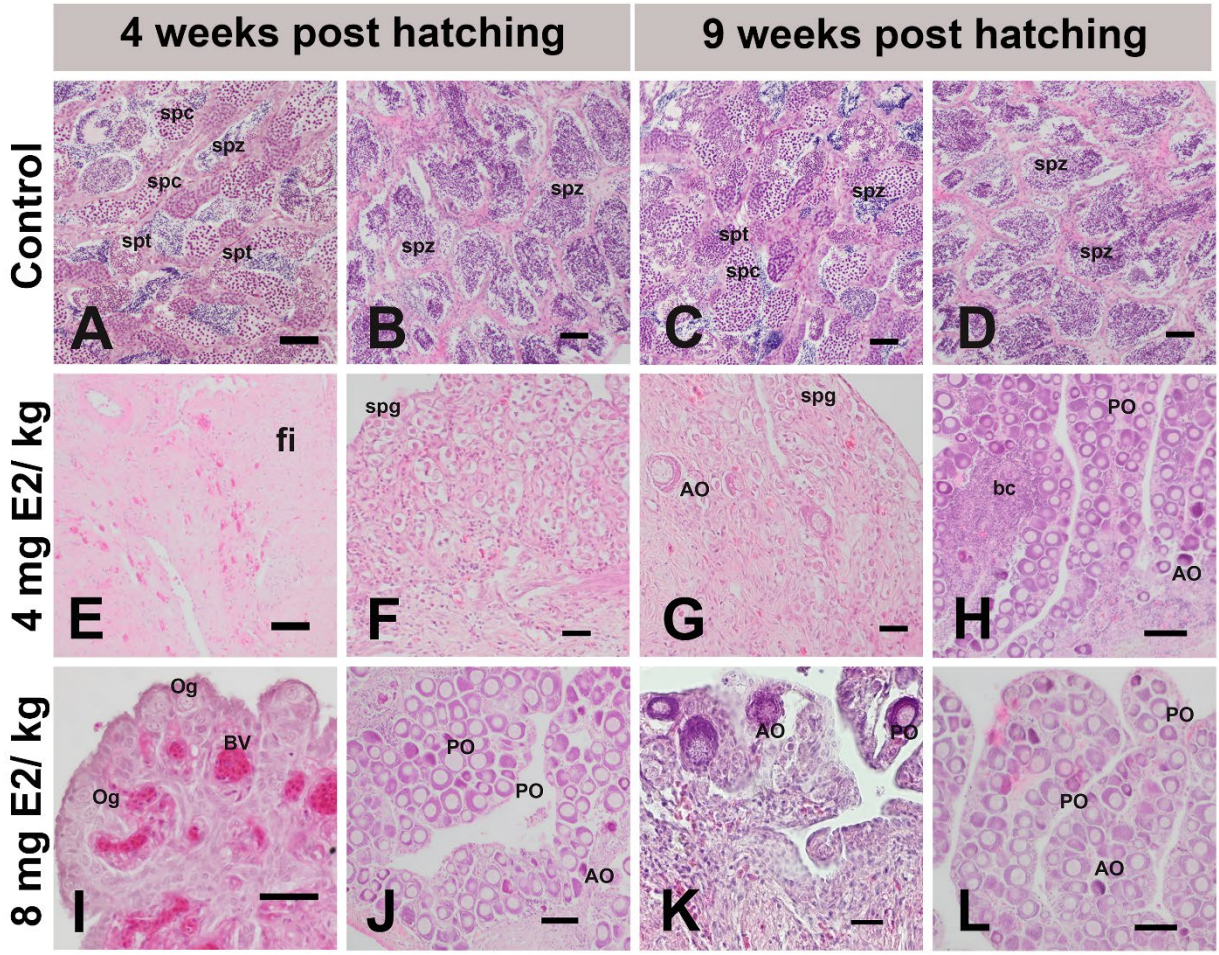


Figure 4.3. Transverse sections of barramundi gonads implanted with E_2 at concentrations of 4 mg kg^{-1} BW or 8 mg kg^{-1} BW at 4 week- and 9 week-post-implantation

Fig. A and **B**: Control barramundi gonads at 4 weeks post-implantation with cholesterol pellets without E_2. **A)** Testis of the control fish at M2 stage. Scale bar 20 µm. **B)** Testis of the control fish at M3 stage. Scale bar 20 µm. **Fig. C** and **D**: Control barramundi gonads at 9 weeks post-implantation with cholesterol pellets contained no hormone. **C)** Testis of the control fish at M3 stage. Scale bar 20 µm. **D)** Testis of the control fish at M3 stage. Scale bar 20 µm. **Fig. E** and **F**: Gonads of barramundi implanted with 4 mg E_2 kg^{-1} BW at 4 weeks post-implantation. Scale bar 50 µm. **Fig. G** and **H**: Gonads of barramundi implanted with 4 mg E_2 kg^{-1} BW at 9 weeks post-implantation. Scale bar 50 µm. **Fig. I** and **J**: Gonads of barramundi implanted with 8 mg E_2 kg^{-1} BW at 4 weeks post-implantation. Scale bar 50 µm. **Fig. K** and **L**: Gonads of barramundi implanted with 8 mg E_2 kg^{-1} BW at 9 weeks post-implantation. **K)** Scale bar 20 µm. **L)** Scale bar 50 µm. Abbreviations: AO, atretic oocyte; BV, blood vessel; bc, basophilic cells; fi, fibrosis; Og, oogonia; PO, previtellogenic oocyte; spc, spermatocyte; spg, spermatogonia; spt, spermatid; spz, spermatozoa.

The phenotypic sex of fish (5/5) on initial sampling was confirmed as being male, as testes contained primarily spermatocytes and spermatids and were classed as stage M2. Likewise, in

all subsequent sampling events, E_2-untreated control fish contained testes at stages M2 to M3. Specifically, at 4 weeks post-implantation two control fish had stage M3 testes, identified as lobules containing large amounts of spermatozoa; the remaining 10 fish had stage M2 testes (Fig. 4.3.A and 4.3.B). At final sampling, 44.4% (4/9) of untreated control fish had stage M3 testes and the remaining 55.6% (5/9) of fish had stage M2 testes (Fig. 4.3.C and 4.3.D).

Four weeks after the administration of E_2, barramundi, in both 'low dose' and 'high dose', treatment groups possessed morphological changes to their gonads. All the individuals in the 'low dose' treatment showed incomplete suppression of spermatogenesis, classified as transitional stage T1, where gonads contained few lobules with spermatogonia distributed along the periphery of the gonadal lamellae and residual spermatocytes (Fig. 4.3.F). In 33.3% (3/9) of gonads, fibrous connective tissue was detected with some dispersed lobules of spermatogonia (Fig. 4.3.E).

Complete testicular regression was observed in 100% (9/9) of 'high dose' E_2-treated fish at 4 weeks post-implantation. Furthermore, 55.6% (5/9) of fish had early stage oocytes (perinucleolar and previtellogenic oocytes) dispersed throughout the gonad (Fig. 4.3.J). Phenotypically, the gonads of the remaining 44.4% (4/9) 'high dose' E_2-treated fish consisted of clusters of oogonia restricted to lamella-like structures and dispersed oocytes (chromatin-nucleolus stage and perinucleolar oocytes) (Fig. 4.3.I). Additionally, there was a high prevalence of vascularity (blood capillary formation) and basophilic cells in 'high dose' E_2-treated fish.

At final sampling, 77.8% (7/9) of individuals in the 'high dose' group showed complete feminisation. Gonadal cross-sections contained exclusively previtellogenic oocytes (20-30 µm diameter) and there was no evidence of residual sperm or testicular tissues (Fig. 4.3.L). Elevated vascularity (blood capillary formation) was also apparent. Comparatively,

individuals in the ‘low dose’ group did not ubiquitously show sex inversion; however, 44.4% (4/9) of gonads of fish in this group contained distinct oocytes (Fig. 4.3.I). The remaining 55.6% (5/9) and 22.2 % (2/9) of ‘low dose’ and ‘high dose’E_2-treated fish, respectively, had transitional gonads staged T2, which contained mainly spermatogonia with some dispersed perinucleolar oocytes.

Histological analysis of the anterior, middle and posterior regions of both the left and right gonads revealed morphological similarities in all untreated control fish and notable differences in the ‘low’ and ‘high’ dose E_2-treated fish. Four weeks post-implantation, the gonads of 100% (9/9) of ‘low dose’ and 33.3% (3/9) of ‘high dose’ E_2-treated fish exhibited complete fibrosis of the anterior region (Fig. 4.3.E). Nine weeks post-implantation, complete fibrosis of the anterior gonadal regions was observed in 66.7% (6/9) of ‘low dose’ and 33.3% (3/9) of ‘high dose’ E_2-treated fish. Otherwise, within both ‘low’ and ‘high’ dose treatments, there were no discernible difference between the middle and posterior gonadal regions.

To examine the potential side effects of exogenous E_2 administration, liver tissues were histologically examined. Livers of untreated control fish were typical of healthy farmed fish (i.e. with the presence of cytoplasmic lipid and vacuolisation). Liver sections showed uniform hepatocytes with distinct nuclei and nucleoli, abundance of cytoplasmic lipid and vacuolisation (Fig. 4.4.D and 4.4.G). No differences were observed between the untreated control and the ‘low dose’ treatment at both samplings (4 and 9 weeks post-hormone-implantation). In the ‘high dose’ E_2-treated group, at 4 weeks post-treatment, 66.7% (6/9) fish had livers with hyperaemia (excess of blood in the vessels supplying an organ) (Fig. 4.4.F). At the final sampling, all sampled livers showed no significant tissue damage regardless of the E_2 dosage (Fig. 4.4.H and 4.4.I).

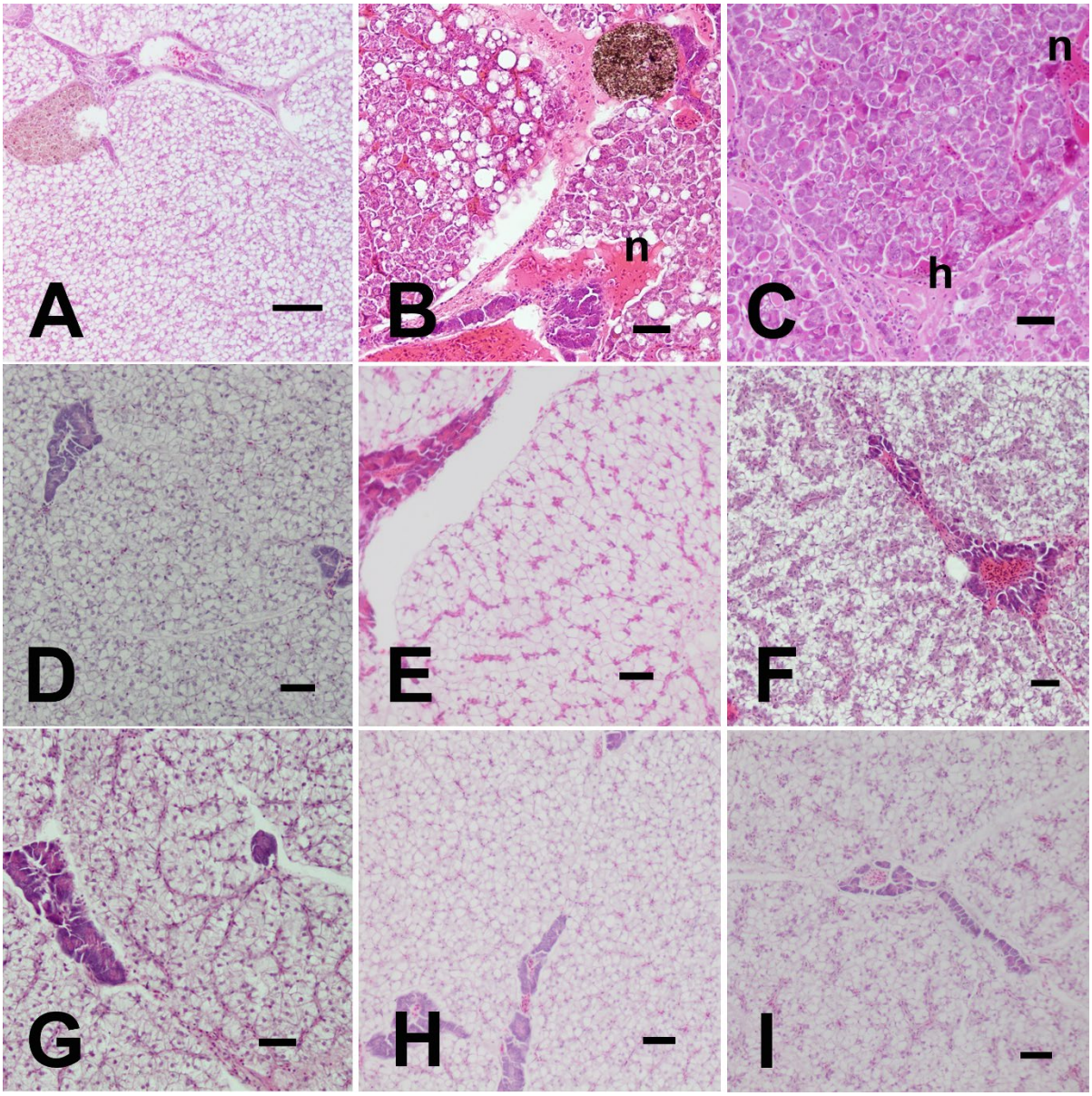


Figure 4.4. Histological images of livers of barramundi implanted with E_2 at different concentrations (10 and 20 mg E_2 kg^{-1} BW)

Fig. A, B and **C**: Livers of barramundi in the E_2 hormone implantation "dosage-range-finding trial" **A)** Liver of the control barramundi implanted with cholesterol pellets without hormone. Scale bar 50 µm. **B)** Liver of the barramundi implanted with 10 mg E_2 kg^{-1} BW. Scale bar 20 µm. **C)** Liver of the barramundi implanted with 20 mg E_2 kg^{-1} BW. Scale bar 20 µm. **Fig. D, E, F, G, H** and **I**: Livers of barramundi in the primary experiment **D)** Liver of the control barramundi implanted with 'dummy" cholesterol pellets at 4 weeks post-implantation. Scale bar 20 µm. **E)** Liver of the barramundi implanted with 4 mg E_2 kg^{-1} BW at 4 weeks post-implantation. Scale bar 20 µm. **F)** Liver of the barramundi implanted with 8 mg E_2 kg^{-1} BW at 4 weeks post-implantation. Scale bar 20 µm. **G)** Liver of the control barramundi implanted with 'dummy" cholesterol pellets at 9 weeks post-implantation. Scale bar 20 µm. **H)** Liver of the barramundi implanted with 4 mg E_2 kg^{-1} BW at 9 weeks post-implantation. Scale bar 20 µm. **I)** Liver of the barramundi implanted with 8 mg E_2 kg^{-1} BW at 9 weeks post-implantation. Scale bar 20 µm. Abbreviations: n, necrosis; h, hyaline.

4.3.2. E_2 altered gene expression profiles within barramundi gonads

The expression of the five genes (*dmrt1*, *cyp11*, *esr1*, *foxl2* and *cyp19a1a*), studied by RT-qPCR, is shown in Fig. 4.5. No significant differences were observed in gene expression of target genes between fish sampled before commencement of the trial and in untreated control fish at final sampling. Conversely, regardless of the dose of exogenous hormone, the expression of all targeted sex-related genes was significantly affected by E_2 implantation ($P < 0.05$).

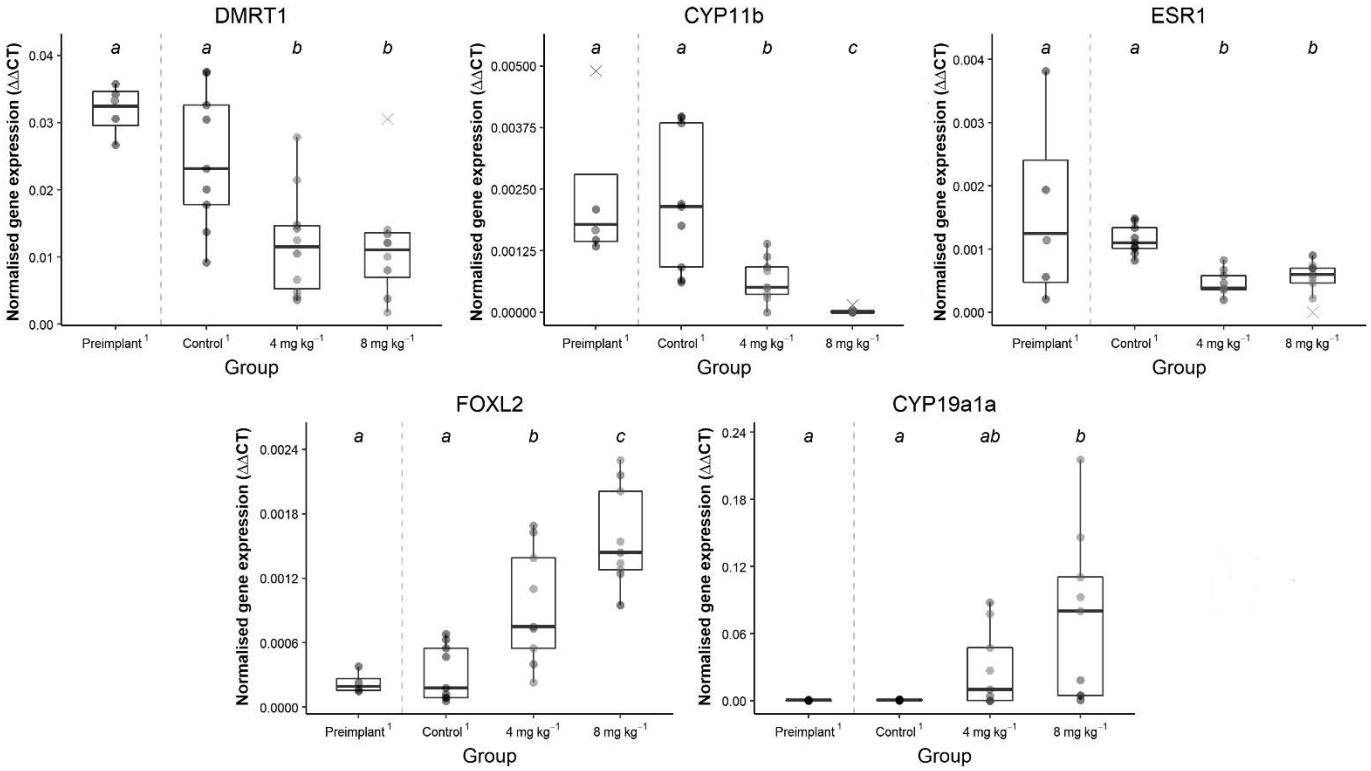


Figure 4.5. Relative gene expression of different sex-related genes measured by RT-qPCR in barramundi gonads implanted with E_2 sampled at 9 weeks post-implantation

(A) *dmrt1*, **(B)** *cyp11b*, **(C)** *esr1*, **(D)** *foxl2* and **(E)** *cyp19a1a*. The values were calibrated with the reference gene *ubq* according to Livak and Schmittgen (2001). Different letters represent statistical differences ($P < 0.05$) between treatments ($n = 9$ for each value, except the pre-implant with $n = 5$).

Specifically, E_2 significantly downregulated the mRNA expression of the known male-biased genes (*dmrt1*, *cyp111b* and *esr1*) in barramundi gonads at final sampling ($P < 0.05$). Gonadal

expression of *dmrt1* and *esr1* was significantly higher (~ two-folds) in untreated control fish than in the gonads of fish in both E_2 treatments. The expression of *dmrt1* and *cyp11b* in gonads of fish in the 'low' and 'high' dose E_2 treatment groups did not differ significantly. 'High dose' E_2 implants resulted in *cyp11b* expression levels below detectable limits of RT-qPCR, suggesting complete suppression of the gene. Meanwhile, 'low dose' E_2 partially suppressed *cyp11b* expression, resulting in only one-third-fold expression compared to the control ($P < 0.05$).

Significantly, the mRNA expression of female-related genes, *cyp19a1a* and *foxl2,* were upregulated in the fish with both 'low' and 'high' dose E_2 implants when compared to the control ($P < 0.05$). Expression of *cyp19a1a* was relatively low in all initial samples and untreated control fish at final sampling. In E_2-treated fish, *cyp19a1a* expression showed a dose-dependent response to E_2; 'high' E_2 dose significantly upregulated *cyp19a1a* expression by approximately three-fold compared to the 'low' E_2 dose. Similar gene expression patterns were observed for *foxl2.* E_2 implantation induced upregulation of *foxl2* expression by three-fold and five-fold in the 'low' and 'high' E_2 dosage groups, respectively, when compared to untreated control fish ($P < 0.05$).

4.4. Discussion

The production of precocious females is critical for the implementation of high-gain selective breeding programs in barramundi. In the present study, gonadal female sex change was achieved using exogenous estradiol pellet implantation in 77.8% of 'high' (8 mg $E_2\,kg^{-1}$) and 44.4% of 'low' (4 mg $E_2\,kg^{-1}$) dose hormone-treated barramundi. Microscopically, the ovaries of fish considered to have undergone sex change consisted of developing lamellae and early stage oocytes (chromatin-nucleolus and pre-vitellogenic stages). Individuals sampled prior to hormone implantation and from the control group were confirmed as male by the presence of

stage M2 to M3 testes. When compared to a previous trial of orally delivered E_2 (*Chapter 3*), this experiment obtained a higher feminisation ratio with shorter treatment duration and preparation of exogenous estradiol pellets was less laborious.

In our preliminary trial, to establish an appropriate hormone dosage-range, poor health outcomes and fatalities were observed in fish implanted with dosage rates of 10 and 20 mg E_2 kg^{-1} BW. Histological analysis of liver tissue revealed evidence of necrotic hepatocytes, hyperaemia and hyaline (as a type of arterial sclerosis referring to hardening of the arteriolar wall). These pathological signs were not observed in the livers of fish sampled just prior to estrogen implantation indicating that both the trial dosages compromised fish health.

In the present study, gonadal sex change was not observed in 100% of individuals in either treatment group at 9 weeks post-implantation; however, all E_2-treated fish showed complete suppression of testicular tissue. Similarly, morphological changes to testicular tissue that inhibit spermatogenesis, such as testicular atrophy, testis involution, spermatogenesis regression and loss of functional maturity, have been documented in fathead minnows (Panter et al., 1998) and abnormal gonadal phenotypes (involute testes, small ovaries and ovaries lacking germ cells) were induced in estuarine killifish *Fundulus heteroclitus* (Urushitani et al., 2002) as a result of early exposure to exogenous E_2. Injection of two year old male summer flounder *P. dentatus* with 1.0 and 10 mg E_2 kg^{-1} BW suppressed testicular development and resulted in regression of spermatogenic cells to primary spermatogonia (Zaroogian et al., 2001). Additionally, in 'high dose' E_2-treated fish, high prevalence of blood capillary formation and basophilic cells was observed, which is commonly considered as a precursor of gonads entering sex transition (Chaves-Pozo et al., 2003; Liarte et al., 2007; Chaves-Pozo et al., 2009).

Significant differences in gene expression profiles of E_2-treated and untreated control fish showed that implantation of exogenous estrogen suppressed expression of male-related genes, *dmrt1, cyp11b* and *esr1*, and increased expression of female-related genes, *cyp19a1a* and *foxl2*. Among the known genes specific to ovarian differentiation (Yao, 2005), *foxl2* is highly conserved across divergent taxonomic groups from fish to humans (Baron et al., 2005; Crespo et al., 2013). In the present study, *foxl2* was minimally expressed in untreated control fish when compared to the 'low' and 'high' dose E_2-treated groups (three- and five-times less, respectively); conversely, administration of exogenous E_2 resulted in the upregulation of *foxl2* during early-stage ovarian development and feminisation of E_2 treated barramundi. This suggests that the ovarian-specific role of *foxl2* has remained conserved in barramundi. Similar sexually dimorphic expression of *foxl2* has been reported in mammals, reptiles (Loffler et al., 2003; Baron et al., 2005; Oshima et al., 2008), and teleost species, including medaka (Nakamoto et al., 2006), rainbow trout (Baron et al., 2004), Nile tilapia (Ijiri et al., 2008), European seabass (Crespo et al., 2013) and Chinese rare minnow *Gobiocypris rarus* (Jiang et al., 2011). Differential expression has also been observed in other hermaphrodites, including protogynous rice field eels *Monopterus albus* (Zhang et al., 2010b), protandrous black porgy (Wu et al., 2010) and the rudimentary hermaphrodite, sparid sharpsnout seabream *Diplodus puntazzo* (Manousaki et al., 2014).

In accordance with *foxl2* expression, transcript levels of *cyp19a1a*, a key gene in estrogen synthesis and ovarian differentiation in various teleosts (Kitano et al., 1999; Leet et al., 2011), were detected at very low levels in untreated male barramundi. Conversely, treatment with exogenous E_2 upregulated *cyp19a1a* expression in the gonadal tissue of both 'low' and 'high' dose E_2-treated fish when compared to the controls. Higher expression of *cyp19a1a* was seen in the 'high dose' E_2-treated fish compared to the 'low dose' (three-fold increase); however, this difference was not statistically significant. The results from our study suggest

the incidence of a positive feedback loop, in which estrogen-induced upregulation of *foxl2* resulted in increased expression of *cyp19a1a* and, in turn, increased the irreversible conversion of endogenous androgens to estrogens (Kazeto et al., 2004; Luckenbach et al., 2009; Guiguen et al., 2010; Piferrer, 2011). This process resulted in the accumulation of endogenous estrogen and lowered endogenous androgens, which is believed to be the determinant for sex differentiation (Piferrer, 2001) and, as a consequence, led to ovarian differentiation in E_2-treated barramundi. The previous study on E_2-feeding (*Chapter 3*) supports this theory, as significantly dimorphic expression of *cyp19a1a* induced by dietary estrogen was maintained (~6 months) after the cessation of hormone treatment. Positive correlation in the *foxl2* and *cyp19a1a* expression has also been observed in the gonads of goat *Capra hircus* spp (Pannetier et al., 2005; Pannetier et al., 2006), chicken (Govoroun et al., 2004), African catfish (Sridevi and Senthilkumaran, 2011; Sridevi et al., 2012), rainbow trout (Baron et al., 2004; Vizziano et al., 2007), medaka (Nakamoto et al., 2006), tilapia (Wang et al., 2007) and black porgy (Wu et al., 2008). *Foxl2* upregulates 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). Further research investigating the expression of these genes is needed to clarify the mechanism by which *foxl2* regulates *cyp19a1a* in the protandrous barramundi.

In confirmation of an antagonistic cascade, our results demonstrated that E_2 administration downregulated the expression of known male-related genes, *dmrt1*, *cyp11b* and *esr1*. *Dmrt1* is a key regulator of male sexual development (Ferguson-Smith, 2007; Smith et al., 2009; Matson and Zarkower, 2012) and has been reported as dominantly or exclusively expressed in testes of humans, chicken, reptile and frog (Raymond et al., 1999; Smith et al., 1999a; Kettlewell et al., 2000; Shibata et al., 2002; Smith and Sinclair, 2004) and teleost fishes

(Marchand et al., 2000; Fernandino et al., 2008; Johnsen et al., 2010; Berbejillo et al., 2012). Suppression of *dmrt1* expression due to exposure to estrogenic compounds has also been recorded in reptiles (Murdock and Wibbels, 2006) and other teleosts (Marchand et al., 2000; Filby et al., 2007; Schulz et al., 2007; Fernandino et al., 2008; Kobayashi et al., 2008). In barramundi, *dmrt1* and *cyp11b* were found to be upregulated during early testicular differentiation (Banh et al., 2017). Male specific expression of *cyp11b*, one of the key steroidogenic enzymes, which catalyses biosynthesis of the potent androgen 11-ketotestosterone (Kime, 1993; Kusakabe et al., 2002), was documented in Nile tilapia (Zhang et al., 2010a), sparid sharpsnout seabream (Manousaki et al., 2014), bluehead wrasse *Thalassoma bifasciatum* (Liu et al., 2015) and barramundi (Ravi et al., 2014). Sexually dimorphic patterns of *esr1* (i.e. higher expression in testis than ovary) were also reported in Nile tilapia (Ijiri et al., 2008; Tao et al., 2013), rainbow trout (Baron et al., 2008; Delalande et al., 2015), European sea bass (Blázquez et al., 2008), and barramundi (Ravi et al., 2014); yet, the opposite expression of *esr1* in other species (Davis et al., 2008; Lynn et al., 2008), and the regulation of other possible estrogen receptor subtypes, such as *esr2* in teleosts (Nelson and Habibi, 2013), suggests there needs to be further study on the mechanism of estrogen hormones and their receptors in sex change/differentiation of barramundi.

Administration of exogenous E_2 has the potential to alter the ratio of endogenous estrogens and androgens (Bjerregaard et al., 2008; Falahatkar et al., 2014; Akhavan et al., 2015). In rice field eel (Yuan et al., 2011), rainbow trout (Depiereux et al., 2014), common snook (Passini et al., 2016) and black porgy (Chang et al., 1995a), a decrease in plasma 11-ketotestosterone in the presence of exogenous E_2 directed sex change and gonadal restructuring (e.g. testicular inhibition). Furthermore, E_2 potentially triggered feminising feedback mechanisms that operate naturally in females by activating *foxl2* and *cyp19a1a* and, as such, interacted with the ovarian development pathway. The gene expression profile seen here in barramundi

supports the genetic pathways proposed by previous studies, in which the female regulatory gene network would suppress the opposing transcriptional network (*dmrt1, cyp11b* and *esr1*) (Ravi et al., 2014; Lamm et al., 2015; Liu et al., 2015; Todd et al., 2016; Capel, 2017). In turn, the downregulation of *dmrt1* resulted in the suppression of *cyp11b* (Wang et al., 2010; Kobayashi et al., 2013). While experimental gene-knockdown has not yet been undertaken in barramundi, *foxl2*-deficient XX tilapia exhibited oocyte degeneration or complete sex reversal (Li et al., 2013). Furthermore, significant upregulation of *dmrt1* and *cyp11b* and downregulation of *cyp19a1a* highlights the role of *foxl2* in supporting the feminising gene pathway.

Feminised barramundi obtained at 9 weeks of E_2 treatment in this study contained gonads with early stages of oogenesis (previtellogenic oocyte). The commercial purpose of this feminisation is to produce precocious females from selected (male) individuals at harvest, which can then be used as female broodstock in genetic improvement programs. Future studies should determine the reproductive potential of E_2-treated females and their capacity to produce mature and viable eggs. E_2-feminised fish were maintained for over 6 months after the end of the study period (unpublished data) demonstrating that once barramundi are sex-changed into females they maintain this sexual state. Currently, it is unclear if the genes necessary for oocyte maturation are also affected by exogenous E_2, or if regulatory mechanisms controlling puberty-like development are present in precocious females. Similarly, the total number of eggs produced by a small precocious female (~500 g-2 kg) is expected to be substantially lower than the number produced by normal females (10+ kg) and needs to be assessed in terms of commercial application of precocious females.

4.5. Conclusions

In summary, this study demonstrated that young male barramundi (~ 6 month post-hatching, ~400 g BW) can be safely sex-changed using E_2 implants. At 9 weeks post-implantation, the feminised rate achieved with a single implant (8 mg E_2 kg^{-1}) was 77.8%. Exogenous E_2 administration influenced barramundi sex-determining networks by inducing upregulation of female (*cyp19a1a* and *foxl2*) and suppression of male genetic pathways (*dmrt1*, *cyp11b* and *esr1*). Moreover, considering the adverse effects on health and survival of fish, E_2 implantation at a maximum dosage of 8 mg kg^{-1} BW is recommended. This result opens the potential for more efficient breeding systems to be applied in barramundi selection programs, which will most importantly include the mating of coetaneous males and females. Further studies are required to assess the breeding potential of E_2-induced, precociously sex-changed females.

Chapter 5. Aromatase inhibitor fadrozole and 17β-estradiol antagonistically affect gonadal development in the protandrous barramundi

5.1. Introduction

The natural sex change of barramundi challenges broodstock management of hatcheries, as high quality male broodfish overtime change sex to female, requiring the recruitment of new males for hatchery broodstock. Alternatively, in selective breeding, sequential sex change of barramundi most often results in a one-generation age difference between sexes, with females older than the males in spawning groups. Inter-generation breeding thereby halves the annualised rate of genetic progress that could otherwise be made through a single-generation selection program (Robinson and Jerry, 2009). Therefore, sex control of sequential hermaphrodites, as in the case of barramundi, through either inducing earlier sex reversal of males into females or preventing the natural sex change to maintain males in the breeding population, is of great importance to the hatchery production of fingerlings in this industry.

Different from higher vertebrates, the course of gonadal development of fish, including barramundi, has been proven to be easily modified by EDC exposure, which can alter the phenotypic sex of individuals (Piferrer, 2001; Devlin and Nagahama, 2002). *Chapters 3* and *4* reported the results of trials to induce male to female sex change of barramundi by exposing either undifferentiated larvae, 6 month-old juveniles, or adult males to exogenous E_2 and showed that sex change to the female gonadal state can be induced when exogenous E_2 is administered. These trials further showed that male-related genes were down-regulated, while female-related genes, including *cyp19a1a*, were significantly up-regulated in feminised fish. *Cyp19a1a* encodes the enzyme aromatase, which catalyses the conversion of testosterone to estradiol and, thus, is the key enzyme involved in female sex control (Guiguen et al., 2010).

This chapter proposes an alternate approach to not sex changing males to females, but to hold fish as males through inhibiting the activity of the aromatase enzyme.

The non-steroidal aromatase inhibitor, fadrozole (FAD), selectively inhibits estrogen production by binding reversibly to the enzyme aromatase (Steele et al., 1987). Its action is based on non-covalent, reversible interaction with the heme domain of aromatase and occupation of its substrate-binding site (Miller et al., 2008). Treatment with FAD has been shown to reduce estrogen biosynthesis in breast cancer patients (Dowsett et al., 1994) and also results in lowered E_2 in other mammals (Shetty et al., 1995; Moudgal et al., 1996). FAD exposure has also been shown to induce complete functional masculinisation in chicken (Elbrecht and Smith, 1992; Abinawanto et al., 1996), turtle (Dorizzi et al., 1994; Richard-Mercier et al., 1995), lizard (Wennstrom and Crews, 1995) and fish (Piferrer et al., 1994b; Kitano et al., 2000; Kwon et al., 2000; Hur et al., 2012; Luzio et al., 2016). Thus, FAD may be a suitable candidate to help hatchery operators maintain certain broodstock as male and prevent sex change to female.

In the previous chapters, expression of the sex-biased genes in barramundi was altered by E_2 administration. Specifically, the examined male-related genes, *dmrt1*, *cyp11b* and *esr1*, were down-regulated at 9 weeks post E_2 implant. Conversely, female biased genes, including *cyp19a1a* and *foxl2*, were significantly upregulated. These results suggest that the elevated E_2 due to exogenous E_2 implant triggers the positive loop of the feminised genetic pathway in barramundi, in which upregulation of *foxl2* may promote *cyp19a1a* expression. It is unknown if inhibiting aromatase with fadrozole would disrupt the female gene expression network in barramundi. Studying the expression of sex-related genes in the gonad of FAD and FAD-and-E_2-treated fish would contribute to our understanding of the genetic mechanisms underlying the important process of sex change of barramundi.

As natural sex changing barramundi are hard to identify without using large numbers of broodfish and through repeated sampling, and given *Chapter 4* showed that fish implanted with 8 mg E_2 kg^{-1} BW 100% sex changed into female, in this study the effects of FAD treatment were examined in 15-month-old fish implanted either with or without exogenous E_2. Additionally, the underlying genetic pathways involving the female (*cyp19a1a* and *foxl2*) and male (*dmrt1*, *cyp11b*, *esr1*) genes were examined by RT-qPCR to further understand genetic mechanisms involved in sex differentiation and sex change of barramundi.

5.2. Material and methods

5.2.1. Experimental design

Fifteen-month-old male barramundi (2102 ± 126 g BW) purchased from a commercial freshwater farm were kept in three 2,500 L tanks maintained under natural conditions of photoperiod and temperature (28-30 ^{o}C). The tanks were connected to a 13,500 L freshwater recirculating aquaculture system (RAS). Fish were fed a commercial barramundi diet (Ridley Corporation) twice daily at 3% BW per day. Fish rearing and water quality monitoring were conducted as demonstrated in *Chapter 2*. The rearing system was also equipped with two cartridges of activated carbon and hormone residual test was conducted as presented in *Chapter 3*. No E_2 was detected in the water supplied to experimental units.

After a week of acclimatisation, fish were anesthetised with AQUI-S (Aqui-S New Zealand Ltd, New Zealand), individually PIT tagged and had their body weight (BW) (g) recorded. The implants were prepared as described previously (*Chapter 4*) and contained either E_2 or fadrozole hydrochloride (FAD, Sigma-Aldrich, F3806) for the treated groups. Briefly, E_2 or FAD chemical stocks were diluted in 80% ethanol before being thoroughly mixed with cholesterol (C8867, Sigma-Aldrich) and coconut oil (5% w/w). The mixture was dried at room temperature in a fumehood until a paste-like consistency was established. Holes (2.34 mm) drilled into a sheet of 15 mm thick plastic acted as a mould for pellet size and shape,

while a similar flat plastic sheet acted as a base. The prepared mixtures of either E_2 or FAD were compressed into the mould and compacted by hand with the flat end of a 2.34 mm drill bit. Once the mould was full, a sharp strike of the drill bit with a hammer expelled a compacted cylindrical pellet. Hormonal pellets were stored at 4 ^{o}C until implantation.

The experiment consisted of three treatments and a control, with 12 individuals in each group. The control fish were implanted a cholesterol pellet without the addition of E_2 or FAD. The three treated groups were implanted with E_2 at 8 mg kg^{-1} (n = 12), FAD 8 mg kg^{-1} (n = 12), and binary treatment with both 8 mg E_2 and 8 mg FAD kg^{-1} (n = 12), respectively. Each fish was implanted with three pellets into the left dorsal musculature using a RalGun pellet injector (Syndel Laboratories Ltd.). Four fish from each group were held in each tank to account for any unexpected potential tank effects on gonadal development. All fish recovered from implantation successfully and survived until completion of the experiment.

Sampling was conducted one day prior to beginning the experiment (n = 5), and a second sampling (n = 12 for each group) at 9 weeks post-implantation when the effects of induced sex change by E_2 implantation on gonadal phenotype are expected, as found in the previous study (*Chapter 4*). For the gonad samplings, left and right gonads of each fish were sectioned into separate pieces for various analyses. Three small pieces (~1 cm long) at the anterior, middle and posterior part of each gonad were preserved separately for histological analysis according to section 5.2.2. below. The remaining parts of the gonad tissue of each fish were preserved in RNAlater^{TM} stabilization solution (ThermoFisher Scientific) at 4 ^{o}C overnight before placed at -20 ^{o}C until RNA extraction.

5.2.2. Histological analysis

Tissues (gonad, kidney and liver) sampled for histological analyses were kept in 10% neutral buffer formalin for 24 h before being processed. Fixed tissues were dehydrated and

histological sections were produced using standard paraffin embedding techniques (*see Section 2.2.2*). Approximately 10-20 slides were obtained from each sample to ensure at least three parts (anterior, middle and posterior) of both left and right gonads were assessed. The slides were examined using an Olympus CelSens Microscope Digital Camera System (Olympus, Japan).

Testicular and ovarian development were categorised according to Guiguen et al. (1994) (*see Chapter 4*). Histopathology of liver and kidney tissues were analysed to examine the health condition of hormone treated fish.

5.2.3. Gene expression analyses by RT-qPCR

The expression of five genes, *dmrt1*, *cyp11b*, *esr1*, *cyp19a1a* and *foxl2*, in the gonad tissues were studied at 9 weeks after implantation. RNA extraction, DNAse treatment, cDNA synthesis and quality control were conducted as described previously (*Chapter 2*).

RT-qPCR was optimised and performed to compare the level of mRNA expression of the target genes (*dmrt1*, *cyp11b*, *esr1*, *cyp19a1a* and *foxl2*) in the gonads of barramundi from 12 fish from each treatment at 9 weeks after the FAD and E_2 implantation. The primer sequences used to amplify the reference gene and all the target genes are shown in Table 4.1.

RT-qPCR efficiencies (E) for each gene were validated using standard curves prepared from five points of 3-fold serially diluted cDNA (E = 0.98-1.03, $R^2 \geq 0.99$). Relative transcript abundance of target genes was normalised to the reference gene *ubq* according to the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).

For each individual gene, two RT-qPCR 100-well rings were prepared, each containing six samples from each of the four treatments at the final sampling, five samples from the initial sampling, one NTC and two standard dilutions of the standard curve, all loaded in triplicate

(three technical replicates). Further to melt curve analysis, RT-qPCR product specificity for each gene was also confirmed by Sanger sequencing (AGRF).

5.2.4. Statistical analysis

Statistical analyses were performed using the SPSS software package (IBM SPSS Statistics 23). Mean C_T value was obtained for each RT-qPCR sample triplicate. Normality and homogeneity of variance were tested using the Kolmogorov-Smirnov and Levene test, respectively. Normalised C_T values that did not meet criteria of either of these tests were log-transformed with outliers removed. Normalised data of *cyp19a1a* and *esr1* expression conformed to parametric assumptions; one-way analysis of variance (ANOVA) with Post-Hoc Tukey's tests were used to test for differences across chemical treatments. As normalised C_T values of the genes *dmrt1*, *foxl2* and *cyp11b* did not meet parametric assumptions, non-parametric Kruskal-Wallis tests, followed by Mann-Whitney U tests for pairwise comparisons were performed. Differences were regarded as statistically significant when $P < 0.05$.

5.3. Results

5.3.1. Altered sex ratio of barramundi induced by administration of E_2 and fadrozole

Samplings for histology were conducted one day prior to implanting the pellets ($n = 5$) and final sampling at 9 weeks after the implantation ($n = 12$ for each group). The percentages of gonadal phenotypes of barramundi are shown in Fig. 5.1.

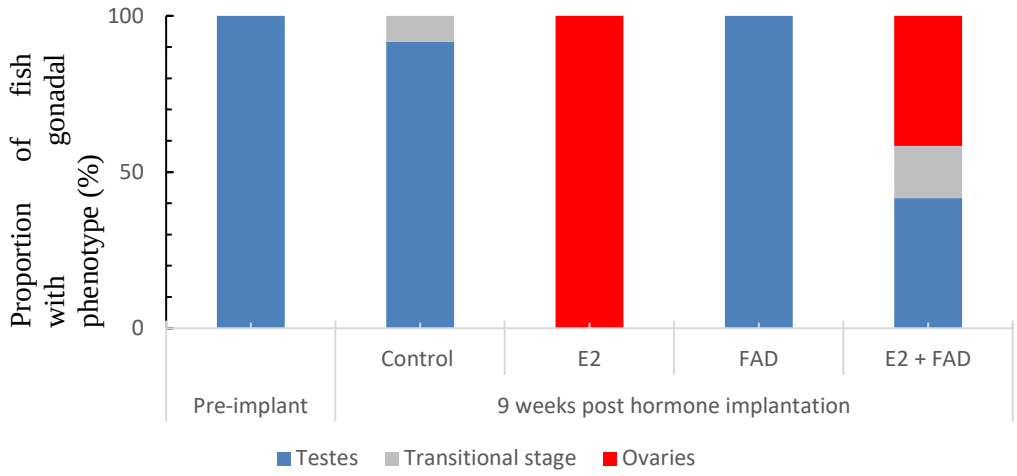


Figure 5.1. Proportion of barramundi that exhibited various gonadal phenotypes (testis, transitional stage and feminised gonad with previtellogenic oocytes) before implant and after 9 weeks of implantation with Estradiol 2, Fadrozole and their binary mixture.

All the fish sampled immediately prior to the implantation (n = 5) were males, with testes at stage M3, containing primarily spermatozoa (Fig. 5.2.A). At the final sampling, 9 weeks after implantation, 92 % (11/12) of the control fish had stage M3 testes (Fig. 5.2.D). One fish in the control exhibited evidence for testicular degeneration, i.e. less spermatozoa and appearance of spermatocytes and fibrous tissue (note: there was no ovarian tissue observed in gonad of this fish; Fig. 5.2.E). According to the classification of Guiguen et al. (1994), this fish appeared to have commenced entering sex change and was a transitional stage T1. All fish (12/12) in the 8 mg E_2 kg^{-1} treatment, as expected, showed complete feminisation and were considered to be female. Their ovaries were filled with pre-vitellogenic oocytes (PO) with a mean diameter of the largest 10 PO per section of 34 ± 11 µm. The POs were distributed along the edges of the lamella structures. Some atretic oocytes were present among the POs. Eosinophilic cells were detected in all E_2 treated gonad. An ovarian cavity was also observed in all the fish of this treatment.

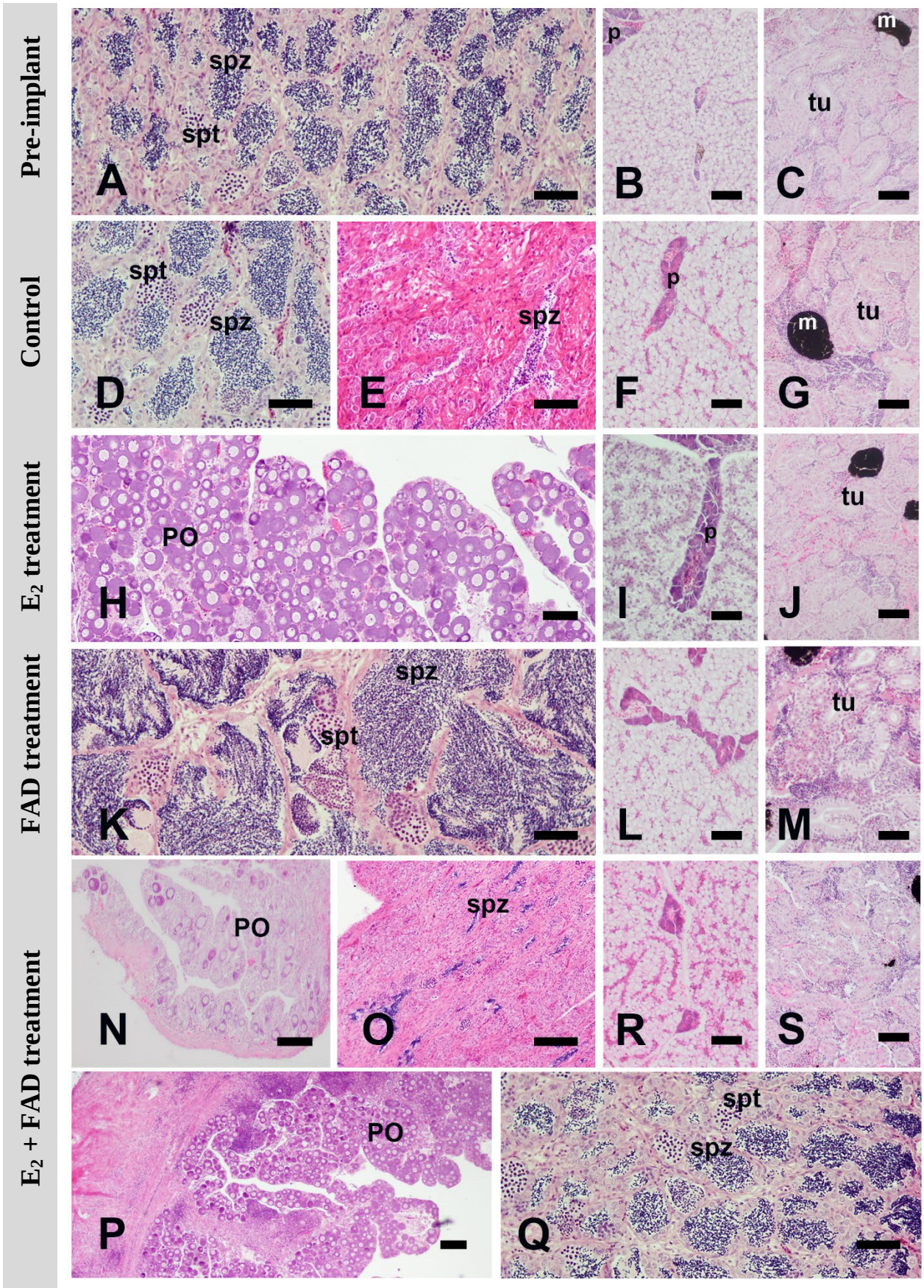

Pre-implant
Control
E2 treatment
FAD treatment
E2 + FAD treatment
spz
spt
A
p
B
m
tu
C
spt
spz
D
spz
E
p
F
m
tu
G
PO
H
p
I
tu
J
spz
spt
K
L
tu
M
PO
N
spz
O
R
S
PO
P
spt
spz
Q

Figure 5.2. Histological images of gonads and livers of barramundi implanted with E_2 (8 mg kg^{-1} BW), FAD (8 mg kg^{-1} BW) and binary of E_2 and FAD (8 mg of E_2 and 8 mg of FAD kg^{-1} BW) at 9 weeks post-implantation

Fig. A, B, and C: Pre-implant barramundi **A)** Testis of pre-implant fish at M3 stage. Scale bar 20 µm. **B)** Liver of pre-implant fish. Scale bar 20 µm. **C)** Kidney of pre-implant fish. Scale bar 20 µm. **Fig. D, E, F** and **G**: Control barramundi **D)** Testis of control fish at M3 stage. Scale bar 20 µm. **E)** One (out of 12) fish in the control group had the gonadal tissue at transitional stage (T1). Scale bar 20 µm. **F)** Liver of control fish. Scale bar 20 µm. **G)** Kidney of control fish. Scale bar 20 µm. **Fig. H, I and J:** Treatment 8 mg E_2 kg^{-1} bw: **H)** Ovary of fish in the treatment 8 mg E_2 kg^{-1} bw. Scale bar 50 µm. **I)** Liver of fish in the treatment 8 mg E_2 kg^{-1} bw. Scale bar 20 µm. **J)** Liver of fish in the treatment 8 mg E_2 kg^{-1} bw. Scale bar 20 µm. **Fig. K, L and M:** Treatment 8 mg FAD kg^{-1} bw. **K)** Testis of fish in the treatment 8 mg FAD kg^{-1} bw at M3 stage. Scale bar 20 µm. **L)** Liver of fish in the treatment 8 mg FAD kg^{-1} bw. Scale bar 20 µm. **M)** Kidney of fish in the treatment 8 mg FAD kg^{-1} bw. Scale bar 20 µm. **Fig. N, O, P, Q, R and S:** Binary treatment of 8 mg E_2 and 8 mg FAD kg^{-1} bw: **N)** Gonad of 1 (out of 12 fish) in the binary treatment of 8 mg E_2 and 8 mg FAD kg^{-1} bw at T3 stage. Scale bar 20 µm. **O)** Gonad of 1 (out of 12 fish) in the binary treatment of 8 mg E_2 and 8 mg FAD kg^{-1} bw at T1 stage. Scale bar 20 µm. **P)** Ovary of fish in the binary treatment of 8 mg E_2 and 8 mg FAD kg^{-1} bw at M3 stage. Scale bar 20 µm. **Q)** Testis of fish in the binary treatment of 8 mg E_2 and 8 mg FAD kg^{-1} bw at M3 stage. Scale bar 20 µm. **R)** Liver of fish in the treatment 8 mg FAD kg^{-1} bw. Scale bar 20 µm. **S)** Kidney of fish in the treatment 8 mg FAD kg^{-1} bw. Scale bar 20 µm. Abbreviations: fi, fibrotic tissue; m, macrophage; p, pancreatic cells; PO, perinucleolar oocytes; spg, spermatogonia; spc, spermatocytes; spt, spermatids; spz, spermatozoa; tu, tubule.

In all (12/12) fish that received the FAD implants (8 mg FAD kg^{-1}), testicular tissues were observed within gonads, containing cysts filled with mostly spermatozoa and some spermatids. The size of these cysts and the interstitial tissues among those cysts were larger in FAD-implanted fish compared to the normal males of the control group (Fig. 5.2.E). Finally, in the fish with implants of both E_2 and FAD (8 mg E_2 and 8 mg FAD kg^{-1}), three types of gonads were observed, with 42% (5/12) being females at stage F1, 42% (5/12) being males at stage M3 (containing mostly spermatozoa) and 17% (2/12) of fish considered transitional at stages T1 and T3. There were no discernible differences or abnormalities in the ovary and

testicular tissues of the females and male fish in the binary treatment, compared to the E_2 treated fish and the control male, respectively.

Histological analysis of liver and kidney tissues was used to examine the potential side effects of E_2 and FAD exposure. In all the groups, including the control, the liver sections showed uniform hepatocytes with distinct nuclei and nucleoli, and abundance of cytoplasmic lipid and vacuolisation, which are typical for healthy farmed fish (Fig. 5.2.B, 5.2.F, 5.2.I., 5.2.L and 5.2.R). Kidney tissues showed normal appearance of nephrons and mesonephric tubules Fig. 5.2.C, 5.2.G, 5.2.J., 5.2.M and 5.2.S).

5.3.2. Fadrozole and E_2 treatments caused alteration of gene expression profiles in barramundi gonads

The expression of the sex-related genes (*dmrt1*, *cyp19a1a*, *foxl2*, *cyp11b* and *esr1*) in the gonadal tissues sampled at the beginning of the experiment (pre-implant) and at 9 weeks after the implantation (control and three treatments) assessed by RT-qPCR displayed significant changes, including time- and endocrine disruptive compound type (E_2, FAD or E_2+FAD)-dependent effects (Fig. 5.3).

The expression of *dmrt1*, *cyp11b* and *cyp19a1a* was not significantly different between the initial fish sampled at the start of the trial and the control fish at final sampling; however, *esr1* and *foxl2* were significantly increased in the untreated control fish compared to the fish sampled at the start of the trial ($P < 0.05$).

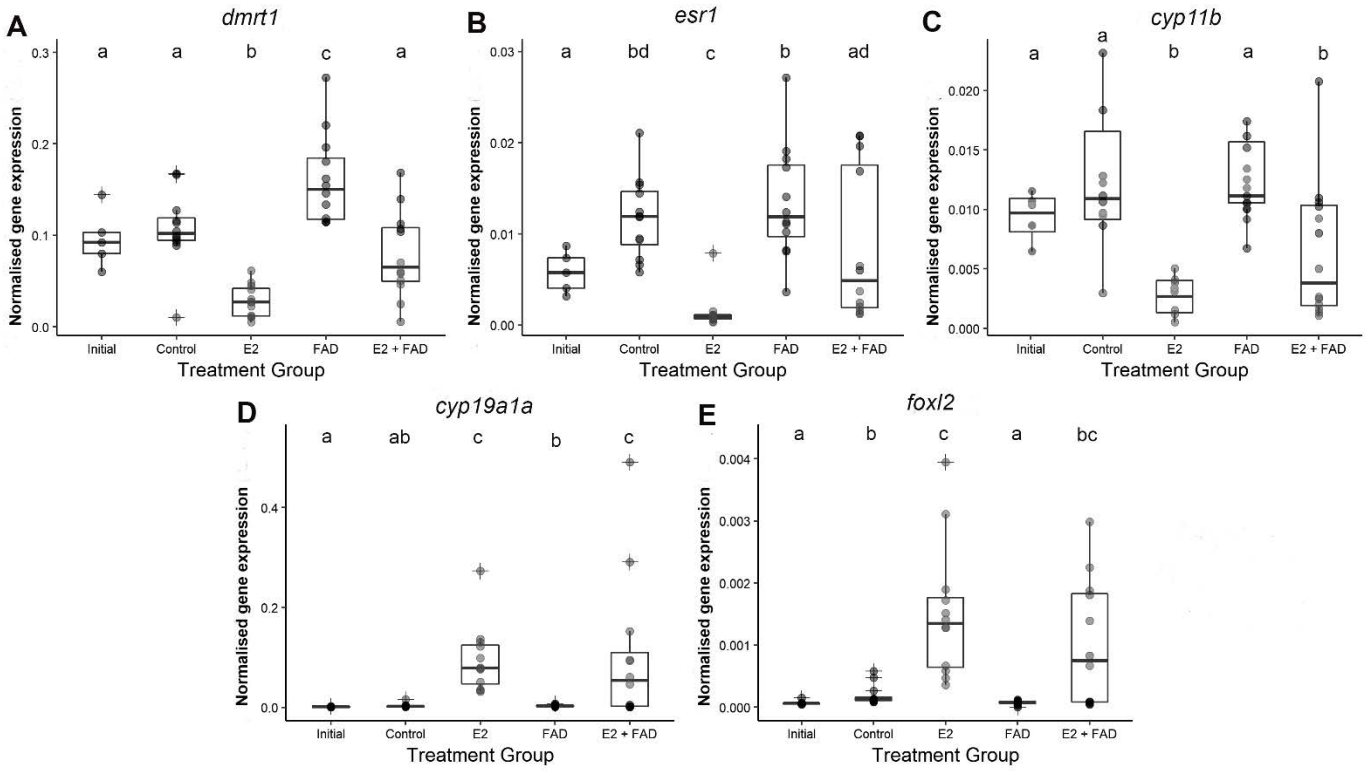


Figure 5.3. Relative gene expression of different sex-related genes measured by RT-qPCR in barramundi gonads implanted with E_2, FAD and binary of E_2 and FAD sampled at 9 weeks post-implant (A) *dmrt1* (B) *cyp11b*, (C) *esr1*, (D) *foxl2* and (E) *cyp19a1a*.

The values were calibrated with the reference gene *ubq* according to Livak and Schmittgen (2001). Different letters represent statistical differences ($P < 0.05$) between treatments (n=12 for each value).

At 9 weeks post-implantation, *dmrt1* expression was significantly up-regulated in the FAD treatment ($P < 0.05$). In contrast, exposing barramundi to E_2 significantly down-regulated the expression of *dmrt1* in their gonads ($P < 0.05$). Expression of *dmrt1* mRNA in the gonads of fish implanted with the mixed E_2+FAD was not significantly different from the control untreated fish. Among all the examined groups, E_2-exposed fish displayed the lowest *dmrt1* expression ($P < 0.05$). Exposure to either E_2 or the combined E_2+FAD treatments induced a significant down-regulation of *cyp11b* ($P < 0.05$). However, there was no significant difference in the *cyp11b* expression between these two treatments. Similarly, *cyp11b*

transcripts in the FAD exposed fish treatment did not significantly differ from the control fish.

At the final sampling, E_2 exposure significantly down-regulated the expression of *esr1* (approx. 8-fold decrease) ($P < 0.05$). The *esr1* expression in the FAD-treated and the E_2+FAD- treated fish were not significantly different from the control group. However, fish exposed to the E_2+FAD treatment had significantly higher expression of *esr1* mRNA than the E_2–treated group, and lower expression than the FAD –treated group ($P < 0.05$).

The expression of the female-biased gene, *cyp19a1a*, was significantly upregulated in the fish exposed to E_2 and E_2+FAD at 9 weeks after implantation ($P<0.05$); however, the difference in *cyp19a1a* expression between FAD-exposed fish and the control group was not significant. *Cyp19a1a* transcripts in the binary E_2+FAD treatment was significantly more abundant than in the gonad of FAD implanted fish ($P < 0.05$). Likewise, *foxl2*, was significantly different in the fish exposed to E_2 and FAD. Specifically, *foxl2* expression was significantly up- and down-regulated by E_2 or FAD implantation, respectively ($P < 0.05$). Although the *foxl2* transcript abundance in the combined E_2+FAD treatment was five-folds more than the control, this difference was not statistically significant. Meanwhile, *foxl2* expression was significantly higher in the combined FAD+E_2 treatment than the fish exposed to FAD only ($P < 0.05$).

5.4. Discussion

Final sampling of this trial was conducted at 9 weeks post-implantation of E_2, FAD and their binary mixture. This time point was chosen for sampling, as E_2 implantation at the same dose (8 mg kg^{-1}) was shown to induce feminisation in male barramundi (*Chapter 4*). Exposure to FAD only was expected to maintain the testicular development of barramundi. Therefore, applying FAD with E_2 at the dose that feminises barramundi allowed for the investigation of

the potential inhibitory effects FAD has on E_2 and how effective FAD is in maintaining the testis and suppressing male to female sex change in barramundi.

In the current study, complete feminisation of barramundi was observed in the E_2 group at 9 weeks post-implantation. This confirms the ability of E_2 in inducing sex reversal in male barramundi as found in the previous trial (*Chapter 4*). Meanwhile, exposure to FAD resulted in 100% fish possessing testicular tissues compared with 92% in controls. The effect of FAD in influencing sex differentiation, and in particular increasing the proportion of males, has been reported for other fishes, including chinook salmon (Piferrer et al., 1994b), Japanese flounder (Kitano et al., 2000), medaka (Paul-Prasanth et al., 2013), and zebrafish (Takatsu et al., 2013). In Nile tilapia, exposure to FAD caused masculinisation in genetically female (XX) fish and also suppressed feminisation in supermale (YY) groups at the higher temperatures (36 ^{o}C), which was previously proven to have feminisation effects (Kwon et al., 2002). FAD also effectively induced female to male sex changes in protogynous fish (female to male sex changing fish), namely, three-spot wrasse *Halichoeres trimaculatus* (Higa et al., 2003), blackeye goby *Coryphopterus nicholsii* (Kroon and Liley, 2000) and honeycomb grouper (Bhandari et al., 2004b; Bhandari et al., 2004a). In the protandrous black porgy, oral administration of aromatase inhibitors (FAD and 1,4,6-androstatriene-3,17-dione, each 10 mg kg^{-1}) suppressed aromatase activity and inhibited the natural sex change from male to female (Lee et al., 2002). Though quality of spermatozoa was not checked in our study, morphology of testicular gonads of FAD treated fish showed advanced spermiation, with enlargement of the seminiferous tubules accompanied by an abundant accumulation of sperm in their lumina relative to controls. This result is in agreement with other studies on FAD exposure (Afonso et al., 2000; Ankley et al., 2002; Takatsu et al., 2013).

Whilst rare, a few research studies have documented the binary effect of endocrine disrupting chemical mixtures with antagonistic effects on teleost sex differentiation (Santos et al., 2006;

Micael et al., 2007; Luzio et al., 2015). In the zebrafish study of Santos et al. (2006), EE_2 blocked the masculinising effects of tributyltin, which acts as an aromatase inhibitor in human tissues and molluscs. Administration of the mixture of FAD and EE_2 to zebrafish at low concentration (50 µg FAD + 4 ng EE_2 per L) showed that FAD still induced zebrafish masculinisation, promoted intersex in testis and neutralised the body and gonad weight gain response to EE_2 (Luzio et al., 2015). In the present study involving the binary treatment of E_2 and FAD, FAD was partially able to neutralise estrogen potency, maintaining the testicular state in ~42% of the fish. These results show the competing and antagonistic effects of estrogens and aromatase inhibitors in the maintenance of the distinct sexual phenotypes in barramundi. This was also confirmed at the genetic level through the assessment of expression levels of key sex-related genes known to influence gonadal maintenance and sex reversal in barramundi (Ravi et al., 2014; Banh et al., 2017; Domingos et al., 2018).

In the current chapter, FAD implants were able to maintain low levels of the pro-female genes *cyp19a1a* and *foxl2*; while maintaining or stimulating levels of the pro-male genes *dmrt1*, *esr1* and *cyp11b.* Similar to barramundi, suppression of the *cyp19a1a* gene following FAD treatment has also been shown in chicken (Elbrecht and Smith, 1992), and fish including Japanese flounder (Kitano et al., 2000) and zebrafish (Fenske and Segner, 2004). Contrastingly, upregulation of the *cyp19a1a* gene was reported in gonads of Murray rainbowfish *Melanotaenia fluviatilis* exposed to bisphenol A, a weak estrogen (at 100 or 500 µg/L) (Shanthanagouda et al., 2014). In addition, where exposure of Murray rainbowfish to the EDC did not affect *cyp19a1a* expression significantly, 50 µg/L of FAD significantly upregulated its expression in the ovary (Shanthanagouda et al., 2014). Similarly, zebrafish immersed and exposed to 100µg/L FAD for 4 days (Villeneuve et al., 2009), medaka to 10 and 100 µg/L FAD for 7 days (Tompsett et al., 2009), and fathead minnows to 100 µg/L FAD from 24 to 96 h (Villeneuve et al., 2009), also upregulated *cyp19a1a* expression in their

gonads. These studies using low concentrations of FAD, which mimic levels currently seen in environmental pollution, possibly resulted in a compensatory response to the estrogen decrease; whereas, higher doses of aromatase inhibitor applied for sex change purposes possibly block this feedback mechanism. Blocking the natural sex change in barramundi by FAD should be tested for its effect on sperm and subsequent offspring quality, and if positive correlations are evident, there is potential of FAD implants to be used in barramundi hatcheries to maintain sires of good quality for broodstock management.

5.5. Conclusions

In conclusion, the administration of the aromatase inhibitor FAD at 8 mg kg^{-1} was shown to promote pro-male genetic pathways including an over-stimulation of the key male gene *dmrt1* and maintain testicular tissues, while suppressing to some extent the potent feminising effects of E_2 in barramundi.

Chapter 6. General discussion

6.1. Significance and major outcomes

Aquaculture contributes significantly to human food security and is expected to help satisfy the fast-growing global population. Increased aquaculture production relies critically on the application of genetically improved animals. Farming for food fish generally focuses on species possessing desirable characteristics, such as hardiness, high growth rate, low production cost and low maintenance for the culture system. Barramundi is a farmed species that possesses all of these positive attributes and is considered as having the potential to be a global tropical aquaculture species. However, although barramundi aquaculture commenced in the 1970s, whole production is still primarily reliant on unimproved or newly selected farmed stocks (FAO, 2018b).

Selective breeding for barramundi has been challenging partly due to the limited ability to control sex of broodstock and in maintaining a desired sex ratio in breeding animals. Being a protandrous hermaphrodite, barramundi gonads first develop as the male phenotype and then change at a later time to female. Most (if not all) barramundi undergo the male phase before entering the irreversible female sex change at 3-5 years of age. Accordingly, same generation pairings during selective breeding are precluded, resulting in decreases of achievable genetic gains due to increased generation interval effects and markedly decreasing returns on breeding program investment. Additionally, due to the large size of female barramundi (> 6 kg at sex change) and expensive infrastructure required to house large numbers of females, the number of brood-fish able to be held for selective breeding is significantly reduced compared to other commercial finfish. This in turn lowers the genetic diversity contained in a breeding system and reduces the rate of possible genetic gains, necessary to avoid inbreeding and any associated loss of biological fitness (Frost et al., 2006). The lack of ability to control

sex of barramundi also results in difficulties in managing broodstock for hatcheries. As natural sex change occurs, male broodfish of high quality constantly change sex into female requiring the recruitment of new male fish for broodstock. Therefore, development of methods to induce sex change, or maintain sex status, is an effective way to facilitate the implementation of genetic-based improvement programs to elicit sustainable gains in commercially appreciable traits.

Sex control for barramundi is proven as feasible thanks to plasticity in the sex differentiation characteristics of teleosts. The responsiveness of fish gonads subject to phenotypic sex modification can be examined by studying cellular development along with expression of the sex-biased genes in gonadal tissues. The most sensitive period where fish gonads are most susceptible to sex manipulation is termed the labile period. In barramundi, the labile period was determined in *Chapter 2* to occur within the first 120 days and is consistent with that generally observed in other fish. The results from this chapter revealed the changes in cellular number and structure in progenial barramundi gonads with signs of early testicular differentiation that have been documented in other teleosts. These morphological changes in gonadal tissues were preceded with the upregulated expression of the important maleness genes, *dmrt1* and *cyp11b*, at corresponding cellular appearances for male differentiation. The presumptively testicular cells responsive for the *dmrt1* expression were the Sertoli cells, as shown by *in situ* hybridisation with a *dmrt1* mRNA probe. The results from this chapter supplemented our knowledge on how a protandrous fish like barramundi sexually differentiate. Practically, determining the labile period provides the window of time when exogenous steroids would be likely to have the highest influence on the phenotypic sex. Therefore, a major outcome of *Chapter 2* was a recommended timing for administration of hormones to influence phenotypic sex differentiation in barramundi.

Based on the determined sex differentiation timing, *Chapter 3* investigated the effect of the two commonly used estrogens (estradiol 2 – E_2 and ethinylestradiol – EE_2), when incorporated into feed, on the sex differentiation of barramundi during their putative labile period. There is limited information on the use of steroidal hormones to induce sex change in barramundi. It is proven that using hormones for sex control is species-specific so the variables in hormone treatment, such as hormone type, dosage, timing, duration, etc., are required to be obtained empirically (Piferrer, 2001). Therefore, different dosages of E_2 and EE_2 were chosen based on the common range of dietary doses used for other teleosts with consideration for adverse effects on fish health (Piferrer, 2001). For instance, in the case of the protandrous common snook *C. undecimalis*, studies on the use of E_2 at different dosages and routes to induce feminisation were reported (Carvalho et al., 2014; Passini et al., 2016). Specifically, 45-day dietary supplement of E_2 at 50 mg E_2 kg^{-1} feed resulted in 26.3% male, 68.4% female and 5.3% intersexed fish; while 100 mg E_2 kg^{-1} feed induced 90% female and 10% male, without compromising fish growth and survival (Carvalho et al., 2014). Implanting common snook with different dosages of E_2 (0.5, 1.0, 4.0 and 8.0 mg kg^{-1} body weight) resulted in 100% feminised fish with perinucleolar oocytes; while at doses higher than 8 mg kg^{-1} all fishes died and showed liver injury (Passini et al., 2016; Hoga et al., 2018). The results of this chapter showed that precocious feminisation was possible in fish exposed to E_2, while there was no phenotypically feminised effect using EE_2. This outcome suggested that E_2 should be used for barramundi sex control purposes and that future work should focus on optimising the protocol for E_2 administration in applied hatchery settings. The morphological changes, including abnormal gonads detected in hormone treated fish and the expression patterns of sex-related genes, obtained from this chapter provided information on the effects of endocrine disruptor chemicals on sex differentiation of barramundi.

Chapter 4 investigated the possibility of inducing feminisation for barramundi outside their labile period, i.e. on already differentiated males. Additionally, alternative routes of hormone administration were tested to reduce the direct and frequent contact with hormones. The results of this chapter demonstrated that it is possible to induce early sex change on male barramundi by applying a single E_2 implant at 8.0 mg kg^{-1} without detrimental effects on fish health, particularly the liver. The expression patterns of the important male- (*dmrt1*, *cyp11b*, *esr1*) and female- (*cyp19a1a*, *foxl2*) biased genes in the gonads of barramundi exposed to E_2 were also studied throughout the process. Although not all the fish treated with hormone were feminised, the obtained female ratio (77.8%) was acceptable for future selective breeding programs and with further optimisation it may be possible to get close to 100% feminisation. Therefore, the empirically determined hormone treatment of this chapter provides an effective tool for early feminisation of barramundi.

Results from *Chapter 3* and *Chapter 4* confirmed the specificity of exogenous steroidal hormones in determining the gonadal sex of a teleost species, barramundi, and the importance of optimising the variables for hormonal treatment, including type, dosage, route and duration. Moreover, results of *Chapter 3* and *Chapter 4* revealed that feminisation of barramundi by exogenous E_2 is possible at both undifferentiated and differentiated male stages, as well as by different routes, such as oral administration through feed and muscular pellet implanting. Considering the practical applications for barramundi, the dietary supplementation of E_2 is not recommended due to lower success rate and longer exposure of fish to hormone versus implanted hormone pellets and potential harmful effects to the environment and handling labourers. Implanting E_2-cholesterol pellets in the fish provides a feasible and more effective approach as *Chapter 4* showed that feminised fish can be achieved after two month treatment with higher successful rate and lower amount of hormone used. The estradiol 2 dose of 8 mg kg^{-1} was then confirmed in *Chapter 5* with 100% of

feminisation achieved for larger/older barramundi males (2102 ± 126 g BW), which had previously only feminised 77.8% of smaller/younger juveniles (405 ± 50 g BW).

Finally, in order to fully and effectively control the sex status of barramundi *Chapter 5* sought for an artificial method to block the natural sex change process and to retain fish as males. Results from *Chapter* 5 revealed that administrating the aromatase inhibitor fadrozole at dosage of 8 mg kg^{-1} through muscular pellet implants may be a suitable strategy to maintain expression status of male genetic pathways (and in the case of *dmrt1*, upregulate) and the male phenotype without causing adverse effects to fish health as shown through normal kidney and liver histological findings. Moreover, when fadrozole was simultaneously administered with E_2 implants, it was able to some extent antagonise female genetic pathways and partially neutralise the potent E_2 feminising effects on barramundi, thus confirming its potential to inhibiting the male to female sex change process of the species. However effective for maintenance of male sexual status and sex change blocking of barramundi, the cost-benefit on the use of fadrozole should be carefully considered, as fadrozole itself is an expensive substance. Furthermore, the long-term effects of fadrozole capacity in blocking the natural sex change process in barramundi should also be studied.

Overall, this thesis elucidates for the first time important knowledge related to several reproductive biological process in barramundi related to gonadal development, sex differentiation and sex change. Specifically, this thesis provides novel information on the biology of male sex differentiation in barramundi, including the timing and duration of morphological changes at a cellular level within gonads, as well as the concomitant expression levels of key genes associated to gonadal differentiation and maintenance. Being protandrous hermaphrodites, barramundi individuals were previously locked within the natural male sex differentiation pathways and would unpredictably sex-change into females, thus complicating selective breeding schemes. Importantly, this study also developed and

validated useful methods for the barramundi aquaculture industry to be able to accelerate either the sex-change of valuable individual broodstock, or keep them as males, using exogenous endocrine disruptors in a safe manner.

6.2. Future directions

The knowledge reported in this thesis contributes to our understanding of what is considered by the aquaculture industry a very important aspect in barramundi farming, that of sexual differentiation, its plasticity, and the use of methods to manipulate gonadal sexual phenotype. Application of the methods developed and tested experimentally will allow hatchery managers an increased ability to effectively deal with the constraints of sex change that the species poses. However, the ability to sex change fish using exogenous hormones is only the first step required in the commercial hatchery process. If the sex-changed fish are to be used as broodstock they will have to produce viable gametes and undergo reproductive behaviours conducive to spawning. In barramundi, it is not known if the eggs in sex-changed precocious females will mature and be fertile, or if fish will undergo female mating behaviours during mass spawning events, as this was outside the scope of work in this thesis. Within the literature, there are examples of both failure and success in reproduction, sex maintenance and performance of offspring from species that have been sexually reversed. For example, a breeding trial of sex-reversed European sea bass with normal fish was successful and produced viable progeny (Blázquez et al., 1999). On the contrary, a small ratio of E_2-induced female black porgy were reversed back to male fish (Chang and Lin, 1998a). In some cases, fish that were exposed to feminising exogenous hormones and had sex changed did not undergo complete maturation, with oocytes remaining at previtellogenic stages. If this is the case for barramundi, further research into appropriate hormone administration may need to be completed.

From an academic perspective, a more comprehensive understanding on the production of endogenous hormones during administration of estrogenic steroids or the aromatase inhibitor fadrozole would be interesting. This would lead to a better appreciation of how the sex steroid feedback mechanism operates and enable researchers to identify the endogenous hormone levels of barramundi during their gonad development with or without exogenous steroids. Dramatic shifts of steroid levels have been detected during important sexual processes of teleost, including barramundi in other studies (Frisch, 2004; Guiguen et al., 2010; Capel, 2017). Sex change, for instance, accompanied with E_2 increase and 11-ketotestosterone decrease were reported in barramundi (Guiguen et al., 1993) and other protandrous fish such as black porgy (Chang et al., 1994). Opposite patterns of these sex steroids were found during sex change of protogynous fish such as saddleback wrasse *Thalassoma duperrey* (Nakamura et al., 1989), black seabass *Centropristis striatus* (Cochran and Grier, 1991) and groupers (Bhandari et al., 2005; Nakamura et al., 2007). However, due to technical, financial and time limitations identified in the current body of research, *in vivo* hormone levels were not able to be measured.

Similarly, there is increasing evidence that DNA methylation of key sex genes plays an important role in regulating sex differentiation and change in fish and other vertebrates, and that addition of steroidal sex hormones differentially influences patterns of methylation (Navarro-Martín et al., 2011; Penaloza et al., 2014; Shao et al., 2014; Matsumoto et al., 2016; Dong et al., 2019), which wouldn't be surprising for barramundi (Domingos et al., 2018). It would be interesting to assay if addition of estrogen or fadrozole changes DNA methylation status of genes such as *dmrt1* and *cyp19a1a*, or other key genes involving in regulation of the sex-inversion pathway in barramundi.

For the future of barramundi selective breeding, hatchery technicians would benefit from accurate and non-destructive tools to determine the gonadal phenotype of barramundi. The

studies in this thesis used cannulation to assist sex identification, but this approach does not always provide a clear answer without extensive histological preparation and staining of tissues. The cannulated tissues are also not fully representative of the whole gonad, especially if the gonadal differentiation or development shows the gradient pattern between the left/right or caudal/cephalo gonad (Yoshikawa and Oguri, 1979; Strüssmann and Ito, 2005; Sarida et al., 2019). Moreover, cannulation is not a fully non-destructive handling technique and causes stress to fish when they are highly vulnerable.

Finally, understanding the mechanism of sex differentiation and sex change of barramundi would be more comprehensive if a complete gene expression analysis using Next Generation Sequencing could be conducted in the studies of this thesis. Although the expression pattern of the most important sex-biased genes reported from other studies have been selected for RT-qPCR analysis, the possibility of other novel important genes, or their putative splicing variants, involved in sex differentiation can be detected with whole transcriptome sequence techniques like RNAseq. Although small-scale transcriptome sequencing on farmed barramundi was conducted in the study of Ravi et al., (2014), which revealed 32 sexually dimorphic genes, there is no information on global genetic levels of hormonal treated barramundi available. Therefore, further studies may want to employ this tool to provide a complete genetic mechanism of barramundi sex development.

REFERENCES

Aberle, H., Bauer, A., Stappert, J., Kispert, A., Kemler, R., 1997. β-catenin is a target for the ubiquitin-proteasome pathway. EMBO J. 16.

Abinawanto, K., S., Yoshida, K., Saito, N., 1996. Effects of aromatase inhibitor on sex differentiation and levels of P45017α and P450arom messenger ribonucleic acid of gonads in chicken embryos. Gen. Comp. Endocrinol. 102, 241-246.

Afonso, L.O.B., Wassermann, G.J., Terezinha de Oliveira, R., 2001. Sex reversal in Nile tilapia (*Oreochromis niloticus*) using a nonsteroidal aromatase inhibitor. J. Exp. Zool. A Ecol. Genet. Physiol. 290, 177-181.

Afonso, L.O.B., Iwama, G.K., Smith, J., Donaldson, E.M., 1999. Effects of the aromatase inhibitor Fadrozole on plasma sex steroid secretion and ovulation rate in female coho salmon, O*ncorhynchus kisutch*, close to final maturation. Gen. Comp. Endocrinol. 113, 221-229.

Afonso, L.O.B., Iwama, G.K., Smith, J., Donaldson, E.M., 2000. Effects of the aromatase inhibitor Fadrozole on reproductive steroids and spermiation in male coho salmon (*Oncorhynchus kisutch*) during sexual maturation. Aquaculture. 188, 175-187.

Akbary, P., Mirvaghefi, A., Akhlaghi, M., Majazi Amiri, B., 2015. Effects of letrozole, non-steroidal aromatase inhibitor, on serum sex steroid levels, oocyte diameter and gonadosomatic index (GSI) in rainbow trout (*Oncorhynchus mykiss*). Iranian Journal of Fisheries Sciences. 14, 289-301.

Akhavan, S.R., Falahatkar, B., Gilani, M.H.T., Lokman, P.M., 2015. Effects of estradiol-17β implantation on ovarian growth, sex steroid levels and vitellogenin proxies in previtellogenic sturgeon *Huso huso*. Anim. Reprod. Sci. 157, 1-10.

Ankley, G., Kahl, M., Jensen, K., Hornung, M., Korte, J., Makynen, E., Leino, R., 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). Toxicol. Sci. 67, 121-130.

Aparicio, S., Chapman, J., Stupka, E., Putnam, N., Chia, J.-m., Dehal, P., Christoffels, A., Rash, S., Hoon, S., Smit, A., 2002. Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. Science. 297, 1301-1310.

Athauda, S., Anderson, T., de Nys, R., 2012. Effect of rearing water temperature on protandrous sex inversion in cultured Asian Seabass (*Lates calcarifer*). Gen. Comp. Endocrinol. 175, 416-423.

Banh, Q.Q., Domingos, J.A., Zenger, K.R., Jerry, D.R., 2017. Morphological changes and regulation of the genes dmrt1 and cyp11b during the sex differentiation of barramundi (*Lates calcarifer* Bloch). Aquaculture. 479, 75-84.

Baroiller, J.-F., Guiguen, Y., Fostier, A., 1999. Endocrine and environmental aspects of sex differentiation in fish. Cell. Mol. Life Sci. 55, 910-931.

Baroiller, J.F., D'Cotta, H., 2001. Environment and sex determination in farmed fish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 130, 399-409.

Baroiller, J.F., D'Cotta, H., Saillant, E., 2009. Environmental effects on fish sex determination and differentiation. Sex. Dev. 3.

Baron, D., Guiguen, Y., 2003. Gene expression during gonadal sex differentiation in rainbow trout (*Oncorhynchus mykiss*): from candidate genes to high throughout genomic approach. Fish Physiol. Biochem. 28.

Baron, D., Houlgatte, R., Fostier, A., Guiguen, Y., 2008. Expression profiling of candidate genes during ovary-to-testis trans-differentiation in rainbow trout masculinized by androgens. General and Comparative Endocrinology. 156, 369-378.

Baron, D., Cocquet, J., Xia, X., Fellous, M., Guiguen, Y., Veitia, R.A., 2004. An evolutionary and functional analysis of *foxL2* in rainbow trout gonad differentiation. J. Mol. Endocrinol. 33.

Baron, D., Batista, F., Chaffaux, S., Cocquet, J., Cotinot, C., Cribiu, E., De Baere, E., Guiguen, Y., Jaubert, F., Pailhoux, E., Pannetier, M., Vaiman, D., Vigier, B., Veitia, R., Fellous, M., 2005.

Foxl2 gene and the development of the ovary: a story about goat, mouse, fish and woman. Reprod. Nutr. Dev. 45.

Beardmore, J.A., Mair, G.C., Lewis, R.I., 2001. Monosex male production in finfish as exemplified by tilapia: applications, problems, and prospects. Aquaculture. 197, 283-301.

Berbejillo, J., Martinez-Bengochea, A., Bedó, G., Vizziano-Cantonnet, D., 2012. Expression of *dmrt1* and *sox9* during gonadal development in the Siberian sturgeon (*Acipenser baerii*). Fish Physiol. Biochem. 39, 91-94.

Bhandari, R.K., Komuro, H., Higa, M., Nakamura, M., 2004a. Sex inversion of sexually immature honeycomb grouper (*Epinephelus merra*) by aromatase inhibitor. Zool. Sci. 21, 305-310.

Bhandari, R.K., Higa, M., Nakamura, S., Nakamura, M., 2004b. Aromatase inhibitor induces complete sex change in the protogynous honeycomb grouper (*Epinephelus merra*). Mol. Reprod. Dev. 67, 303-307.

Bhandari, R.K., Alam, M.A., Higa, M., Soyano, K., Nakamura, M., 2005. Evidence that estrogen regulates the sex change of honeycomb grouper (*Epinephelus merra*), a protogynous hermaphrodite fish. J Exp Zool A Ecol Genet Physiol. 303A, 497-503.

Billard, R., Breton, B., Richard, M., 1981. On the inhibitory effect of some steroids on spermatogenesis in adult rainbow trout (*Salmo gairdneri)*. Can. J. Zool. 59, 1479-1487.

Bjerregaard, L.B., Lindholst, C., Korsgaard, B., Bjerregaard, P., 2008. Sex hormone concentrations and gonad histology in brown trout (*Salmo trutta*) exposed to 17β-estradiol and bisphenol A. Ecotoxicology. 17, 252-263.

Blázquez, M., Zanuy, S., Carrillo, M., Piferrer, F., 1998. Structural and functional effects of early exposure to estradiol-17β and 17α-ethynylestradiol on the gonads of the gonochoristic teleost *Dicentrarchus labrax*. Fish Physiol. Biochem. 18, 37-47.

Blázquez, M., Carrillo, M., Zanuy, S., Piferrer, F., 1999. Sex ratios in offspring of sex-reversed sea bass and the relationship between growth and phenotypic sex differentiation. J. Fish Biol. 55, 916-930.

Blázquez, M., Piferrer, F., Zanuy, S., Carrillo, M., Donaldson, E.M., 1995. Development of sex control techniques for European sea bass (*Dicentrarchus labrax* L.) aquaculture: effects of dietary 17 α-methyltestosterone prior to sex differentiation. Aquaculture. 135, 329-342.

Blázquez, M., González, A., Papadaki, M., Mylonas, C., Piferrer, F., 2008. Sex-related changes in estrogen receptors and aromatase gene expression and enzymatic activity during early development and sex differentiation in the European sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 158, 95-101.

Brion, F., Tyler, C.R., Palazzi, X., Laillet, B., Porcher, J.M., Garric, J., Flammarion, P., 2004. Impacts of 17β-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*). Aquat. Toxicol. 68, 193-217.

Brunner, B., Hornung, U., Shan, Z., Nanda, I., Kondo, M., Zend-Ajusch, E., Haaf, T., Ropers, H.H., Shima, A., Schmid, M., Kalscheuer, V.M., Schartl, M., 2001. Genomic organization and expression of the doublesex-related gene cluster in vertebrates and detection of putative regulatory regions for *dmrt1*. Genomics. 77.

Budd, A., Banh, Q., Domingos, J., Jerry, D., 2015. Sex control in fish: Approaches, challenges and opportunities for aquaculture. J. Mar. Sci. Eng. 3, 329.

Cabas, I., Chaves-Pozo, E., Alcázar, A.G., Meseguer, J., Mulero, V., García-Ayala, A., 2011. Dietary intake of 17α-ethinylestradiol promotes leukocytes infiltration in the gonad of the hermaphrodite gilthead seabream. Mol. Immunol. 48, 2079-2086.

Capel, B., 2017. Vertebrate sex determination: evolutionary plasticity of a fundamental switch. Nat. Rev. Genet. 18, 675.

Cardwell, J., Liley, N., 1991. Hormonal control of sex and color change in the stoplight parrotfish, *Sparisoma viride*. Gen. Comp. Endocrinol. 81, 7-20.

Caruso, C.C., Breton, T.S., Berlinsky, D.L., 2016. The effects of temperature on ovarian aromatase (*cyp19a1a*) expression and sex differentiation in summer flounder (*Paralichthys dentatus*). Fish Physiol. Biochem. 42, 795-805.

Carvalho, C.V.A.d., Passini, G., Costa, W.d.M., Vieira, B.N., Cerqueira, V.R., 2014. Effect of estradiol-17 on the sex ratio, growth and survival of juvenile common snook (*Centropomus undecimalis*). Acta Scientiarum. Animal Sciences. 36, 239-245.

Chaboissier, M.C., Kobayashi, A., Vidal, V.I.P., Lützkendorf, S., van de Kant, H.J.G., Wegner, M., de Rooij, D.G., Behringer, R.R., Schedl, A., 2004. Functional analysis of Sox8 and Sox9 during sex determination in the mouse. Development. 131.

Chakraborty, T., Shibata, Y., Zhou, L.-Y., Katsu, Y., Iguchi, T., Nagahama, Y., 2011. Differential expression of three estrogen receptor subtype mRNAs in gonads and liver from embryos to adults of the medaka, *Oryzias latipes*. Mol. Cell. Endocrinol. 333, 47-54.

Chang, C.-F., Lee, M.-F., Chen, G.-R., 1994. Estradiol-17β associated with the sex reversal in protandrous black porgy, *Acanthopagrus schlegeli*. J. Exp. Zool. A Ecol. Genet. Physiol. 268, 53-58.

Chang, C.-F., Lau, E.-L., Lin, B.-Y., 1995a. Estradiol-17β suppresses testicular development and stimulates sex reversal in protandrous black porgy, *Acanthopagrus schlegeli*. Fish Physiol. Biochem. 14, 481-488.

Chang, C.F., Lin, B.Y., 1998a. Estradiol-17 beta stimulates aromatase activity and reversible sex change in protandrous black porgy, *Acanthopagrus schlegeli*. J. Exp. Zool. 280, 165-173.

Chang, C.F., Lin, B.Y., 1998b. Estradiol-17 beta stimulates aromatase activity and reversible sex change in protandrous black porgy, *Acanthopagrus schlegeli*. J. Exp. Zool. 280.

Chang, C.F., Lau, E.L., Lin, B.Y., 1995b. Stimulation of spermatogenesis or of sex reversal according to the dose of exogenous estradiol-17 beta in juvenile males of protandrous black porgy, *Acanthopagrus schlegeli*. Gen. Comp. Endocrinol. 100.

Chatain, B., Saillant, E., Peruzzi, S., 1999. Production of monosex male populations of European seabass, *Dicentrarchus labrax* L. by use of the synthetic androgen 17α-methyldehydrotestosterone. Aquaculture. 178, 225-234.

Chaves-Pozo, E., Pelegrín, P., Mulero, V., Meseguer, J., García-Ayala, A., 2003. A role for acidophilic granulocytes in the testis of the gilthead seabream (*Sparus aurata* L., Teleostei). J. Endocrinol. 179.

Chaves-Pozo, E., Liarte, S., Mulero, I., Abellan, E., Meseguer, J., Garcia-Ayala, A., 2009. Early presence of immune cells in the developing gonad of the gilthead seabream (*Sparus aurata* Linnaeus, 1758). J. Reprod. Dev. 55, 440-445.

Chaves-Pozo, E., Liarte, S., Vargas-Chacoff, L., García-Lopez, A., Mulero, V., Meseguer, J., Mancera, J.M., García-Ayala, A., 2007. 17β-estradiol triggers postspawning in spermatogenically active gilthead seabream (*Sparus aurata* L.) males. Biol. Reprod. 76.

Chen, H., Zhang, Y., Li, S., Lin, M., Shi, Y., Sang, Q., Liu, M., Zhang, H., Lu, D., Meng, Z., 2011. Molecular cloning, characterization and expression profiles of three estrogen receptors in protogynous hermaphroditic orange-spotted grouper (*Epinephelus coioides*). Gen. Comp. Endocrinol. 172, 371-381.

Cheshenko, K., Brion, F., Le Page, Y., Hinfray, N., Pakdel, F., Kah, O., Segner, H., Eggen, R.I., 2007. Expression of zebra fish aromatase *cyp19a* and *cyp19b* genes in response to the ligands of estrogen receptor and aryl hydrocarbon receptor. Toxicol. Sci. 96.

Cheskis, B.J., Greger, J.G., Nagpal, S., Freedman, L.P., 2007. Signaling by estrogens. Journal of Cellular Physiology. 213, 610-617.

Chiba, H., Miura, T., Nakamura, M., Yamauchi, K., 1997. Differentiation and development of Leydig cell, and induction of spermatogenesis during testicular differentiation in the Japanese eel, *Anguilla japonica*. Fish Physiol. Biochem. 16, 187-195.

Choi, C., Habibi, H., 2003. Molecular cloning of estrogen receptor α and expression pattern of estrogen receptor subtypes in male and female goldfish. Mol. Cell. Endocrinol. 204, 169-177.

Christiansen, T., Korsgaard, B., Jespersen, A., 1998. Effects of nonylphenol and 17 beta-oestradiol on vitellogenin synthesis, testicular structure and cytology in male eelpout *Zoarces viviparus*. J. Exp. Biol. 201, 179-192.

Cochran, R.C., Grier, H.J., 1991. Regulation of sexual succession in the protogynous black sea bass, Centropristis striatus (Osteichthyes: Serranidae). Gen. Comp. Endocrinol. 82, 69-77.

Condeça, J.B., Canario, A.V., 1999. The effect of estrogen on the gonads and on in vitro conversion of androstenedione to testosterone, 11-ketotestosterone, and estradiol-17beta in *Sparus aurata* (Teleostei, Sparidae). Gen. Comp. Endocrinol. 116.

Coyle, S.D., Durborow, R.M., Tidwell, J.H., 2004. Anesthetics in aquaculture. Southern Regional Aquaculture Center Texas.

Crespo, B., Lan-Chow-Wing, O., Rocha, A., Zanuy, S., Gómez, A., 2013. foxl2 and foxl3 are two ancient paralogs that remain fully functional in teleosts. Gen. Comp. Endocrinol. 194, 81-93.

Crim, L.W., 1985. Methods for acute and chronic hormone administration in fish. in: Lee, C.S., Liao, I.C. (Eds.), Reproduction and culture of milkfish, Hawaii pp. 1-13.

Crim, L.W., Sherwood, N.M., Wilson, C.E., 1988. Sustained hormone release. II. Effectiveness of LHRH analog (LHRHa) administration by either single time injection or cholesterol pellet implantation on plasma gonadotropin levels in a bioassay model fish, the juvenile rainbow trout. Aquaculture. 74, 87-95.

Crisponi, L., Deiana, M., Loi, A., Chiappe, F., Uda, M., Amati, P., Bisceglia, L., Zelante, L., Nagaraja, R., Porcu, S., Ristaldi, M.S., Marzella, R., Rocchi, M., Nicolino, M., Lienhardt-Roussie, A., Nivelon, A., Verloes, A., Schlessinger, D., Gasparini, P., Bonneau, D., Cao, A., Pilia, G., 2001. The putative forkhead transcription factor foxl2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat. Genet. 27.

Cunningham, L., 2005. Assessing the contribution of aquaculture to food security: a survey of methodologies. in: 1010, F.F.C.N. (Ed.). FAO, Rome, pp. 25.

D'Cotta, H., Fostier, A., Guiguen, Y., Govoroun, M., Baroiller, J.-F., 2001. Aromatase plays a key role during normal and temperature-induced sex differentiation of tilapia *Oreochromis niloticus*. Mol. Reprod. Dev. 59, 265-276.

Davis, K.B., Ludwig, G.M., 2004. Hormonal effects on sex differentiation and growth in sunshine bass *Morone chrysops×Morone saxatilis*. Aquaculture. 231, 587-596.

Davis, K.B., Simco, B.A., Goudie, C.A., Parker, N.C., Cauldwell, W., Snellgrove, R., 1990. Hormonal sex manipulation and evidence for female homogamety in channel catfish. Gen. Comp. Endocrinol. 78, 218-223.

Davis, L.K., Pierce, A.L., Hiramatsu, N., Sullivan, C.V., Hirano, T., Grau, E.G., 2008. Gender-specific expression of multiple estrogen receptors, growth hormone receptors, insulin-like growth factors and vitellogenins, and effects of 17β-estradiol in the male tilapia (*Oreochromis mossambicus*). Gen. Comp. Endocrinol. 156, 544-551.

Davis, T.L.O., 1982. Maturity and sexuality in Barramundi, *Lates calcarifer* (Bloch), in the Northern Territory and south-eastern Gulf of Carpentaria. Aust. J. Mar. Freshw. Res. . 33, 529-545.

Davis, T.L.O., 1984. A population of sexually precocious barramundi, *Lates calcarifer*, in the Gulf of Carpentaria, Australia. Copeia. 1984, 144-149.

De Santis, C., Smith-Keune, C., Jerry, D.R., 2011. Normalizing RT-qPCR data: Are we getting the right answers? An appraisal of normalization approaches and internal reference genes from a case study in the finfish *Lates calcarifer*. Mar. Biotechnol. 13, 170-180.

Delalande, C., Goupil, A.-S., Lareyre, J.-J., Gac, F., 2015. Differential expression patterns of three aromatase genes and of four estrogen receptors genes in the testes of trout (*Oncorhynchus mykiss*). Mol. Reprod. Dev. 82.

Deloffre, L.A.M., Martins, R.S.T., Mylonas, C.C., Canario, A.V.M., 2009. Alternative transcripts of dmrt1 in the European sea bass: Expression during gonadal differentiation. Aquaculture. 293, 89-99.

Depiereux, S., Liagre, M., Danis, L., De Meulder, B., Depiereux, E., Segner, H., Kestemont, P., 2014. Intersex occurrence in rainbow trout (*Oncorhynchus mykiss*) male fry chronically exposed to ethynylestradiol. PloS ONE. 9.
Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture. 208, 191-364.
Dietrich, D.R., Krieger, H.O., 2009. Histological analysis of endocrine discruptive effects in small laboratory fish. John Wiley & Sons, Inc., New Jersey, United State of America.
Domingos, J.A., Budd, A.M., Banh, Q.Q., Goldsbury, J.A., Zenger, K.R., Jerry, D.R., 2018. Sex-specific dmrt1 and cyp19a1 methylation and alternative splicing in gonads of the protandrous hermaphrodite barramundi. PLoS ONE. 13.
Donaldson, E.M., Hunter, G.A., 1982. Sex control in fish with particular reference to salmonids. Can. J. Fish Aquat. Sci. 39.
Dong, J., Xiong, L., Ding, H., Jiang, H., Zan, J., Nie, L., 2019. Characterization of DNA Methylation and Transcript Abundance of Sex-related Genes during Temperature-dependent Sex Determination in Mauremys reevesii. Biol. Reprod.
Dorizzi, M., Richard-Mercier, N., Desvages, G., Girondot, M., Pieau, C., 1994. Masculinization of gonads by aromatase inhibitors in a turtle with temperature-dependent sex determination. Differentiation. 58, 1-8.
Dowsett, M., Smithers, D., Moore, J., Trunet, P., Coombes, R., Powles, T., Rubens, R., Smith, I., 1994. Endocrine changes with the aromatase inhibitor fadrozole hydrochloride in breast cancer. Eur. J. Cancer. 30A, 1453-1458.
Eckstein, B., Spira, M., 1965. Effect of sex hormones on gonadal differentiation in a cichlid, *Tilapia aurea*. Biol. Bull. 129, 482-489.
Elbrecht, A., Smith, R.G., 1992. Aromatase enzyme activity and sex determination in chickens. Science. 255.
Falahatkar, B., Poursaeid, S., Meknatkhah, B., Khara, H., Efatpanah, I., 2014. Long-term effects of intraperitoneal injection of estradiol-17β on the growth and physiology of juvenile stellate sturgeon *Acipenser stellatus*. Fish Physiol. Biochem. 40, 365-373.
Falconer, D., Mackay, T., 1996. Introduction to quantitative genetics, 4 ed. Longman, Essex.
Falconer, D.S., 1975. Introduction to quantitative genetics. Pearson Education India.
FAO, 2009. How to feed the world in 2050, FAO Headquarters, Rome.
FAO, 2018a. http://www.fao.org/fishery/culturedspecies/Lates_calcarifer/en#tcNA00EA.
FAO, 2018b. The State of the World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals, Rome.
Fenske, M., Segner, H., 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (*Danio rerio*). Aquat. Toxicol. 67, 105-126.
Ferguson-Smith, M., 2007. The evolution of sex chromosomes and sex determination in vertebrates and the key role of DMRT1. Sex. Dev. 1.
Fernandino, J.I., Hattori, R.S., Shinoda, T., Kimura, H., Strobl-Mazzulla, P.H., Strüssmann, C.A., Somoza, G.M., 2008. Dimorphic expression of *dmrt1* and *cyp19a1* (ovarian aromatase) during early gonadal development in pejerrey, *Odontesthes bonariensis*. Sex. Dev. 2, 316-324.
Filby, A.L., Tyler, C.R., 2007. Cloning and characterization of cDNAs for hormones and/or receptors of growth hormone, insulin-like growth factor-I, thyroid hormone, and corticosteroid and the gender-, tissue-, and developmental-specific expression of their mRNA transcripts in fathead minnow (*Pimephales promelas*). Gen. Comp. Endocrinol. 150, 151-163.
Filby, A.L., Thorpe, K.L., Maack, G., Tyler, C.R., 2007. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. Aquat. Toxicol. 81, 219-231.
Fitzpatrick, M.S., Gale, W.L., Schreck, C.B., 1994. Binding characteristics of an androgen receptor in the ovaries of coho salmon, *Oncorhynchus kisutch*. Gen. Comp. Endocrinol. 95, 399-408.

Frisch, A., 2004. Sex-change and gonadal steroids in sequentially-hermaphroditic teleost fish. Rev. Fish Biol. Fish. 14, 481-499.
Frost, L.A., Evans, B.S., Jerry, D.R., 2006. Loss of genetic diversity due to hatchery culture practices in barramundi (*Lates calcarifer*). Aquaculture. 261, 1056-1064.
George, T., Pandian, T., 1996. Hormonal induction of sex reversal and progeny testing in the zebra cichlid *Cichlasoma nigrofasciatum*. J. Exp. Zool. 275, 374-382.
Gimeno, S., Komen, H., Jobling, S., Sumpter, J., Bowmer, T., 1998. Demasculinisation of sexually mature male common carp, *Cyprinus carpio*, exposed to 4-tert-pentylphenol during spermatogenesis. Aquat. Toxicol. 43, 93-109.
Gjedrem, T., 1997. Selective breeding to improve aquaculture production. World Aquaculture. 28, 33-46.
Gjedrem, T., Robinson, N., Rye, M., 2012. The importance of selective breeding in aquaculture to meet future demands for animal protein: A review. Aquaculture. 350-353, 117-129.
Gjerde, B., 1999. Realized selection differentials for growth rate and early sexual maturity in Atlantic salmon. Aquacult. Eur. 27, 73-74.
Goetz, F.W., Donaldson, E.M., Hunter, G.A., Dye, H.M., 1979. Effects of estradiol-17β and 17α-methyltestosterone on gonadal differentiation in the coho salmon, *Oncorhynchus kisutch*. Aquaculture. 17, 267-278.
Gorshkov, S., Gorshkova, G., Colorni, B., Gordin, H., 2004. Effects of natural estradiol-17β and synthetic 17α- ethynylestradiol on direct feminization of European sea bass *Dicentrarchus labrax*. J. World Aquacult. Soc. 35, 167-177.
Govoroun, M., McMeel, O.M., D'Cotta, H., Ricordel, M.-J., Smith, T., Fostier, A., Guiguen, Y., 2001. Steroid enzyme gene expressions during natural and androgen-induced gonadal differentiation in the rainbow trout, *Oncorhynchus mykiss*. J. Exp. Zool. A Ecol. Genet. Physiol. 290, 558-566.
Govoroun, M.S., Pannetier, M., Pailhoux, E., Cocquet, J., Brillard, J.P., Couty, I., Batellier, F., Cotinot, C., 2004. Isolation of chicken homolog of the *foxl2* gene and comparison of its expression patterns with those of aromatase during ovarian development. Dev. Dyn. 231.
Gray, M.A., Teather, K.L., Metcalfe, C.D., 1999. Reproductive success and behavior of Japanese medaka (*Oryzias latipes*) exposed to 4-tert-octylphenol. Environ. Toxicol. Chem. 18, 2587-2594.
Guan, G., Kobayashi, T., Nagahama, Y., 2000. Sexually dimorphic expression of two types of DM (Doublesex/Mab-3)-domain genes in a teleost fish, the Tilapia (*Oreochromis niloticus*). Biochem. Biophys. Res. Commun. 272.
Guiguen, Y., Jalabert, B., Thouard, E., Fostier, A., 1993. Changes in plasma and gonadal steroid hormones in relation to the reproductive cycle and the sex inversion process in the protandrous seabass, *Lates calcarifer*. Gen. Comp. Endocrinol. 92, 327-338.
Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F., 2010. Ovarian aromatase and estrogens: A pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol. 165.
Guiguen, Y., Cauty, C., Fostier, A., Fuchs, J., Jalabert, B., 1994. Reproductive cycle and sex inversion of the seabass, *Lates calcarifer*, reared in sea cages in French Polynesia: histological and morphometric description. Environ. Biol. Fish. 39.
Guiguen, Y., Baroiller, J.-F., Ricordel, M.-J., Iseki, K., McMeel, O.M., Martin, S.A.M., Fostier, A., 1999. Involvement of estrogens in the process of sex differentiation in two fish species: The rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). Mol. Reprod. Dev. 54, 154-162.
Guo, Y.Q., Cheng, H.H., Huang, X., Gao, S., Yu, H.S., Zhou, R.J., 2005. Gene structure, multiple alternative splicing, and expression in gonads of zebrafish *dmrt1*. Biochem. Biophys. Res. Commun. 330.
Guraya, S.S., 1994. Gonadal development and production of gametes in fish. Proc. Indian Natl. Sci. Acad. B Biol. Sci. 60, 15-15.

Hackmann, E., Reinboth, R., 1974. Delimitation of the critical stage of hormone-influenced sex differentiation in *Hemihaplochromis multicolor* (Hilgendorf) (Cichlidae). Gen. Comp. Endocrinol. 22, 42-53.

Halm, S., Martınez-Rodrıguez, G., Rodrıguez, L., Prat, F., Mylonas, C., Carrillo, M., Zanuy, S., 2004. Cloning, characterisation, and expression of three oestrogen receptors (*ERα*, *ERβ1* and *ERβ2*) in the European sea bass, *Dicentrarchus labrax*. Mol. Cell. Endocrinol. 223, 63-75.

Hamaguchi, S., 1992. Sex differentiation of germ cells and their supporting cells in *Oryzias latipes*. The fish biology journal Medaka-Nagoya University. 4, 11-17.

Hanson, A.M., Kittilson, J.D., McCormick, S.D., Sheridan, M.A., 2012. Effects of 17β-estradiol, 4-nonylphenol, and β-sitosterol on the growth hormone-insulin-like growth factor system and seawater adaptation of rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 362–363, 241-247.

Hardisty, M., 1978. Primordial germ cells and the vertebrate germ line. The Vertebrate Ovary, 1-45.

Hatakeyama, R., Shirafuji, N., Nishimura, D., Kawamura, T., Watanabe, Y., 2005. Gonadal development in early life stages of *Spratelloides gracilis*. Fish. Sci. 71, 1201-1208.

Haugen, T., Almeida, F.F., Andersson, E., Bogerd, J., Male, R., Skaar, K.S., Schulz, R.W., Sørhus, E., Wijgerde, T., Taranger, G.L., 2012. Sex differentiation in Atlantic cod (*Gadus morhua* L.): morphological and gene expression studies. Reprod. Biol. Endocrinol. 10, 1-13.

Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., Thomas, P., 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc. Natl. Acad. Sci. U. S. A. 97, 10751-10756.

He, C.-L., Du, J.-L., Wu, G.-C., Lee, Y.-H., Sun, L.-T., Chang, C.-F., 2003. Differential *dmrt1* transcripts in gonads of the protandrous black porgy, *Acanthopagrus schlegeli*. Cytogenet. Genome Res. 101, 309-313.

Hendry, C.I., Martin-Robichaud, D.J., Benfey, T.J., 2003. Hormonal sex reversal of Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture. 219, 769-781.

Herman, R.L., Kincaid, H.L., 1988. Pathological effects of orally administered estradiol to rainbow trout. Aquaculture. 72, 165-172.

Herpin, A., Schartl, M., 2011. *Dmrt1* genes at the crossroads: a widespread and central class of sexual development factors in fish. FEBS Journal. 278, 1010-1019.

Herpin, A., Adolfi, M.C., Nicol, B., Hinzmann, M., Schmidt, C., Klughammer, J., 2013. Divergent expression regulation of gonad development genes in medaka shows incomplete conservation of the downstream regulatory network of vertebrate sex determination. Mol. Biol. Evol. 30.

Higa, M., Ogasawara, K., Sakaguchi, A., Nagahama, Y., Nakamura, M., 2003. Role of steroid hormones in sex change of protogynous wrasse. Fish Physiol. Biochem. 28, 149-150.

Hill Jr, R.L., Janz, D.M., 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio*): I. Effects on sex ratio and breeding success. Aquat. Toxicol. 63, 417-429.

Hoga, C.A., Almeida, F.L., Reyes, F.G., 2018. A review on the use of hormones in fish farming: Analytical methods to determine their residues. CyTA-Journal of Food. 16, 679-691.

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 496, 498.

Hunter, G.A., Donaldson, E.M., Stoss, J., Baker, I., 1983. Production of monosex female groups of chinook salmon (*Oncorhynchus tshawytscha*) by the fertilization of normal ova with sperm from sex-reversed females. Aquaculture. 33, 355-364.

Hunter, G.A., Solar, I.I., Baker, I.J., Donaldson, E.M., 1986. Feminization of coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha*) by immersion of alevins in a solution of estradiol-17β. Aquaculture. 53, 295-302.

Hur, S.-P., Lim, B.-S., Hwang, I.-J., Kim, S.-J., Ryu, Y.-W., Hur, S.-W., Song, Y.-B., Jeong, H.-B., Baek, H.-J., Takemura, A., Lee, Y.-D., 2012. Masculinization in juvenile longtooth grouper, *Epinephelus*

bruneus, with aromatase inhibitor: changes in GtH subunit mRNA expression and steroids hormone levels. Animal Cells and Systems. 16, 127-134.
Ijiri, S., Kaneko, H., Kobayashi, T., Wang, D.S., Sakai, F., Paul-Prasanth, B., Nakamura, M., Nagahama, Y., 2008. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. Biol. Reprod. 78.
Itahashi, H., Kawase, H., 1973. Normal development of gonad in the mosquitofish, *Gambusia affinis*. Bull. Aichi Uni. Education. 22, 63-71.
Janssen, K., Chavanne, H., Berentsen, P., Komen, H., 2017. Impact of selective breeding on European aquaculture. Aquaculture. 472, 8-16.
Jerry, D.R., 2013. Biology and Culture of Asian Seabass *Lates calcarifer*. CRC Press.
Jiang, W., Yang, Y., Zhao, D., Liu, X., Duan, J., Xie, S., Zhao, H., 2011. Effects of sexual steroids on the expression of foxl2 in *Gobiocypris rarus*. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 160, 187-193.
Jianga, J.-Q., Kobayashi, T., Ge, W., Kobayashi, H., Tanaka, M., Okamoto, M., Nonaka, Y., Nagahama, Y., 1996. Fish testicular 11β-hydroxylase: cDNA cloning and mRNA expression during spermatogenesis. FEBS Lett. 397, 250-252.
Johnsen, H., Tveiten, H., Torgersen, J.S., Andersen, Ø., 2013. Divergent and sex-dimorphic expression of the paralogs of the *Sox9-Amh-Cyp19a1* regulatory cascade in developing and adult atlantic cod (*Gadus morhua* L.). Mol. Reprod. Dev. 80, 358-370.
Johnsen, H., Seppola, M., Torgersen, J.S., Delghandi, M., Andersen, Ø., 2010. Sexually dimorphic expression of dmrt1 in immature and mature Atlantic cod (*Gadus morhua* L.). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 156, 197-205.
Johnstone, R., Simpson, T.H., Walker, A.F., 1979. Sex reversal in salmonid culture: Part III. The production and performance of all-female populations of brook trout. Aquaculture. 18, 241-252.
Kang, I.J., Yokota, H., Oshima, Y., Tsuruda, Y., Yamaguchi, T., Maeda, M., Imada, N., Tadokoro, H., Honjo, T., 2002. Effect of 17β-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). Chemosphere. 47, 71-80.
Kasahara, M., Naruse, K., Sasaki, S., Nakatani, Y., Qu, W., Ahsan, B., Yamada, T., Nagayasu, Y., Doi, K., Kasai, Y., 2007. The medaka draft genome and insights into vertebrate genome evolution. Nature. 447, 714.
Kavumpurath, S., Pandian, T., 1993a. Production of a YY female guppy, Poecilia reticulata, by endocrine sex reversal and progeny testing. Aquaculture. 118, 183-189.
Kavumpurath, S., Pandian, T., 1993b. Determination of labile period and critical dose for sex reversal by oral administration of estrogens in *Betta splendens* (Regan). Indian J. Exp. Biol. . 31, 16-20.
Kawamura, T., Omura, S., Sakai, S., Yamashita, I., 2003. No effects of estrogen receptor overexpression on gonadal sex differentiation and reversal in medaka fish. Zool. Sci. 20, 43-48.
Kazeto, Y., Place, A.R., Trant, J.M., 2004. Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (*Danio rerio*) juveniles. Aquat. Toxicol. 69, 25-34.
Kettlewell, J.R., Raymond, C.S., Zarkower, D., 2000. Temperature-dependent expression of turtle *dmrt1* prior to sexual differentiation. Genesis. 26, 174-178.
Kime, D.E., 1993. 'Classical' and 'non-classical' reproductive steroids in fish. Rev. Fish Biol. Fish. 3, 160-180.
Kitano, T., Takamune, K., Nagahama, Y., Abe, S.-I., 2000. Aromatase inhibitor and 17α-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus)*. Mol. Reprod. Dev. 56, 1-5.
Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., Abe, S.I., 1999. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female

larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J. Mol. Endocrinol. 23.
Klinge, C.M., 2000. Estrogen receptor interaction with co-activators and co-repressors. Steroids. 65, 227-251.
Kobayashi, T., Nagahama, Y., 2009. Molecular aspects of gonadal differentiation in a teleost fish, the Nile tilapia. Sex. Dev. 3, 108-117.
Kobayashi, T., Kajiura-Kobayashi, H., Nagahama, Y., 2003. Induction of XY sex reversal by estrogen involves altered gene expression in a teleost, tilapia. Cytogenet. Genome Res. 101, 289-294.
Kobayashi, T., Kajiura-Kobayashi, H., Guan, G., Nagahama, Y., 2008. Sexual dimorphic expression of *dmrt1* and *Sox9a* during gonadal differentiation and hormone-induced sex reversal in the teleost fish Nile tilapia (*Oreochromis niloticus*). Dev. Dyn. 237, 297-306.
Kobayashi, T., Matsuda, M., Kajiura-Kobayashi, H., Suzuki, A., Saito, N., Nakamoto, M., Shibata, N., Nagahama, Y., 2004. Two DM domain genes, *dmy* and *dmrt1*, involved in testicular differentiation and development in the medaka, *Oryzias latipes*. Dev. Dyn. 231, 518-526.
Kobayashi, Y., Nagahama, Y., Nakamura, M., 2013. Diversity and plasticity of sex determination and differentiation in fishes. Sex. Dev. 7, 115-125.
Kojima, Y., Bhandari, R., Kobayashi, Y., Nakamura, M., 2008. Sex change of adult initial-phase male wrasse, *Halichoeres trimaculatus* by estradiol-17β treatment. Gen. Comp. Endocrinol. 156, 628-632.
Komatsu, T., Nakamura, S., Nakamura, M., 2006. Masculinization of female golden rabbitfish *Siganus guttatus* using an aromatase inhibitor treatment during sex differentiation. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 143, 402-409.
Koopman, P., Loffler, K.A., 2003. Sex determination: the fishy tale of *dmrt1*. Curr. Biol. 13.
Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., Lovellbadge, R., 1991. Male development of chromosomally female mice transgenic for *Sry*. Nature. 351.
Kroon, F.J., Liley, N.R., 2000. The role of steroid hormones in protogynous sex change in the blackeye goby, *Coryphopterus nicholsii* (Teleostei: Gobiidae). Gen. Comp. Endocrinol. . 118, 273-283.
Kusakabe, M., Kobayashi, T., Todo, T., Mark Lokman, P., Nagahama, Y., Young, G., 2002. Molecular cloning and expression during spermatogenesis of a cDNA encoding testicular 11β-hydroxylase (P45011β) in rainbow trout (*Oncorhynchus mykiss*). Mol. Reprod. Dev. 62, 456-469.
Kwon, J.Y., McAndrew, B.J., Penman, D.J., 2001. Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia *Oreochromis niloticus*. Mol. Reprod. Dev. 59, 359-370.
Kwon, J.Y., McAndrew, B.J., Penman, D.J., 2002. Treatment with an aromatase inhibitor suppresses high-temperature feminization of genetic male (YY) Nile tilapia. J. Fish Biol. 60, 625-636.
Kwon, J.Y., Haghpanah, V., Kogson-Hurtado, L.M., McAndrew, B.J., Penman, D.J., 2000. Masculinization of genetic female Nile tilapia (*Oreochromis niloticus*) by dietary administration of an aromatase inhibitor during sexual differentiation. J. Exp. Zool. . 287, 46-53.
Lafont, A.-G., Rousseau, K., Tomkiewicz, J., Dufour, S., 2016. Three nuclear and two membrane estrogen receptors in basal teleosts, *Anguilla* sp.: Identification, evolutionary history and differential expression regulation. Gen. Comp. Endocrinol. 235, 177-191.
Lai, K., Scrimshaw, M., Lester, J., 2002. The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit. Rev. Toxicol. 32, 113-132.
Lamm, M.S., Liu, H., Gemmell, N.J., Godwin, J.R., 2015. The need for speed: neuroendocrine regulation of socially-controlled sex change. Integr. Comp. Biol. 55.
Länge, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P., 2001. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20, 1216-1227.

Lee, C.S., Tamaru, C.S., Kelley, C.D., 1986. Technique for making chronic-release LHRH-a and 17α-methyltestosterone pellets for intramuscular implantation in fishes. Aquaculture. 59, 161-168.

Lee, Y.-H., Yueh, W.-S., Du, J.-L., Sun, L.-T., Chang, C.-F., 2002. Aromatase inhibitors block natural sex change and induce male function in the protandrous black porgy, *Acanthopagrus schlegeli* Bleeker: possible mechanism of natural sex change. Biol. Reprod. 66, 1749-1754.

Lee, Y.-H., Du, J.-L., Yueh, W.-S., Lin, B.-Y., Huang, J.-D., Lee, C.-Y., Lee, M.-F., Lau, E.-L., Lee, F.-Y., Morrey, C., Nagahama, Y., Chang, C.-F., 2001. Sex change in the protandrous black porgy, *Acanthopagrus schlegeli*: A review in gonadal development, estradiol, estrogen receptor, aromatase activity and gonadotropin. J. Exp. Zool. 290, 715-726.

Lee, Y.-M., Seo, J.S., Kim, I.-C., Yoon, Y.-D., Lee, J.-S., 2006. Endocrine disrupting chemicals (bisphenol A, 4-nonylphenol, 4-tert-octylphenol) modulate expression of two distinct cytochrome P450 aromatase genes differently in gender types of the hermaphroditic fish *Rivulus marmoratus*. Biochem. Biophys. Res. Commun. 345, 894-903.

Leet, J.K., Gall, H.E., Sepúlveda, M.S., 2011. A review of studies on androgen and estrogen exposure in fish early life stages: effects on gene and hormonal control of sexual differentiation. J Appl Toxicol. 31, 379-398.

Leonard, S., 1995. In vitro transcription of cRNA from PCR-generated DNA fragments. in: Gobinda, S. (Ed.), Methods in Neurosciences. Academic Press, pp. 243-251.

Lessman, C.A., Habibi, H.R., 1987. Estradiol-17β silastic implants suppress oocyte development in the brook trout, *Salvelinus fontinalis*. Gen. Comp. Endocrinol. 67, 311-323.

Li, G., Liu, X., Lin, H., 2005. Aromatase inhibitor letrozole induces sex inversion in the protogynous red spotted grouper (*Epinephelus akaara*). Acta Physiol. Sinica. 57, 473-479.

Li, M.-H., Yang, H.-H., Li, M.-R., Sun, Y.-L., Jiang, X.-L., Xie, Q.-P., 2013. Antagonistic roles of Dmrt1 and Foxl2 in sex differentiation via estrogen production in tilapia as demonstrated by TALENs. Endocrinol. 154.

Liarte, S., Chaves-Pozo, E., García-Alcazar, A., Mulero, V., Meseguer, J., García-Ayala, A., 2007. Testicular involution prior to sex change in gilthead seabream is characterized by a decrease in *dmrt1* gene expression and by massive leukocyte infiltration. Reprod. Biol. Endocrinol. 5, 1-15.

Lim, L., Wong, C., 1996. Fry production of freshwater ornamental fish in Singapore. in: LeRoy, R. (Ed.), World Aquaculture '96, Bangkok, Thailand, pp. 228.

Lindeman, Robin E., Gearhart, Micah D., Minkina, A., Krentz, Anthony D., Bardwell, Vivian J., Zarkower, D., 2015. Sexual cell-fate reprogramming in the ovary by *dmrt1*. Curr. Biol. 25, 764-771.

Liu, H., Lamm, M.S., Rutherford, K., Black, M.A., Godwin, J.R., Gemmell, N.J., 2015. Large-scale transcriptome sequencing reveals novel expression patterns for key sex-related genes in a sex-changing fish. Biol Sex Differ. 6, 26.

Liu, J.-F., Guiguen, Y., Liu, S.-J., 2008. Aromatase (P450arom) and 11β-hydroxylase (P45011β) genes are differentially expressed during the sex change process of the protogynous rice field eel, *Monopterus albus*. Fish Physiol. Biochem. 35, 511-518.

Liu, S., Govoroun, M., D'Cotta, H., Ricordel, M.-J., Lareyre, J.-J., McMeel, O.M., Smith, T., Nagahama, Y., Guiguen, Y., 2000. Expression of cytochrome P45011β (11β-hydroxylase) gene during gonadal sex differentiation and spermatogenesis in rainbow trout, *Oncorhynchus mykiss*. J. Steroid Biochem. Mol. Biol. 75, 291-298.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 25, 402-408.

Loffler, K.A., Zarkower, D., Koopman, P., 2003. Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome: FOXL2 is a conserved, early-acting gene in vertebrate ovarian development. Endocrinology. 144, 3237-3243.

Luckenbach, J.A., Fairgrieve, W.T., Hayman, E.S., 2017. Establishment of monosex female production of sablefish (*Anoplopoma fimbria*) through direct and indirect sex control. Aquaculture.

Luckenbach, J.A., Borski, R.J., Daniels, H.V., Godwin, J., 2009. Sex determination in flatfishes: Mechanisms and environmental influences. Semin. Cell Dev. Biol. 20.

Luzio, A., Monteiro, S.M., Rocha, E., Fontaínhas-Fernandes, A.A., Coimbra, A.M., 2016. Development and recovery of histopathological alterations in the gonads of zebrafish (*Danio rerio*) after single and combined exposure to endocrine disruptors (17α-ethinylestradiol and fadrozole). Aquat. Toxicol. 175, 90-105.

Luzio, A., Monteiro, S.M., Garcia-Santos, S., Rocha, E., Fontaínhas-Fernandes, A.A., Coimbra, A.M., 2015. Zebrafish sex differentiation and gonad development after exposure to 17α-ethinylestradiol, fadrozole and their binary mixture: A stereological study. Aquat. Toxicol. 166, 83-95.

Lynn, S.G., Birge, W.J., Shepherd, B.S., 2008. Molecular characterization and sex-specific tissue expression of estrogen receptor α (esr1), estrogen receptor βa (esr2a) and ovarian aromatase (cyp19a1a) in yellow perch (Perca flavescens). Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 149, 126-147.

Maack, G., Segner, H., Tyler, C., 2003. Ontogeny of sexual differentiation in different strains of zebrafish (*Danio rerio*). Fish Physiol. Biochem. 28, 125-128.

Malison, J.A., Best, C.D., Kayes, T.B., Amundson, C.H., Wentworth, B.C., 1985. Hormonal growth promotion and evidence for a size-related difference in response to estradiol-17β in yellow perch (*Perca flavescens*). Can. J. Fish. Aquat. Sci. 42, 1627-1633.

Manousaki, T., Tsakogiannis, A., Lagnel, J., Sarropoulou, E., Xiang, J.Z., Papandroulakis, N., 2014. The sex-specific transcriptome of the hermaphrodite sparid sharpsnout seabream (*Diplodus puntazzo*). BMC Genomics. 15.

Marchand, O., Govoroun, M., D'Cotta, H., McMeel, O., Lareyre, J.-J., Bernot, A., Laudet, V., Guiguen, Y., 2000. *Dmrt1* expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. Biochim. Biophys. Acta. 1493, 180-187.

Masuyama, H., Yamada, M., Kamei, Y., Fujiwara-Ishikawa, T., Todo, T., Nagahama, Y., Matsuda, M., 2012. *Dmrt1* mutation causes a male-to-female sex reversal after the sex determination by *Dmy* in the medaka. Chromosome Res. 20, 163-176.

Matson, C.K., Zarkower, D., 2012. Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. Nat. Rev. Genet. 13.

Matson, C.K., Murphy, M.W., Sarver, A.L., Griswold, M.D., Bardwell, V.J., Zarkower, D., 2011. *Dmrt1* prevents female reprogramming in the postnatal mammalian testis. Nature. 476, 101-104.

Matsuda, M., 2003. Sex determination in fish: Lessons from the sex-determining gene of the teleost medaka, *Oryzias latipes*. Dev. Growth Differ. 45, 397-403.

Matsuda, M., 2005. Sex determination in the teleost medaka, *Oryzias latipes*. Annu. Rev. Genet. 39.

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C.E., Shibata, N., Asakawa, S., Shimizu, N., 2002. *Dmy* is a Y-specific DM-domain gene required for male development in the medaka fish. Nature. 417.

Matsumoto, Y., Hannigan, B., Crews, D., 2016. Temperature shift alters DNA methylation and histone modification patterns in gonadal aromatase (cyp19a1) gene in species with temperature-dependent sex determination. PloS one. 11, e0167362.

Meijide, F.J., Nostro, F.L.L., Guerrero, G.A., 2005. Gonadal development and sex differentiation in the cichlid fish Cichlasoma dimerus (*Teleostei, perciformes*): A light- and electron-microscopic study. J. Morphol. 264, 191-210.

Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F., 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol. Reprod. 66, 1881-1892.

Micael, J., Reis-Henriques, M., Carvalho, A., Santos, M., 2007. Genotoxic effects of binary mixtures of xenoandrogens (tributyltin, triphenyltin) and a xenoestrogen (ethinylestradiol) in a partial life-cycle test with zebrafish (*Danio rerio*). Environ. Int. 33, 1035-1039.

Miller, W.R., Bartlett, J., Brodie, A.M.H., Brueggemeier, R.W., di Salle, E., Lønning, P.E., Llombart, A., Maass, N., Maudelonde, T., Sasano, H., Goss, P.E., 2008. Aromatase inhibitors: Are there differences between steroidal and nonsteroidal aromatase inhibitors and do they matter? The Oncologist. 13, 829-837.

Miura, S., Nakamura, S., Kobayashi, Y., Piferrer, F., Nakamura, M., 2008. Differentiation of ambisexual gonads and immunohistochemical localization of P450 cholesterol side-chain cleavage enzyme during gonadal sex differentiation in the protandrous anemonefish, *Amphiprion clarkii*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 149, 29-37.

Moore, R., 1979. Natural sex inversion in the giant perch (*Lates calcarifer*). Austr. J. Mar. Freshw. Res. 30, 803-813.

Moudgal, N.R., Shetty, G., Selvaraj, N., Bhatnagar, A.S., 1996. Use of a specific aromatase inhibitor for determining whether there is a role for oestrogen in follicle/oocyte maturation, ovulation and preimplantation embryo development. J. Reprod. Fertil. Suppl. 50, 69-81.

Murdock, C., Wibbels, T., 2006. *Dmrt1* expression in response to estrogen treatment in a reptile with temperature-dependent sex determination. J. Exp. Zool. B Mol. Dev. Evol. 306.

Nagahama, Y., Kagawa, H., Young, G., 1982. Cellular sources of sex steroids in teleost gonads. Can. J. Fish. Aquat. Sci. 39, 56-64.

Nagler, J.J., Krisfalusi, M., Cyr, D.G., 2000. Quantification of rainbow trout (*Oncorhynchus mykiss*) estrogen receptor-alpha messenger RNA and its expression in the ovary during the reproductive cycle. J. Mol. Endocrinol. 25, 243-251.

Nagler, J.J., Cavileer, T., Sullivan, J., Cyr, D.G., Rexroad III, C., 2007. The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel *ERα2* and both *ERβ* isoforms. Gene. 392, 164-173.

Nakamoto, M., Matsuda, M., Wang, D.S., Nagahama, Y., Shibata, N., 2006. Molecular cloning and analysis of gonadal expression of *foxl2* in the medaka, *Oryzias latipes*. Biochem. Biophys. Res. Commun. 344.

Nakamura, M., 1974. Sex differentiation of the gonad in the masu salmon (*Oncorhynchus masou*). Sci. Rep. Hokkaido Salmon Hatchery. 28, 1-8.

Nakamura, M., 1975. Dosage-dependent changes in the effect of oral administration of methyltestosterone on gonadal sex differentiation in *Tilapia mossambica*. Bull. Faculty Fish., Hokkaido University. 26, 99-108.

Nakamura, M., 1982. Gonadal sex differentiation in whitespotted char, *Salvelinus leucomaenis*. Japanese Journal of Ichthyology (Japan). 28, 431-436.

Nakamura, M., 1984. Effects of estradiol-17β on gonadal sex differentiation in two species of salmonids, the masu salmon, *Oncorhynchus masou*, and the chum salmon, *O. keta*. Aquaculture. 43, 83-90.

Nakamura, M., 2013. Morphological and physiological studies on gonadal sex differentiation in teleost fish. Aqua-BioScience Monographs. 6, 1-47.

Nakamura, M., Takahashi, H., 1973. Gonadal sex differentiation in *Tilapia mossambica*, with special regard to the time of estrogen treatment effective in inducing complete feminization of genetic males. Bull. Faculty Fish., Hokkaido University. 24.

Nakamura, M., Nagahama, Y., 1985. Steroid producing cells during ovarian differentiation of the tilapia, *Sarotherodon niloticus*. Dev. Growth Differ. 27, 701-708.

Nakamura, M., Nagahama, Y., 1989. Differentiation and development of Leydig cells, and changes of testosterone levels during testicular differentiation in tilapia *Oreochromis niloticus*. Fish Physiol. Biochem. 7, 211-219.

Nakamura, M., Nagahama, Y., 1993. Ultrastructural study on the differentiation and development of steroid-producing cells during ovarian differentiation in the amago salmon, *Oncorhynchus rhodurus*. Aquaculture. 112, 237-251.

Nakamura, M., Bhandari, R.K., Higa, M., 2003. The role estrogens play in sex differentiation and sex changes of fish. Fish Physiol. Biochem. 28, 113-117.

Nakamura, M., Kobayashi, T., Chang, X.T., Nagahama, Y., 1998. Gonadal sex differentiation in teleost fish. J. Exp. Zool. A Ecol. Genet. Physiol. 281.

Nakamura, M., Alam, M.A., Kobayashi, Y., Bhandari, R.K., 2007. Role of sex hormones in sex change of grouper. J. Mar. Sci. Technol., 23-27.

Nakamura, M., Hourigan, T.F., Yamauchi, K., Nagahama, Y., Grau, E.G., 1989. Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse Thalassoma duperrey. Environ. Biol. Fish. 24, 117-136.

Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., Schmid, M., Schartl, M., 2002. A duplicated copy of *dmrt1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. Proc. Natl. Acad. Sci. USA. 99, 11778-11783.

Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Croce, L., 2011. DNA methylation of the gonadal aromatase (cyp19a) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. PLoS Genet. 7.

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15-24.

Nelson, J.S., Grande, T.C., Wilson, M.V., 2016. Fishes of the World, fifth ed. John Wiley & Sons.

Ng, T.B., Idler, D.R., 1983. Yolk Formation and Differentiation in Teleost Fishes. in: Hoar, W.S., Randall, D.J., Donaldson, E.M. (Eds.), Fish physiology. Academic Press, New York, pp. 373-404.

Nilsson, S., Mäkelä, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., Gustafsson, J.-Å., 2001. Mechanisms of estrogen action. Physiol. Rev. 81, 1535-1565.

Norris, D.O., 1987. Regulation of male gonaducts and sex accessory structures, Hormones and Reproduction in Fishes, Amphibians, and Reptiles. Springer, pp. 327-354.

Nozu, R., Kojima, Y., Nakamura, M., 2009. Short term treatment with aromatase inhibitor induces sex change in the protogynous wrasse, Halichoeres trimaculatus. Gen. Comp. Endocrinol. 161, 360-364.

Olito, C., Brock, I., 1991. Technical notes: Sex reversal of rainbow trout: Creating an all-female population. The Progressive Fish-Culturist. 53, 41-44.

Oropesa, A.L., Jiménez, B., Gil, M.C., Osswald, J., Fallola, C., Pula, H.J., Cuesta, J.M., Gómez, L., 2014. Histological alterations in the structure of the testis in tench (*Tinca tinca*) after exposure to 17 alpha-ethynylestradiol. Environ.Toxicol. 29, 1182-1192.

Oshima, Y., Uno, Y., Matsuda, Y., Kobayashi, T., Nakamura, M., 2008. Molecular cloning and gene expression of *foxl2* in the frog *Rana rugosa*. Gen. Comp. Endocrinol. 159.

Osterburg, H.H., Allen, J.K., Finch, C.E., 1975. The use of ammonium acetate in the precipitation of ribonucleic acid. Biochem. J. 147, 367-368.

Pandian, T.J., Sheela, S.G., 1995. Hormonal induction of sex reversal in fish. Aquaculture. 138, 1-22.

Pandian, T.J., Kirankumar, S., 2003. Recent advances in hormonal induction of sex-reversal in fish. J. Appl. Aquaculture. 13, 205-230.

Pannetier, M., Renault, L., Jolivet, G., Cotinot, C., Pailhoux, E., 2005. Ovarian-specific expression of a new gene regulated by the goat PIS region and transcribed by a FOXL2 bidirectional promoter. Genomics. 85, 715-726.

Pannetier, M., Fabre, S., Batista, F., Kocer, A., Renault, L., Jolivet, G., Mandon-Pepin, B., Cotinot, C., Veitia, R., Pailhoux, E., 2006. FOXL2 activates P450 aromatase gene transcription: towards a

better characterization of the early steps of mammalian ovarian development. J. Mol. Endocrinol. 36.
Panter, G., Thompson, R., Sumpter, J., 1998. Adverse reproductive effects in male fathead minnows (Pimephales promelas) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. Aquat. Toxicol. 42, 243-253.
Panter, G.H., Hutchinson, T.H., Hurd, K.S., Bamforth, J., Stanley, R.D., Duffell, S., Hargreaves, A., Gimeno, S., Tyler, C.R., 2006. Development of chronic tests for endocrine active chemicals: Part 1. An extended fish early-life stage test for oestrogenic active chemicals in the fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 77, 279-290.
Papadaki, M., Piferrer, F., Zanuy, S., Maingot, E., Divanach, P., Mylonas, C.C., 2005. Growth, sex differentiation and gonad and plasma levels of sex steroids in male- and female-dominant populations of *Dicentrarchus labrax* obtained through repeated size grading. J. Fish Biol. 66, 938-956.
Passini, G., Carvalho, C.V.A., Sterzelecki, F.C., Cerqueira, V.R., 2016. Induction of sex inversion in common snook (*Centropomus undecimalis*) males, using 17-β oestradiol implants. Aquac. Res. 47, 1090-1099.
Patiño, R., Davis, K.B., Schoore, J.E., Uguz, C., Strüssmann, C.A., Parker, N.C., Simco, B.A., Goudie, C.A., 1996. Sex differentiation of channel catfish gonads: normal development and effects of temperature. J. Exp. Zool. 276, 209-218.
Paul-Prasanth, B., Nakamura, M., Nagahama, Y., 2011. Sex determination in fishes, Hormones and reproduction of vertebrates. Elsevier, pp. 1-14.
Paul-Prasanth, B., Bhandari, R., Kobayashi, T., Horiguchi, R., Kobayashi, Y., Nakamoto, M., Shibata, Y., Sakai, F., Nakamura, M., Nagahama, Y., 2013. Estrogen oversees the maintenance of the female genetic program in terminally differentiated gonochorists. Sci. Rep. . 3, 2862.
Penaloza, C.G., Estevez, B., Han, D.M., Norouzi, M., Lockshin, R.A., Zakeri, Z., 2014. Sex-dependent regulation of cytochrome P450 family members Cyp1a1, Cyp2e1, and Cyp7b1 by methylation of DNA. The FASEB Journal. 28, 966-977.
Pérez, M.R., Fernandino, J.I., Carriquiriborde, P., Somoza, G.M., 2012. Feminization and altered gonadal gene expression profile by ethinylestradiol exposure to pejerrey, *Odontesthes bonariensis*, a South American teleost fish. Environ. Toxicol. Chem. 31, 941-946.
Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture. 197, 229-281.
Piferrer, F., 2011. Hormonal control of reproduction and growth - endocrine control of sex differentiation in Fish in: Farrell, A.P. (Ed.), Encyclopedia of Fish Physiology: From Genome to Environment. Academic Press, San Diego, pp. 1490-1499.
Piferrer, F., Donaldson, E.M., 1989. Gonadal differentiation in coho salmon, *Oncorhynchus kisutch*, after a single treatment with androgen or estrogen at different stages during ontogenesis. Aquaculture. 77, 251-262.
Piferrer, F., Donaldson, E.M., 1992. The comparative effectiveness of the natural and a synthetic estrogen for the direct feminization of chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture. 106, 183-193.
Piferrer, F., Guiguen, Y., 2008. Fish Gonadogenesis. Part II: Molecular Biology and Genomics of Sex Differentiation. Reviews in Fisheries Science. 16, 35-55.
Piferrer, F., Baker, I.J., Donaldson, E.M., 1993. Effects of natural, synthetic, aromatizable, and nonaromatizable androgens in inducing male sex differentiation in genotypic female chinook salmon (*Oncorhynchus tshawytscha*). Gen. Comp. Endocrinol. 91, 59-65.
Piferrer, F., Benfey, T.J., Donaldson, E.M., 1994a. Production of female triploid coho salmon (*Oncorhynchus kisutch*) by pressure shock and direct estrogen treatment. Aquat. Living Resour. 7, 127-131.

Piferrer, F., Blázquez, M., Navarro, L., González, A., 2005. Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). Gen. Comp. Endocrinol. 142.

Piferrer, F., Zanuy, S., Carrillo, M., Solar, I.I., Devlin, R.H., Donaldson, E.M., 1994b. Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males. J. Exp. Zool. 270, 255-262.

Pinto, P.I., Passos, A.L., Martins, R.S., Power, D.M., Canário, A.V., 2006. Characterization of estrogen receptor βb in sea bream (Sparus auratus): phylogeny, ligand-binding, and comparative analysis of expression. Gen. Comp. Endocrinol. 145, 197-207.

Pongthana, N., Penman, D.J., Baoprasertkul, P., Hussain, M.G., Shahidul Islam, M., Powell, S.F., McAndrew, B.J., 1999. Monosex female production in the silver barb (*Puntius gonionotus* Bleeker). Aquaculture. 173, 247-256.

Quillet, E., Labbe, L., Queau, I., 2004. Asymmetry in sexual development of gonads in intersex rainbow trout. J. Fish Biol. 64, 1147-1151.

Raghuveer, K., Garhwal, R., Wang, D.S., Bogerd, J., Kirubagaran, R., Rasheeda, M.K., Sreenivasulu, G., Bhattachrya, N., Tarangini, S., Nagahama, Y., Senthilkumaran, B., 2005. Effect of methyl testosterone- and ethynyl estradiol-induced sex differentiation on catfish, *Clarias gariepinus:* expression profiles of *dmrt1,* Cytochrome P450aromatases and 3 β-hydroxysteroid dehydrogenase. Fish Physiol. Biochem. 31, 143.

Rasmussen, T.H., Jesperesen, Å., Korsgaard, B., 2006. Gonadal morphogenesis and sex differentiation in intraovarian embryos of the viviparous fish *Zoarces viviparous* (Teleostei, Perciformes, Zoarcidae): A histological and ultrastructural study. J. Morphol. 267.

Ravi, P., Jiang, J., Liew, W.C., Orbán, L., 2014. Small-scale transcriptomics reveals differences among gonadal stages in Asian seabass (*Lates calcarifer*). Reprod. Biol. Endocrinol. 12, 1-14.

Raymond, C.S., Kettlewell, J.R., Hirsch, B., Bardwell, V.J., Zarkower, D., 1999. Expression of *dmrt1* in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. Dev. Biol. 215.

Raymond, C.S., Shamu, C.E., Shen, M.M., Seifert, K.J., Hirsch, B., Hodgkin, J., Zarkower, D., 1998. Evidence for evolutionary conservation of sex-determining genes. Nature. 391, 691-695.

Reyhanian Caspillo, N., Volkova, K., Hallgren, S., Olsson, P.-E., Porsch-Hällström, I., 2014. Short-term treatment of adult male zebrafish (*Danio Rerio*) with 17α-ethinyl estradiol affects the transcription of genes involved in development and male sex differentiation. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 164, 35-42.

Richard-Mercier, N., Dorizzi, M., Desvages, G., Girondot, M., Pieau, C., 1995. Endocrine sex reversal of gonads by the aromatase inhibitor letrozole (CGS 20267) in *Emys orbicularis,* a turtle with temperature-dependent sex determination. Gen. Comp. Endocrinol. 100, 314-326.

Rinchard, J., Dabrowski, K., Garcia–Abiado, M.A., Ottobre, J., 1999. Uptake and depletion of plasma 17α-methyltestosterone during induction of masculinization in muskellunge, *Esox masquinongy:* Effect on plasma steroids and sex reversal. Steroids. 64, 518-525.

Roberts Jr, D.E., Schlieder, R.A., 1983. Induced sex inversion, maturation, spawning and embryogeny of the protogynous grouper, *Mycteroperca microlepis*. J. World Maricult. Soc. 14, 637-649.

Robinson, N.A., Jerry, D.R., 2009. Development of a genetic management and improvement strategy for Australian cultured barramundi, Final Report to the Australian Seafood CRC, Project No. 2008/758, Flinders University, pp. 58.

Robinson, N.A., Schipp, G., Bosmans, J., Jerry, D.R., 2010. Modelling selective breeding in protandrous, batch-reared Asian sea bass (Lates calcarifer, Bloch) using walkback selection. Aquaculture research. 41, e643-e655.

Ruan, H., Wu, G., Huang, R., 1996. Induced sex reversal of black sea bream (*Sparus macrocephalus*). Stud. Mar. Sin, 151-161.

Sahafi, H.H., khaje Rahimi, A., Askari, F., Bashti, T., Zargham, D., 2011. Dietary effects of 17-β estradiol on sex reversal of rainbow trout (*Oncorhynchus mykiss*) in early larval stage. Adv. Environ. Biol., 2100-2107.

Santi, S., Gennotte, V., Toguyeni, A., Mélard, C., Antoine, N., Rougeot, C., 2016. Thermosensitivity of the sex differentiation process in the African catfish, *Clarias gariepinus*: Determination of the thermosensitive period. Aquaculture. 455, 73-80.

Santos, M., Micael, J., Carvalho, A., Morabito, R., Booy, P., Massanisso, P., Lamoree, M., Reis-Henriques, M., 2006. Estrogens counteract the masculinizing effect of tributyltin in zebrafish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 142, 151-155.

Sarida, M., Hattori, R.S., Zhang, Y., Yamamoto, Y., Strüssmann, C.A., 2019. Spatiotemporal Correlations between amh and cyp19a1a Transcript Expression and Apoptosis during Gonadal Sex Differentiation of Pejerrey, Odontesthes bonariensis. Sexual Development. 13, 99-108.

Sarter, K., Papadaki, M., Zanuy, S., Mylonas, C.C., 2006. Permanent sex inversion in 1-year-old juveniles of the protogynous dusky grouper (*Epinephelus marginatus*) using controlled-release 17α-methyltestosterone implants. Aquaculture. 256, 443-456.

Schäfers, C., Teigeler, M., Wenzel, A., Maack, G., Fenske, M., Segner, H., 2007. Concentration- and time-dependent effects of the synthetic estrogen, 17α-ethinylestradiol, on reproductive capabilities of the zebrafish, *Danio rerio*. J. Toxicol. Environ. Health A. 70, 768-779.

Schipp, G., Bosmans, J., Humphrey, J., 2007. Barramundi farming handbook. in: Schipp, G. (Ed.), Department of Primary Industry, Fisheries and Mines, Northern Territory Government, Northern Territory Government.

Schmidt, D., Ovitt, C.E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treier, A.-C., Treier, M., 2004. The murine winged-helix transcription factor *foxl2* is required for granulosa cell differentiation and ovary maintenance. Development. 131.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods. 9, 671-675.

Scholz, S., Gutzeit, H.O., 2000. 17-α-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias latipes*). Aquat. Toxicol. 50, 363-373.

Schulz, R.W., Bogerd, J., Male, R., Ball, J., Fenske, M., Olsen, L.C., Tyler, C.R., 2007. Estrogen-induced alterations in amh and *dmrt1* expression signal for disruption in male sexual development in the zebrafish. Environ. Sci. Technol. Lett. 41, 6305-6310.

Seki, M., Yokota, H., Matsubara, H., Tsuruda, Y., Maeda, M., Tadokoro, H., Kobayashi, K., 2002. Effect of ethinylestradiol on the reproduction and induction of vitellogenin and testis-ova in medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 21, 1692-1698.

Shanthanagouda, A., Nugegoda, D., Patil, J., 2014. Effects of bisphenol A and fadrozole exposures on cyp19a1 expression in the Murray rainbowfish, *Melanotaenia fluviatilis*. Arch. Environ. Con. Toxicol. 67, 270-280.

Shao, C., Li, Q., Chen, S., Zhang, P., Lian, J., Hu, Q., Sun, B., Jin, L., Liu, S., Wang, Z., 2014. Epigenetic modification and inheritance in sexual reversal of fish. Genome Research. 24, 604-615.

Shen, Z.-G., Wang, H.-P., 2014. Molecular players involved in temperature-dependent sex determination and sex differentiation in Teleost fish. Genet. Sel. Evol. 46, 1-21.

Sherwood, N.M., Crim, L., Carolsfeld, J., Walters, S.M., 1988. Sustained hormone release. I. Characteristics of in vitro release of gonadotropin-releasing hormone analogue (GnRH-A) from pellets. Aquaculture. 74, 75-86.

Shetty, G., Bhatnagar, A.S., Moudgal, N.R., 1995. Blockade of estrogen synthesis with an aromatase inhibitor affects luteal function of the pseudopregnant rat. J. Steroid Biochem. Mol. Biol. 55, 347-353.

Shibata, K., Takase, M., Nakamura, M., 2002. The *dmrt1* expression in sex-reversed gonads of amphibians. Gen. Comp. Endocrinol. 127, 232-241.

Shved, N., Berishvili, G., D'Cotta, H., Baroiller, J.-F., Segner, H., Eppler, E., Reinecke, M., 2007. Ethinylestradiol differentially interferes with IGF-I in liver and extrahepatic sites during development of male and female bony fish. J. Endocrinol. 195, 513-523.

Slanchev, K., Stebler, J., de la Cueva-Méndez, G., Raz, E., 2005. Development without germ cells: the role of the germ line in zebrafish sex differentiation. Proc. Nat. Acad. Sci. 102, 4074-4079.

Smith, C.A., Sinclair, A.H., 2004. Sex determination: insights from the chicken. Bioessays. 26.

Smith, C.A., McClive, P.J., Western, P.S., Reed, K.J., Sinclair, A.H., 1999a. Evolution: Conservation of a sex-determining gene. Nature. 402, 601-602.

Smith, C.A., McClive, P.J., Western, P.S., Reed, K.J., Sinclair, A.H., 1999b. Conservation of a sex-determining gene. Nature. 402.

Smith, C.A., Roeszler, K.N., Ohnesorg, T., Cummins, D.M., Farlie, P.G., Doran, T.J., Sinclair, A.H., 2009. The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature. 461.

Smith, E.K., Guzmán, J.M., Luckenbach, J.A., 2013. Molecular cloning, characterization, and sexually dimorphic expression of five major sex differentiation-related genes in a Scorpaeniform fish, sablefish (*Anoplopoma fimbria*). Comp. Biochem. Physiol. B Biochem Mol. Biol. 165, 125-137.

Socorro, S., Power, D., Olsson, P., Canario, A., 2000. Two estrogen receptors expressed in the teleost fish, *Sparus aurata*: cDNA cloning, characterization and tissue distribution. J. Endocrinol. 166, 293-306.

Socorro, S., Martins, R.S., Deloffre, L., Mylonas, C.C., Canario, A.V.M., 2007. A cDNA for European sea bass (*Dicentrarchus labrax*) 11β-hydroxylase: Gene expression during the thermosensitive period and gonadogenesis. Gen. Comp. Endocrinol. 150, 164-173.

Solar, I., Donaldson, E., 1991. A comparison of the economic aspects of monosex chinook salmon production versus mixed sex stocks for aquaculture. Bull. Aquacul. Assoc. Can. 91, 28-30.

Sridevi, P., Senthilkumaran, B., 2011. Cloning and differential expression of *foxl2* during ovarian development and recrudescence of the catfish, *Clarias gariepinus*. Gen. Comp. Endocrinol. 174.

Sridevi, P., Chaitanya, R.K., Dutta-Gupta, A., Senthilkumaran, B., 2012. FTZ-F1 and FOXL2 up-regulate catfish brain aromatase gene transcription by specific binding to the promoter motifs. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms. 1819, 57-66.

Steele, R.E., Mellor, L.B., Sawyer, W.K., Wasvary, J.M., Browne, L.J., 1987. In vitro and in vivo studies demonstrating potent and selective estrogen inhibition with the nonsteroidal aromatase inhibitor CGS 16949A. Steroids. 50, 147-161.

Strüssmann, C.A., Nakamura, M., 2002. Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. Fish Physiol. Biochem. 26, 13-29.

Strüssmann, C.A., Ito, L.S., 2005. Where does gonadal sex differentiation begin? Gradient of histological sex differentiation in the gonads of pejerrey, *Odontesthes bonariensis* (Pisces, Atherinidae). J. Morphol. 265, 190-196.

Strüssmann, C.A., Calsina Cota, J.C., Phonlor, G., Higuchi, H., Takashima, F., 1996. Temperature effects on sex differentiation of two South American atherinids, *Odontesthes argentinensis* and *Patagonina hatcheri*. Environ. Biol. Fish. . 47, 143-154.

Sudhakumari, C.C., Kobayashi, T., Kajiura-Kobayashi, H., Wang, D.S., Yoshikuni, M., Nagahama, Y., Senthilkumaran, B., 2005. Ontogenic expression patterns of several nuclear receptors and cytochrome P450 aromatases in brain and gonads of the Nile tilapia *Oreochromis niloticus* suggests their involvement in sex differentiation. Fish Physiol. Biochem. 31.

Sun, P., You, F., Liu, M., Wu, Z., Wen, A., Li, J., Xu, Y., Zhang, P., 2010. Steroid sex hormone dynamics during estradiol-17β induced gonadal differentiation in *Paralichthys olivaceus* (Teleostei). Chin. J. Oceanol. Limnol. 28, 254-259.

Svingen, T., Koopman, P., 2013. Building the mammalian testis: origins, differentiation, and assembly of the component cell populations. Genes Dev. 27, 2409-2426.

Szentes, K., Mészáros, E., Szabó, T., Csorbai, B., Borbély, G., Bernáth, G., Urbányi, B., Horváth, Á., 2012. Gonad development and gametogenesis in the Asian sea bass (*Lates calcarifer*) grown in an intensive aquaculture system. J. Appl. Ichthyol. 28, 883-885.

Takahashi, H., 1977. Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*. Bull. Fac. Fish. Hokkaido Univ. 28.

Takatsu, K., Miyaoku, K., Roy, S.R., Murono, Y., Sago, T., Itagaki, H., Nakamura, M., Tokumoto, T., 2013. Induction of female-to-male sex change in adult zebrafish by aromatase inhibitor treatment. Sci. Rep. 3, 3400.

Tan-Fermin, J.D., 1992. Withdrawal of exogenous 17-alpha methyl-testosterone causes reversal of sex-inversed male grouper *Epinephelus suillus* (Bloch and Schneider). Philipp. Sci. 29, 33-39.

Tao, W., Yuan, J., Zhou, L., Sun, L., Sun, Y., Yang, S., 2013. Characterization of gonadal transcriptomes from Nile tilapia (*Oreochromis niloticus*) reveals differentially expressed genes. PLoS ONE. 8.

Todd, E.V., Liu, H., Muncaster, S., Gemmell, N.J., 2016. Bending genders: The biology of natural sex change in fish. Sex. Dev. 10, 223-241.

Tompsett, A., Park, J., Zhang, X., Jones, P., Newsted, J., Au, D., Chen, E., Yu, R., Wu, R., Kong, R., 2009. In situ hybridization to detect spatial gene expression in medaka. Ecotoxicol. Environ. Safety. 72, 1257-1264.

Trudeau, V.L., Wade, M.G., Kraak, G.V.D., Peter, R.E., 1993. Effects of 17β-estradiol on pituitary and testicular function in male goldfish. Can. J. Zool. 71, 1131-1135.

Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2002. Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. J. Exp. Biol. 205.

Uda, M., Ottolenghi, C., Crisponi, L., Garcia, J.E., Deiana, M., Kimber, W., Forabosco, A., Cao, A., Schlessinger, D., Pilia, G., 2004. *Foxl2* disruption causes mouse ovarian failure by pervasive blockage of follicle development. Hum. Mol. Genet. 13.

Uhlenhaut, N.H., Jakob, S., Anlag, K., Eisenberger, T., Sekido, R., Kress, J., Treier, A.-C., Klugmann, C., Klasen, C., Holter, N.I., 2009. Somatic sex reprogramming of adult ovaries to testes by *foxl*2 ablation. Cell. 139, 1130-1142.

Urushitani, H., Shimizu, A., Katsu, Y., Iguchi, T., 2002. Early estrogen exposure induces abnormal development of Fundulus heteroclitus. J. Exp. Zool. . 293, 693-702.

Van den Belt, K., Berckmans, P., Vangenechten, C., Verheyen, R., Witters, H., 2004. Comparative study on the in vitro/in vivo estrogenic potencies of 17β-estradiol, estrone, 17α-ethynylestradiol and nonylphenol. Aquat. Toxicol. 66, 183-195.

Van Den Hurk, R., Richter, C.J.J., Janssen-Dommerholt, J., 1989. Effects of 17α-methyltestosterone and 11β-hydroxyandrostenedione on gonad differentiation in the African catfish, *Clarias gariepinus*. Aquaculture. 83, 179-191.

Veith, A.-M., Froschauer, A., Körting, C., Nanda, I., Hanel, R., Schmid, M., Schartl, M., Volff, J.-N., 2003. Cloning of the dmrt1 gene of *Xiphophorus maculatus*: *dmY/dmrt1Y* is not the master sex-determining gene in the platyfish. Gene. 317, 59-66.

Villeneuve, D.L., Knoebl, I., Kahl, M.D., Jensen, K.M., Hammermeister, D.E., Greene, K.J., Blake, L.S., Ankley, G.T., 2006. Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 76, 353-368.

Villeneuve, D.L., Wang, R.L., Bencic, D.C., Biales, A.D., Martinović, D., Lazorchak, J.M., Toth, G., Ankley, G.T., 2009. Altered gene expression in the brain and ovaries of zebrafish (*Danio rerio*) exposed to the aromatase inhibitor fadrozole: microarray analysis and hypothesis generation. Environ. Toxicol. Chem. 28, 1767-1782.

Vizziano-Cantonnet, D., Baron, D., Mahè, S., Cauty, C., Fostier, A., Guiguen, Y., 2008. Estrogen treatment up-regulates female genes but does not suppress all early testicular markers during rainbow trout male-to-female gonadal transdifferentiation. J. Mol. Endocrinol. 41, 277-288.

Vizziano, D., Randuineau, G., Baron, D., Cauty, C., Guiguen, Y., 2007. Characterization of early molecular sex differentiation in rainbow trout, *Oncorhynchus mykiss*. Dev. Dyn. 236, 2198-2206.

Wang, D.s., Kobayashi, T., Zhou, L.y., Nagahama, Y., 2004. Molecular cloning and gene expression of f*oxl2* in the Nile tilapia, *Oreochromis niloticus*. Biochem. Biophys. Res. Commun. 320, 83-89.

Wang, D.S., Zhou, L.Y., Kobayashi, T., Matsuda, M., Shibata, Y., Sakai, F., Nagahama, Y., 2010. Doublesex- and Mab-3-related transcription factor-1 repression of aromatase transcription, a possible mechanism favoring the male pathway in tilapia. Endocrinology. 151.

Wang, D.S., Kobayashi, T., Zhou, L.Y., Paul-Prasanth, B., Ijiri, S., Sakai, F., Okubo, K., Morohashi, K., Nagahama, Y., 2007. *Foxl2* up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. Mol. Endocrinol. 21.

Wang, W., Li, A., Cai, T., Wang, J., 2005. Effects of intraperitoneal injection of cortisol on non-specific immune functions of *Ctenopharyngodon idella*. J. Fish Biol. 67, 779-793.

Weber, L.P., Hill Jr, R.L., Janz, D.M., 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio*): II. Histological evaluation of gametogenesis and organ toxicity. Aquat. Toxicol. 63, 431-446.

Weber, L.P., Balch, G.C., Metcalfe, C.D., Janz, D.M., 2004. Increased kidney, liver, and testicular cell death after chronic exposure to 17α-ethinylestradiol in medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 23, 792-797.

Webster, K.A., Schach, U., Ordaz, A., Steinfeld, J.S., Draper, B.W., Siegfried, K.R., 2017. *Dmrt1* is necessary for male sexual development in zebrafish. Dev. Biol. 422, 33-46.

Wen, A.Y., You, F., Sun, P., Li, J., Xu, D.D., Wu, Z.H., Ma, D.Y., Zhang, P.J., 2014. CpG methylation of *dmrt1* and *cyp19a* promoters in relation to their sexual dimorphic expression in the Japanese flounder *Paralichthys olivaceus*. J. Fish Biol. 84, 193-205.

Wennstrom, K.A., Crews, D., 1995. Making males from females: the effects of aromatase inhibitors on a Parthenogenetic species of whiptail lizard. Gen. Comp. Endocrinol. 99, 316-322.

Wu, G.-C., Tomy, S., Nakamura, M., Chang, C.-F., 2008. Dual roles of *cyp19a1a* in gonadal sex differentiation and development in the protandrous black porgy, *Acanthopagrus schlegeli*. Biol. Reprod. 79, 1111-1120.

Wu, G.-C., Chiu, P.-C., Lin, C.-J., Lyu, Y.-S., Lan, D.-S., Chang, C.-F., 2012. Testicular *dmrt1* is involved in the sexual fate of the ovotestis in the protandrous black porgy. Biol. Reprod. 86, 41.

Wu, G.-C., Tomy, S., Lee, M.-F., Lee, Y.-H., Yueh, W.-S., Lin, C.-J., Lau, E.-L., Chang, C.-F., 2010. Sex differentiation and sex change in the protandrous black porgy, *Acanthopagrus schlegeli*. Gen. Comp. Endocrinol. 167, 417-421.

Xia, W., Zhou, L., Yao, B., Li, C.-J., Gui, J.-F., 2007. Differential and spermatogenic cell-specific expression of *dmrt1* during sex reversal in protogynous hermaphroditic groupers. Mol. Cell. Endocrinol. 263, 156-172.

Yamada, H., Satoh, R.-i., Yamashita, T., Kambegawa, A., Iwata, M., 1997. Development of a time-resolved fluoroimmunoassay (TR-FIA) for testosterone: measurement of serum testosterone concentrations after testosterone treatment in the rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 106, 181-188.

Yamaguchi, T., Yamaguchi, S., Hirai, T., Kitano, T., 2007. Follicle-stimulating hormone signaling and *foxl2* are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem. Biophys. Res. Commun. 359.

Yamamoto, T.-O., 1969. Sex Differentiation. in: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology. Academic Press, New York, pp. 117-175.

Yao, H.H., 2005. The pathway to femaleness: current knowledge on embryonic development of the ovary. Mol. Cell. Endocrinol. 230.

Yoshikawa, H., Oguri, M., 1979. Gonadal sex differentiation in the medaka, *Oryzias latipes,* with special regard to the gradient of the differentiation of testes. Bulletin of the Japanese Society of Scientific Fisheries. 45, 1115-1121.

Yoshiura, Y., Senthilkumaran, B., Watanabe, M., Oba, Y., Kobayashi, T., Nagahama, Y., 2003. Synergistic expression of Ad4BP/SF-1 and cytochrome P-450 aromatase (ovarian type) in the ovary of Nile tilapia, *Oreochromis niloticus*, during vitellogenesis suggests transcriptional interaction. Biol. Reprod. 68, 1545-1553.

Yuan, H.W., Xu, Q.Q., Gong, S.Y., Yuan, Y.C., Chu, Z.J., Yang, D.Q., 2011. Effects of different exogenous estradiol contents on steroid hormones, GSI, survival rate and sex reversal in the Asian swamp eel. Adv. Mat. Res. 382, 481-485.

Zaroogian, G., Gardner, G., Borsay Horowitz, D., Gutjahr-Gobell, R., Haebler, R., Mills, L., 2001. Effect of 17β-estradiol, o,p'-DDT, octylphenol and p,p'-DDE on gonadal development and liver and kidney pathology in juvenile male summer flounder (*Paralichthys dentatus*). Aquat. Toxicol. 54, 101-112.

Zeder, M.A., Smith, B.D., 2009. A conversation on agricultural origins: talking past each other in a crowded room. Curr. Anthropol. 50, 681-691.

Zha, J., Wang, Z., Wang, N., Ingersoll, C., 2007. Histological alternation and vitellogenin induction in adult rare minnow (*Gobiocypris rarus*) after exposure to ethynylestradiol and nonylphenol. Chemosphere. 66, 488-495.

Zhang, W.-L., Zhou, L.-Y., Senthilkumaran, B., Huang, B.-F., Sudhakumari, C.C., Kobayashi, T., Nagahama, Y., Wang, D.-S., 2010a. Molecular cloning of two isoforms of 11β-hydroxylase and their expressions in the Nile tilapia, *Oreochromis niloticus*. Gen. Comp. Endocrinol. 165, 34-41.

Zhang, W., Yang, Y., Peng, Y., Zhang, S., Zhang, Y., Wu, C., Zhang, L., 2010b. Differential synergism of *Ftz-f1* homologues and *foxl2* on the activation of *cyp19a1a* gene from rice field eel *Monopterus albus*, a protogynous hermaphroditic teleost. Biol. Reprod. 83.