Banh, Quyen Q.T., Domingos, Jose A., Pinto, Ricardo C.C., Nguyen, Kien T., and Jerry, Dean R. (2021) ***Dietary 17 β-oestradiol and 17 α-ethinyloestradiol alter gonadal morphology and gene expression of the two sex-related genes, dmrt1 and cyp19a1a, in juvenile barramundi (Lates calcarifer Bloch).*** **Aquaculture Research, 52 (4) pp. 1414-1430.**

https://doi.org/10.1111/are.14996

Title: 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 (*Lates calcarifer* Bloch)

Quyen Q. T. Banh[*,1,4], Jose A. Domingos[1,3], Ricardo C. C. Pinto[2], Kien T. Nguyen[1], Dean R. Jerry[1,3]

[1]Centre for Sustainable Tropical Fisheries and Aquaculture, College of Science and Engineering, James Cook University, Townsville, 4811, Queensland, Australia

[2]Federal University of Ceará, Marine Science Institute, Labomar, Avenida Abolição, 3207, Meireles 60165-081 - Fortaleza, Ceará State, Brazil

[3]Current address: Tropical Futures Institute, James Cook University Singapore, 149 Sims Drive, 387380, Singapore

[4]Current address: Institute of Aquaculture, Nha Trang University, 2 Nguyen Dinh Chieu, Nha Trang, Vietnam

[*]Correspondence: Quyen Q. T. Banh, Centre for Sustainable Tropical Fisheries and Aquaculture, College of Science and Engineering, James Cook University, 1 Angus Smith Dr, Douglas, QLD 4811, Australia

Tel: +61 7 4781 5585

Fax: +61 7 4781 4585

Email: thiquyenquyen.banh@my.jcu.edu.au

Co-author email:

Jose A. Domingos: jose.domingos1@jcu.edu.au

Ricardo C. C. Pinto: ricardoccpinto@gmail.com

Kien T. Nguyen: trungkien.nguyen@my.jcu.edu.au

Dean R. Jerry: dean.jerry@jcu.edu.au

Abstract

This study investigated the effect of 17 β-estradiol (E_2) and 17 α-ethinylestradiol (EE_2) on the feminization of barramundi (*Lates calcarifer*). 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 days post-hatch (dph), which covers the period of initial male gonad differentiation. The effect of E_2 and EE_2 treatment on gonadal morphology and *dmrt1* and *cyp19a1a* gene expression was analyzed by histology and RT-qPCR.

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} 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} treatment did not induce feminization, 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 feminization of barramundi, and when delivered through the feed it has a dose-dependent feminizing effect.

Keywords: gene expression; sex reversal; Asian seabass; estradiol 2; feminization

1. INTRODUCTION

Barramundi *Lates calcarifer* 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 & Jerry, 2009). Mature females are 90-120 cm in length (10-20 kg) (Davis, 1982; Robinson & Jerry, 2009) requiring large-volume tank facilities.

Moreover, as the rate of genetic progress in breeding programs is factored by generation interval (Falconer & Mackay, 1996), only half the potential genetic gain can be made as males and females of the same age (generation) cannot be mated (Robinson & Jerry, 2009). Developing methods to precociously feminize male barramundi and, therefore, produce both sexes simultaneously would be beneficial for selective breeding of barramundi.

Induced feminization 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 & 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 (Budd et al., 2015; Piferrer, 2001; Strüssmann & Nakamura, 2002). One of the most common and effective estrogenic hormones for inducing feminization is the natural estrogen compound estradiol 2 (E_2) or 17 β-estradiol (Nakamura et al., 2003). E_2 is known to play a key role in growth, promotion of gonadal development and hepatic vitellogenin production (Christiansen et al., 1998; Klinge, 2000; Ng & Idler, 1983; Nilsson et al., 2001). The synthetic type of estradiol 2, namely ethinylestradiol (or EE_2), is also used for hormone treatment in aquaculture breeding programs due to its two- to three-fold potency in feminizing fish when compared to E_2 (Blázquez et al., 1998; Piferrer, 2001; Yamamoto, 1969). Both E_2 and EE_2 have been documented as effective hormone treatments for feminizing teleosts such as coho salmon *Oncorhynchus kisutch* (Goetz et al., 1979; Piferrer et al., 1994), common snook *Centropomus undecimalis* (Carvalho et al., 2014; Passini et al., 2016), black porgy *Acanthopagrus schlegelii* (Chang et al., 1995; Chang & Lin, 1998), and sablefish *Anoplopoma fimbria* (Luckenbach et al., 2017).

Masculinization or feminization of the undifferentiated gonad, as influenced by hormones, is determined by the balance between a masculinizing or feminizing molecular pathways (Capel, 2017; Todd et al., 2016). One of

the key male-supporting genes, *dmrt1* (doublesex- and mab-3-related transcription factor 1), plays an important role in the testicular development and differentiation of many fish species (Guan et al., 2000; Guo et al., 2005; Johnsen et al., 2010; Kettlewell et al., 2000; Masuyama et al., 2012; Shen & Wang, 2014; Smith et al., 1999). *Dmrt1* expression is significantly higher in male pejerrey *Odontesthes bonariensis* (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 (days post hatch), 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 (Domingos et al., 2018; Ravi et al., 2014). In an antagonistic fashion to *dmrt1*, the gene *cyp19a1* (cytochrome P450 aromatase) 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). *Cyp19a1* is responsible for the provision of all endogenous estrogens by controlling steroidogenic conversion of androgens and, as a result, is regarded as a key determinant for ovarian differentiation and development (Kitano et al., 1999; Kobayashi et al., 2003; Piferrer & Guiguen, 2008; Smith et al., 2013). Fishes have two gene copies for this enzyme, *cyp19a1a* and *cyp19a1b*, which are usually highly expressed in the gonad or in the brain, respectively (Böhne et al., 2013; Piferrer & Blázquez, 2005). *Cyp19a1a* is upregulated in ovarian tissues of various teleost species (Blázquez et al., 2008; Caruso et al., 2016; Ijiri et al., 2008; Kwon et al., 2001; Wen et al., 2014; Wu et al., 2008) 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 feminization. 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 real-time qPCR at 160 dph and 12 mph.

2. MATERIALS AND METHODS

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).

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, 6000 fish of average 9.1 ± 0.4 mm total length (TL) were distributed into fifteen 40 L tanks (i.e. 400 individuals per unit). From 80 dph 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 β-estradiol (E_2; Sigma-Aldrich, E8875) at 10 mg ($10E_2$) or 20 mg kg^{-1} ($20E_2$), or ethinylestradiol (EE_2; Sigma-Aldrich, E4876) at 5 mg ($5EE_2$) or 10 mg kg^{-1} ($10EE_2$). The hormone dosage for feminisation is highly species-specific and as this was the first effort to orally deliver estrogen to barramundi dosage ranges were chosen based on those applied to other fish species and as generalized by Piferrer (2001). Due to the hormone duration treatment being 130 days and with fish health in mind doses of estrogen were therefore chosen to be at a maximum of 20 mg kg^{-1} feed. EE_2 is more potent than E_2, hence, EE_2 dosages were 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 months post hatch (mph).

Fish were reared in recirculated seawater (30-35 ppt) at 28-30 ^{o}C, with a stable 12 h light:12 h dark photoperiod (Schipp et al., 2007). Feed rations varied depending on the age and body weight (BW) of juveniles (e.g. age 30-90 dph fed 7% BW, 90 – 140 dph fed 5% BW, and 140 dph onwards fed 3% BW). Water quality parameters were checked daily and maintained within suitable limits for fish development (DO > 5 mg/L, TAN < 1 mg/L; NO_2^- < 2 mg/L; pH 7.8-8.0). 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 17 β-Estradiol (E_2, quantitative analysis ranges from 0.05 μg/L to1 μg/L) and Ethynylestradiol (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.

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 euthanized 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 millimeter 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 histology as

described in 2.3. Gonad tissues were sampled for gene expression analyses (as described in 2.4) 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.

2.3. Histological analyses

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. In brief, the tissues were dehydrated through a series of graded ethanol steps, cleared in xylene and impregnated in paraffin Histosec® pastilles (Merck Millipore, Germany). After processing, tissues were embedded in paraffin, transverse sectioned at a 5-µm thickness and mounted on slides. After dewaxing and rehydration, the sections were stained with hematoxylin-eosin. Approximately 20-40 slides were obtained from each sample to cover at least three parts (anterior, middle and posterior) of both left and right gonads. The slides were examined using an Olympus CelSens Microscope Digital Camera System (Olympus, Japan). Gonad surface areas were measured using Thresholding in the ImageJ software.

Testicular development during spermatogenesis was categorized 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.

2.4. Reverse transcription (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™ 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.

2.4.1. RNA extraction, DNAse treatment and cDNA synthesis

RNA extraction, DNAse treatment and cDNA synthesis were conducted as described previously (Banh et al., 2017). Briefly, total RNA was extracted from approximately 0.1 g of barramundi gonadal tissue using Trizol® RNA Isolation Reagents (Thermo Fisher Scientific, USA) following the instruction of the manufacturer. The RNA yield of all samples were measured with a NanoDrop 1800 spectrophotometer (Nanodrop Technologies, Wilmington, USA). For DNAse treatment, 4 µg RNA extracts were processed for each sample using a TURBO DNA-free™ kit (Invitrogen™, USA) according to the manufacturer's protocol. DNAse-treated RNA was then treated with an ammonium acetate precipitation protocol (Osterburg et al., 1975) for cleaning. The yield and purity of all RNA extracts were monitored with the NanoDrop 1800. The A260/A280 values of the samples that were used for further analysis ranged from 1.89 to 2.03. Integrity of RNA extracts were detected by electrophoresis on 1.5% agarose gel (in 1x TBE made with DEPC treated water) with GelGreen™ (Biotium Inc, USA). Only RNA extracts that showed no smear and two clear bands (28S:18S) were included for subsequent cDNA synthesis.

cDNA was synthesized using a Tetro cDNA synthesis kit (Bioline, USA). In short, 2 µg of DNAse-treated RNA was put in a RNAse-free 200 µL tube before adding 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 volume of 20 µL. Residual DNA within DNAse treated RNA samples were not present in any samples confirmed by performing the no amplification control (NAC) reactions with an aliquot of DNAse-treated RNA diluted to the same concentration as the RNA used in the real cDNA syntheses without reverse transcriptase. All vials (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

°C for 10 min, followed by 45 °C for 30 min before a final termination cycle by incubating at 85 °C for 5 min. The cDNA was then stored at -20 °C until RT-qPCR.

2.4.2. Quantitative reverse transcription PCR (RT-qPCR)

RT-qPCR was routinely optimized 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; Domingos et al., 2018; Ravi et al., 2014) (Table 1). RT-qPCR was performed in triplicate 15 µL reactions (7.5 µL of SsoFast™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). Reactions were conducted by an automatic pipetting system (Corbett Robotics, Qiagen, Germany) into a 100-well ring (Qiagen) and run on a Corbett Rotor-Gene 6000 thermocycler (Qiagen). Cycle conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 58 °C (*dmrt1* and *cyp19a1a*) or 61 °C (*ubq*) for 10 s, followed by a melt curve analysis (65 °C to 95 °C in 0.5 °C increments) for monitoring target specificity. RT-qPCR efficiencies (E) for each gene were validated using standard curves prepared from five points of 3-fold serially diluted cDNA (Table 1).

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 normalize possible variability between runs. RT-qPCR product specificity for each gene was confirmed by analysis of melting curves, visualization of the size of products on a 1.5% agarose gel, and Sanger sequencing (Australian Genome Research Facility).

2.5.Statistical analyses

Statistical analyses were conducted using the SPSS software package (IBM SPSS Statistics 23, USA). A mean C_T value was obtained for each RT-qPCR sample triplicate. The relative abundance of the target genes (*dmrt1* and *cyp19a1a*) were normalized using the reference gene *ubq* according to the $2^{-\Delta Ct}$ method of Livak and

Schmittgen (2001). The stability of the reference gene for normalization was examined using GeNorm (Primer Design, Ltd., UK). Normality and homogeneity of variance were tested using the Kolmogorov-Smirnov and Levene tests, respectively. Normalized 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 examine differences among hormone treatments on fish growth (body weight and length). Data that could not be normalized, or that presented heterogeneous variances after transformation were analyzed 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. RESULTS

3.1. Growth performance and survival

The effects of oral administration of E_2 and EE_2 on the growth performance (body weight and total length) and survival of barramundi are shown in Figure 1. Treatment commenced at 30 dph at body weight 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 caused poor 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.

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

Morphological changes in barramundi gonads induced by dietary E_2 and EE_2 are summarized in Table 2. 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. 2A). 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. 2B); 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 the 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 elongations of the cell aggregations fused together to form the ovarian cavity (Fig. 2D). 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. 2C). 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. 2E) 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. 2F), 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 enlarged proliferation of fibroblasts as a result of increased deposition of collagen leading to an appearance of thickened connective tissue (Dietrich & Krieger, 2009; Gray et al., 1999).

At 100 dph, lobular structures containing strips of primary germ cells (PGCs) were observed in control fish (Fig. 2G). 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 organization, consisting of multiple lobular structures containing large cysts of undifferentiated germ cells surrounded by dense connective tissues (Fig. 2H). Ten fish of the $20E_2$ treatment (83%) had a developed ovarian cavity in the middle of the gonad, representing feminization of gonads (Fig. 2J). 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. 2I). 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.2I and 2J). 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. 2K). 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. 2L). Some normal germ cells also existed and were surrounded by fibrotic connective tissue.

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. 3A). 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. 3B). In 50% (6/12) of fish, a few spermatozoa were detected. 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. 3I). Oogonia are typically recognized 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. 3H). 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. 3H).

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. 3J and 3M). 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 organized lumen (Fig. 3N). In the enlarged tubular posterior region, testes were devoid of spermatogonia and spermatocytes. Only advanced testicular germ cells (spermatids and spermatozoa) were present within enlarged lobules (Fig. 3L and 3P). Gonads of the remaining fish (33%) of the $10EE_2$ treatment displayed complete fibrosis along the gonad without any presence of germinal cells.

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. 4A and 4B). 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. 4D). 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. 4F). 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 and atresic oogonia (Fig. 4G). 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. 4L). 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. 4I and 4J).

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 treatments at all samplings. In the sampled liver of these groups, liver sections showed uniform hepatocytes with distinct nuclei and nucleoli, abundance of cytoplasmic lipid and vacuolization. These signs are typical of healthy farmed fish. However, liver sections in both EE_2 treatments showed some hyaline material as a sign of damaged health. Compared to other treatments, vitellogenin level was putatively low in the 10EE2 as evidenced by a reduction in protein stained (hematoxylin-eosin stains vitellogenin pink-red). 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. 4 C, E, H, K and N, respectively.

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. 5). The results of Sanger sequencing on the RT-qPCR products of *dmrt1* and *cyp19a1a* were aligned and confirmed there correct mapping 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 ($p < 0.05$).

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 & Jerry, 2009). Sex control for aquaculture species, particularly the success of feminizing fish through the application of exogenous estrogens, shows diverse results depending on fish species, hormone used, treatment timing and duration (Budd et al., 2015; Piferrer, 2001). These treatment variables generally need to be determined empirically for each species as often different responses to hormonal manipulation are 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 feminizing 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 kg^{-1} 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 feminized effects such as the presence of an ovarian cavity and/or oocytes (Blázquez et al., 1998; Brion et al., 2004; Filby et al., 2007; Nakamura & Takahashi, 1973; Piferrer & Donaldson, 1989). 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 on barramundi gonads reflecting the divergence in estrogenic potencies and toxicity of these two steroids, being stronger for EE_2 than E_2 (Blázquez et al., 1998; Piferrer & Donaldson, 1992). In the studies testing both E_2 and EE_2 on feminization of fish, EE_2 has generally been shown to be more efficient. For example, in chinook salmon *Oncorhynchus tshawytscha* EE_2 produced feminizing effects when fish were exposed to shorter treatment durations than E_2 (Piferrer & Donaldson, 1992), or at lower hormone concentrations in the guppy *Poecilia reticulate* (Kavumpurath & Pandian, 1993). In European seabass, *Dicentrarchus labrax*, EE_2 resulted in a higher feminizing rate than E_2 when using the same concentration and duration treatment (Blázquez et al., 1998). Contrarily, in the present study, feminized fish were only evident in the fish fed 20 mg E_2 kg^{-1} of feed; fish in the control and other treatments, including the fish fed 10 mg E_2 kg^{-1} of feed and both EE_2 treatments, did not exhibit significant evidence of feminization. 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. Higher toxicity of EE_2, which may be species-specific to barramundi, induced abnormal gonad development with suppressed sex-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, acellularity (absence of cells), and delayed spermatogenesis. Germ cell apoptosis in estrogen exposed fish was reported in medaka *Oryzias latipes* (Weber et al., 2004) and fathead minnows *Pimephales promelas* (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; Oropesa et al., 2014; Weber et al., 2003). 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; Panter et al., 2006; Weber et al., 2003). 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 *Danio rerio* (Schäfers et al., 2007; Weber et al., 2003). 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-characterized 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 as a result 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, *Sparus aurata*, E_2 and EE_2 promoted an inflammatory process as indicated by the appearance of immune cells in the exposed gonads (Cabas et al., 2011; Chaves-Pozo et al., 2007). 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 (Condeça & Canario, 1999; Davis & Ludwig, 2004; Hendry et al., 2003; Johnstone et al., 1979; Kojima et al., 2008). Interestingly, feeding sea bream (~40 g BW) with a diet containing 15 EE_2 mg kg^{-1} resulted in poor appetite and growth; however, replacing the treatment with E_2 at the same dosage did not reduce appetite and growth (Condeça & Canario, 1999).

Investigation of the molecular effects of estrogen exposure in rainbow trout, *Oncorhynchus mykiss*, 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 & 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 reduced survival and/or growth rate (Donaldson & Hunter, 1982; Hu et al., 2017; Piferrer & 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 feed for two moths (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*. On the other hand, 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 *cyp19a1*, respectively (Fernandino et al., 2008; Pérez et al., 2012). Exposure of the sexually undifferentiated juvenile catfish *Clarias gariepinus* to exogenous 17α-methyltestosterone, an aromatizable hormone which can convert to some extent to methylestradiol, repressed *dmrt1* despite up-regulated *cyp19a1* expression (Raghuveer et al., 2005). Dietary fadrozole, an aromatase inhibitor, induced 100% male Nile tilapia *Oreochromis niloticus* and upregulated the gene *cyp19a1* and downregulated *dmrt1* (Kobayashi et al., 2003; Kobayashi et al., 2008; Kobayashi & Nagahama, 2009). Likewise, estrogen treatment induced the down-regulation of *dmrt1* of zebrafish (Reyhanian Caspillo et al., 2014; Schulz et al., 2007), fathead minnow (Filby et al., 2007), and rainbow trout (Marchand et al., 2000). The findings in the present

435 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
440 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 shown 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
445 sexually maturing fathead minnows of mixed sex to EE_2 at 10 ng per liter resulted in upregulated expression of gonadal aromatase (Filby et al., 2007; Scholz & Gutzeit, 2000). Similar expreesion pattern were recoreded when feeding Japanese flounder females with 1µg E_2 g^{-1} diet upregulating *cyp19a1a* expression; while antagonizing estrogen resulted in masculinization by downregulating *cyp19a1a* mRNA transcripts (Kitano et al., 2007). Aromatase was detectable in testis of XY males zebrafish exposed to 10 ng L^{-1} EE_2 (Scholz &
450 Gutzeit, 2000). Contrarily, exposure to 100 nmol L^{-1} EE_2 for a 3-day duration downregulated the transcription of c*yp19a1a* in zebrafish juveniles (Kazeto et al., 2004). In the study of Ravi et al. (2014), *cyp19a1a* was reported to be expressed higher in the testes than in ovaries of barramundi. The present study showed 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 feminization effects compared to other
455 treatments. This may be because the 5' flanking region of *cyp19a1a* was detected containing estrogen responsive elements (Yoshiura et al., 2003), explaining the up-regulation of *cyp19a1a* by administration of exogenous estrogen. The results from the present study confirm the important role of *cyp19a1a* in ovarian differentiation of teleost.

Estrogens, including E_2 and EE_2, have been known as having high toxicity towards many studied teleosts (Kazeto et al., 2004; Van den Belt et al., 2004). As a synthetic estradiol, EE_2 is proved to be more estrogenic potent and toxic compared to the natural E_2 (Piferrer, 2001). The toxicity of EE_2 is amplified when the exposed animals are in the very early life stage (embryonic or larval stages) and longer treatment duration/ higher treatment doses (Piferrer, 2001). EE_2 significantly suppressed the early gonadal development in both sexes of zebrafish (Hill Jr & Janz, 2003; Weber et al., 2003). Hu et al. (2017) reported high mortality (>60.0%) in larval clearhead icefish *Protosalanx hyalocranius* after 23-day continuous exposure to 1 mg L^{-1} EE_2 or E_2. Serious damages to fish health such as generalized edema, injured gill epithelia, damaged liver and fibrous testicular tissue were evident in the sheephead minnow exposed to EE_2 at concentration from 200 ng L^{-1} to 3,200 ng L^{-1} (Zillioux et al., 2001). In our current study, possible high doses of EE_2 were used during early stages of the barramundi lifecycle caused irreversible damage to the hepatic and 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.

5. CONCLUSIONS

In this study E_2 treatment induced some feminizing effects when fed to juveniles at a higher dose (20 mg E_2 kg^{-1} feed), and inhibited spermatogenesis at a lower dose (10 mg E_2 kg^{-1} feed). Higher dosage and/or longer treatment duration should be tested to optimize 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 EE_2 kg^{-1} feed) suppressed the expression of important feminizing 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.

ACKNOWLEDGEMENT

This project was supported by the Australian Research Council (ARC) Linkage Project LP130100007 in partnership with Mainstream Aquaculture Pty Ltd. We gratefully acknowledge Edmund Tan, Jared Huk, Jarrod Guppy and Julie Goldsbury for their kind assistance in samplings. PhD scholarship for Quyen Banh was granted by the Australian Awards Scholarship Scheme (Australian Government).

CONFLICT OF INTEREST

No conflict of interest is present among the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are provided as supplementary materials.

Tables

Table 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 estradiol 2 (E_2) and ethinylestradiol (EE_2)

Target gene	Accession	Nucleotide sequences (5'-3')	Efficiency	R^2	References
dmrt1	KR232516.1	Forward: GTGACTCTGACTGGCCCAGAG Reverse: CAGCAGGTCGGACGTTCC	0.98	0.99	Ravi et al. (2014)
cyp19a1a	KR492506.1	Forward:CACTGTTGTAGGTGAGAGACA Reverse: CTGTAGCCGTCTATGATGTCA	1.00	0.99	Domingos et al. (2018)
ubq	XM_018704769	Forward: ACGCACACTGTCTGACTAC Reverse: TGTCGCAGTTGTATTTCTGG	1.03	0.99	De Santis et al. (2011)

500 **Table 2. 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)**

Gonadal description	Treatment group				
	Control	10 mg E_2 kg^{-1} feed	20 mg E_2 kg^{-1} feed	5 mg EE_2 kg^{-1} feed	10 mg EE_2 kg^{-1} feed
Average gonad surface at 50 dph (μm^2)	$1049.5^a \pm 213.3$ μm^2	2022.3b ± 337.1 µm2	$1823.6^b \pm 198.7$ μm^2	$997.2^a \pm 112.4$ μm^2	$724.9^c \pm 102.5$ μm^2
Gonad changes at 100 dph	100% fish had visible efferent duct. Lobular structures contained strips of primary germ cells distributed along the epithelial layer.	100% fish had larger and more complicated gonads with multiple lobes containing large cysts of undifferentiated germ cells.	83% fish had ovarian lumen; 17% fish had gonads with dual lobes, indicating that the enclosing to form ovarian cavity was not complete.	100% fish had gonads containing large lumen, mild fibrosis and degenerative material of the somatic cells.	100% fish had gonads containing apoptotic germ cells, cell shrinkage and condensation of the nucleus. Some normal germ cells also existed and surrounding by fibrotic connective tissue.
Gonadal phenotype at 160 dph	Testis stage M1 and M2 observed in 9 of 12 (75%) and 6 of 12 (25%) fish, respectively.	100% of fish had gonads containing cysts of spermatogonia with some acellular areas. Mild fibrosis and basophilic cells were also noticed in gonads.	67% of fish had gonads containing oogonia along the periphery at the ventral side of gonads. 33% of the fish had few perinucleolar oocytes.	100% fish had abnormal gonads with fibrosis in the anterior part; and spermatozoa and spermatids were present only in enlarged lobules.	100% fish had abnormal gonads (in which 33% fish had complete fibrotic gonad) with fibrosis in the anterior part; and spermatozoa and spermatids were present only in enlarged lobules.
Gonadal phenotype at 12 mph	100% male (83% at M2 and 17% at M3 stages).	100% treated fish had gonads as inhibited/abnormal (acellular) testicular tissues.	100% feminized fish at early transitional stages (containing perinucleolar oocytes; oogonia, and atretic oogonia).	100% fish had abnormal gonads with some remained testicular cells.	100% fish had abnormal gonads with extreme fibrosis.

* Different letters represent statistical differences ($p < 0.05$) of the value (mean ± standard deviation).

Supplementary table

505 Table S1. Actual dose of the hormone E_2 and EE_2 given to barramundi via feed at the different ages per day from day 40 to day 160

Stages	Feed mount (g/kg fish bw/day)	10 mg E_2		20 mg E_2		5 mg EE_2		10 mg EE_2	
		Hormone amount given per day for							
		1 kg fish (mg)	1 fish (mg)	1 kg fish (mg)	1 fish (mg)	1 kg fish (mg)	1 fish (mg)	1 kg fish (mg)	1 fish (mg)
From 40 dph to 79 dph (fed with 7% bw)	70	0.7	8.99E-06	1.4	1.8E-05	0.35	4.494E-06	0.7	8.99E-06
From 80 dph to 119 dph (fed with 5% bw)	50	0.5	1.76E-05	1	1.3E-05	0.25	8.945E-06	0.5	1.8E-05
From 120 dph to 160 dph (fed with 3% bw)	30	0.3	2.72E-05	0.6	7.7E-06	0.15	1.652E-05	0.3	2.97E-05

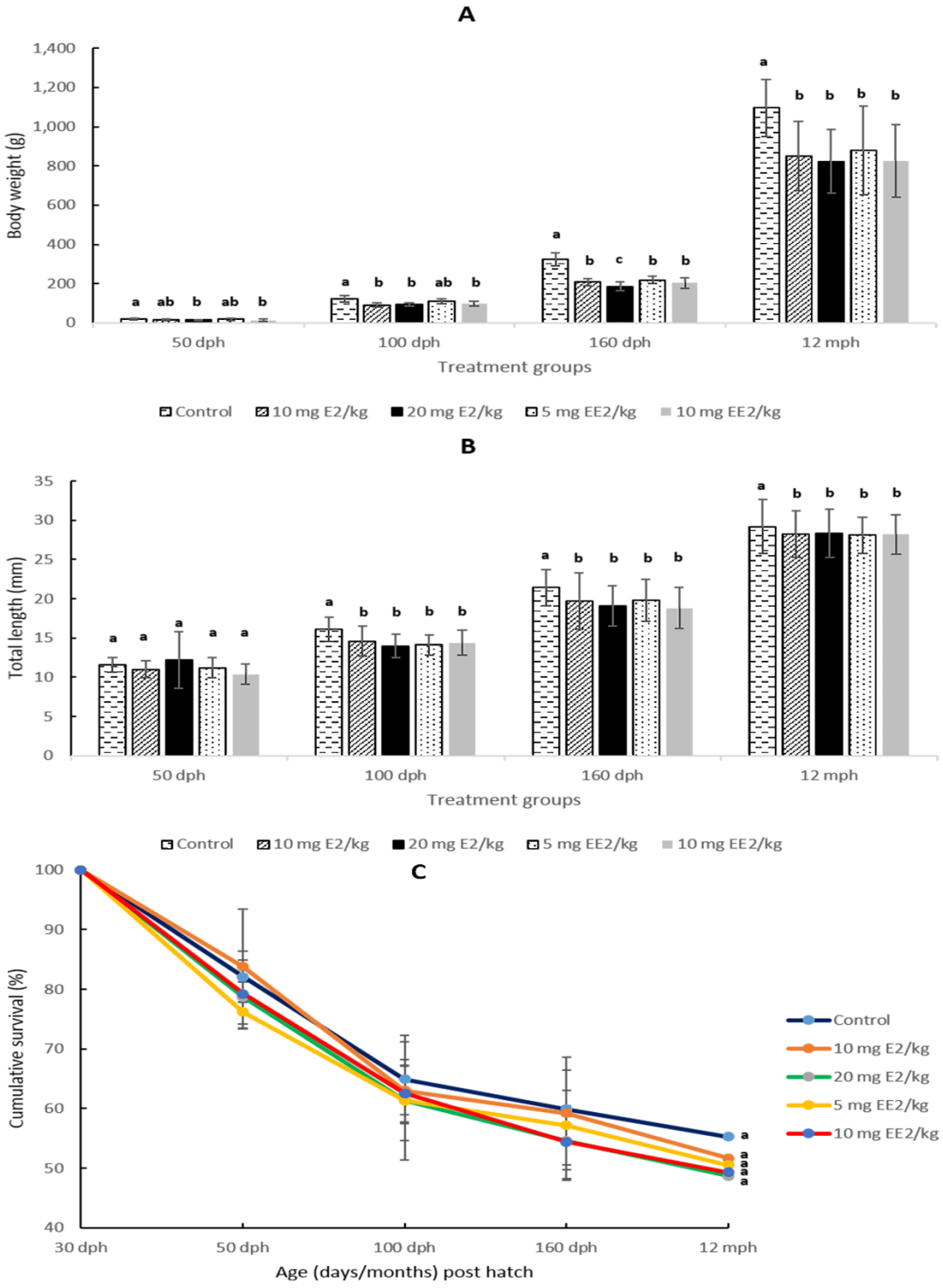

Figure 1. Effects of estradiol 2 (E_2) and ethinylestradiol (EE_2) at various dosages on growth **(A)** as body weight (g) and **(B)** as total length (mm) and **(C)** survival of barramundi during hormone treatment (from 30 to 160 dph) and after termination of hormone supplement (at 12 mph). At 12 mph, survival of each group was only recorded from one common tank. Different superscript letters represent statistical differences ($p < 0.05$) of the value (mean ± standard deviation) between treatments at the same sampling time-point ($n = 12$ for each value). No superscript indicates non-significant difference ($p > 0.05$).

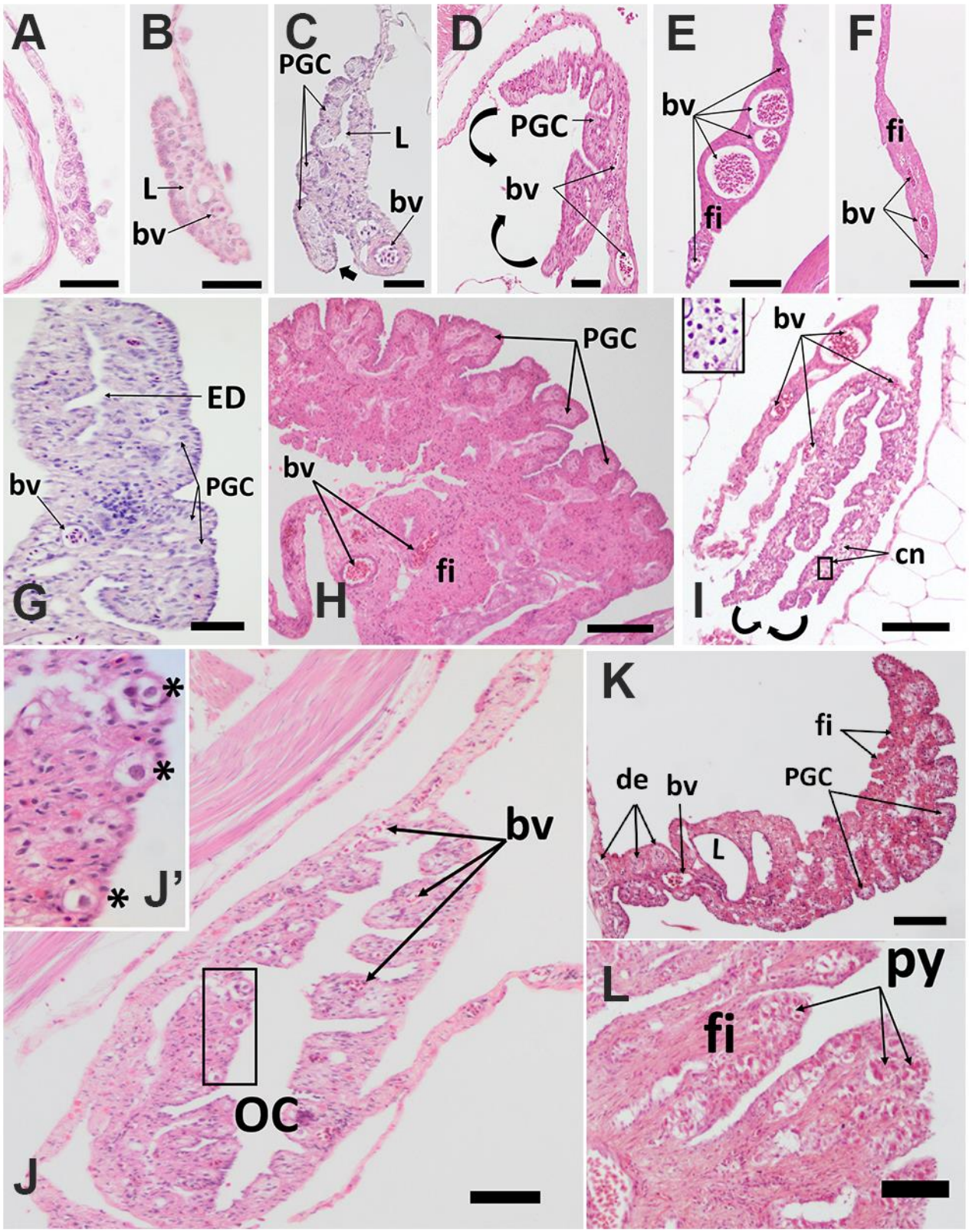

Figure 2. Transverse sections of barramundi gonads at different treatments of dietary E_2 and EE_2 at 50 and 100 dph. **(A)** Undifferentiated gonad of barramundi larvae at 30 dph. Scale bar 20 µm. **(B)** Control barramundi at 50 dph showing a narrow slit-like lumen as a presumptive efferent duct. Scale bar 20 µm. **(C)** At 50 dph, barramundi gonad of the treatment 10 mg E_2 kg^{-1} diet had a large trilobular lumen in the gonad tissue at the dorsal side, and, at the ventral side an outgrowth of tissue. Scale bar 20 µm. **(D)** At 50 dph, treatment 20 mg E_2 kg^{-1} diet, barramundi gonad showing paired, elongated aggregations of somatic cells in the proximal and distal portions of the gonads. 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 treatment 5 mg EE_2 kg^{-1} diet. Scale bar 20 µm. **(L)** Barramundi gonad at 100 dph 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.

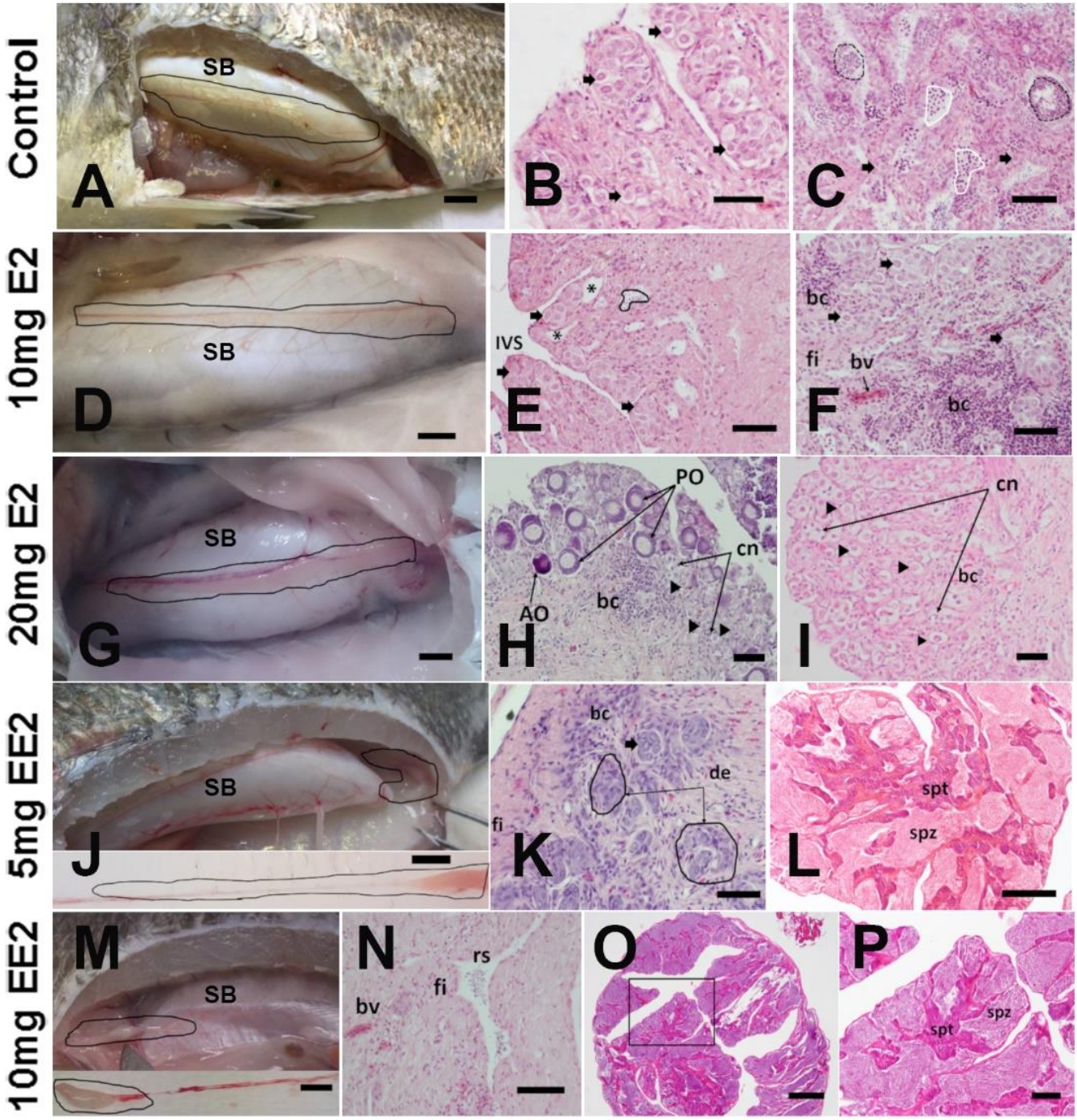

Figure 3. Gonadal histology of 160 days post hatching (dph) barramundi exposed to different treatments of dietary E_2 and EE_2. **Fig A, B, and C**: Control barramundi gonads **(A)** Anatomically, gonads of control fish were thin and attached to the side of the swim bladder. Scale bar 1 cm. **(B)** Testis of the control fish at M1 stage. Scale bar 20 µm. **(C)** Testis of the control fish at M2 stage. 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 when observed with naked eye. Scale bar 1 cm. **(E)** Black arrows indicate spermatozoa 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 color with clear blood vein. Scale bar 1 cm. **(H)** Scale bar 20 µm. **(I)** Scale bar 20 µm. **Fig J, K and L**: Gonad of barramundi in the treatment 5 mg EE_2 kg^{-1} diet. **(J)** Gonad of barramundi in the treatment 5 mg EE_2 kg^{-1} diet had enlarged tubular form in the posterior part, enclosed by the black line. Scale bar 1 cm. **(K)** Anterior filiform part of the gonad. Scale bar 20 µm. **(L)** Posterior part of the gonad. Scale bar 50 µm. **Fig M, N, O and P**: Barramundi gonad of the treatment 20 mg E_2 kg^{-1} diet. **(M)** Gonad of barramundi in the treatment 10 mg EE_2 kg^{-1} 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.

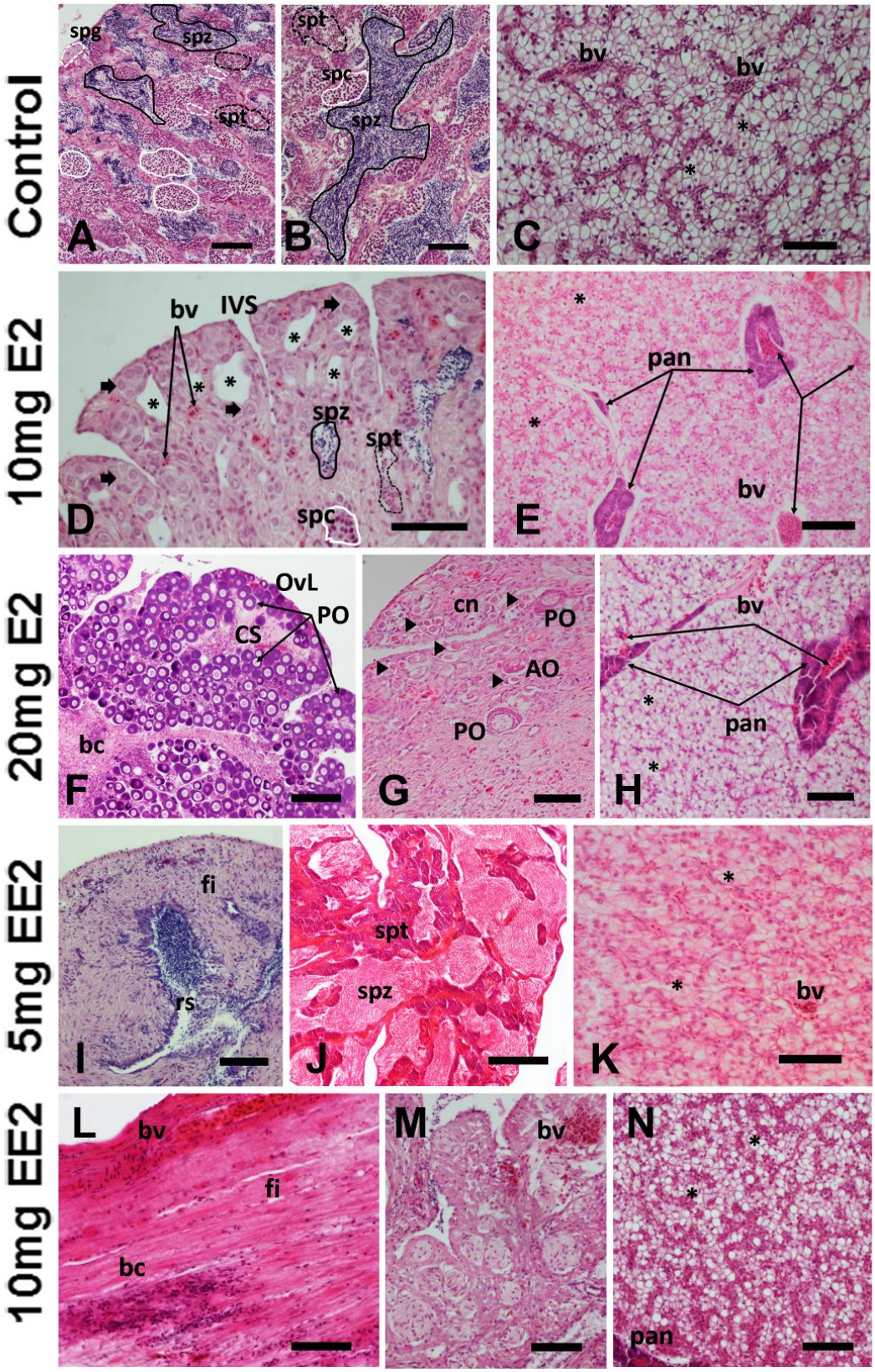

Figure 4. Histological images of gonads and livers of barramundi exposed to different treatments of dietary E_2 and EE_2 at 12 months post hatch (mph). **Fig A, B, and C**: Control barramundi: **(A)** Testis of control fish at M2 stage. Scale bar 20 µm. **(B)** Testis of control fish at M3 stage. Scale 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} diet: **(D)** Black arrows indicate spermatozoa distributed along the periphery at the ventral side of

the gonad. 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} diet: **(F)** Scale bar 50 µm. **(G)** Scale bar 20 µm. **(H)** Liver of $20E_2$ fish. Asterisks indicate vacuoles. Scale bar 50 µm. **Fig I, J and K**: Treatment 5 mg EE_2 kg^{-1} diet. **(I)** Anterior part of the gonad. Scale bar 50 µm. **(J)** Posterior part of the gonad. Scale bar 20 µm. **(K)** Liver of control fish. Asterisks indicate vacuoles. Scale bar 20 µm. **Fig L, M and N:** Treatment 10 mg EE_2 kg^{-1} diet: **(L)** Most fish in treatment 10 EE_2 had severe fibrosis in tissue as represented. Scale bar 20 µm. **(M)** Some parts of the gonads in the treatment 10 EE_2 had blood vessels and degenerative cells (*). 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.

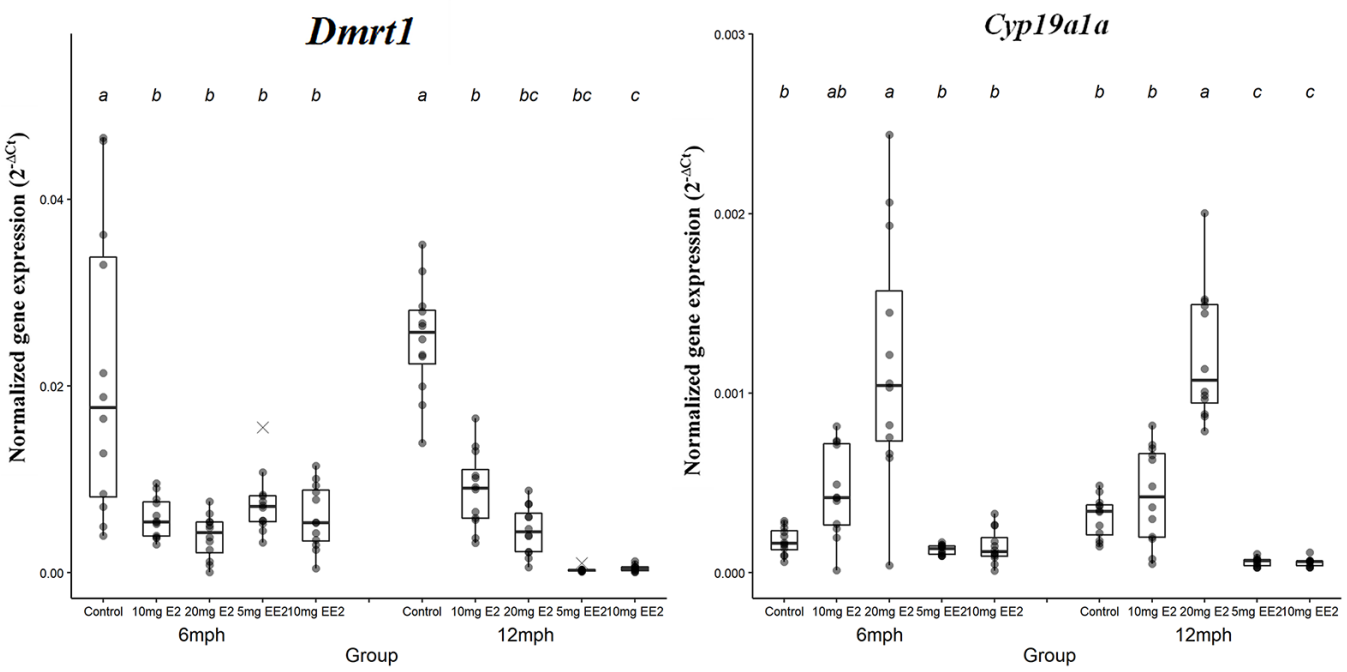

Figure 5. Relative *dmrt1* and *cyp19a1a* expression measured by RT-qPCR in barramundi gonads fed with E_2 and EE_2 at different dosages sampled at 160 dph and 12 mph. The values were calibrated with the reference gene *ubq* according to Livak and Schmittgen (2001). Different letters represent statistical differences ($p < 0.05$) between treatments at the same time-point and different time-points of the same treatment ($n = 12$ for each value).

REFERENCES

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*(6), 910-931. doi:10.1007/s000180050344

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*(1), 95-101. doi:http://dx.doi.org/10.1016/j.ygcen.2008.06.001

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*(1), 37-47. doi:10.1023/a:1007736110663

Böhne, A., Heule, C., Boileau, N., & Salzburger, W. (2013). Expression and sequence evolution of aromatase *cyp19a1* and other sexual development genes in east African cichlid fishes. *Mol. Biol. Evol., 30*. doi:10.1093/molbev/mst124

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*(3), 193-217. doi:http://dx.doi.org/10.1016/j.aquatox.2004.01.022

Budd, A., Banh, Q., Domingos, J., & Jerry, D. (2015). Sex control in fish: Approaches, challenges and opportunities for aquaculture. *J. Mar. Sci. Eng., 3*(2), 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*(15), 2079-2086.

Capel, B. (2017). Vertebrate sex determination: evolutionary plasticity of a fundamental switch. *Nat. Rev. Genet., 18*(11), 675.

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*(2), 795-805. doi:10.1007/s10695-015-0176-0

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.

Chang, C.-F., Lau, E.-L., & Lin, B.-Y. (1995). Estradiol-17β suppresses testicular development and stimulates sex reversal in protandrous black porgy, *Acanthopagrus schlegeli*. *Fish Physiol. Biochem., 14*(6), 481-488. doi:10.1007/bf00004348

Chang, C. F., & Lin, B. Y. (1998). Estradiol-17 beta stimulates aromatase activity and reversible sex change in protandrous black porgy, *Acanthopagrus schlegeli*. *J. Exp. Zool., 280*. doi:3.0.co;2-m

Chaves-Pozo, E., Liarte, S., Vargas-Chacoff, L., García-Lopez, A., Mulero, V., Meseguer, J., . . . García-Ayala, A. (2007). 17β-estradiol triggers postspawning in spermatogenically active gilthead seabream (*Sparus aurata* L.) males. *Biol. Reprod., 76*. doi:10.1095/biolreprod.106.056036

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*(2), 179-192.

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*. doi:10.1006/gcen.1999.7338

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*(3), 265-276. doi:10.1002/mrd.1031

Davis, K. B., & Ludwig, G. M. (2004). Hormonal effects on sex differentiation and growth in sunshine bass *Morone chrysops×Morone saxatilis*. *Aquaculture, 231*(1), 587-596. doi:http://dx.doi.org/10.1016/j.aquaculture.2003.10.031

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.

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*(2), 170-180. doi:10.1007/s10126-010-9277-z

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*(1–2), 89-99. doi:http://dx.doi.org/10.1016/j.aquaculture.2009.03.048

Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture, 208*(3–4), 191-364. doi:http://dx.doi.org/10.1016/S0044-8486(02)00057-1

Dietrich, D. R., & Krieger, H. O. (2009). *Histological analysis of endocrine discruptive effects in small laboratory fish*. New Jersey, United State of America: John Wiley & Sons, Inc.

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*(9).

Donaldson, E. M., & Hunter, G. A. (1982). Sex control in fish with particular reference to salmonids. *Can. J. Fish Aquat. Sci., 39*. doi:10.1139/f82-012

Falconer, D., & Mackay, T. (1996). *Introduction to quantitative genetics* (4 ed.). Essex: Longman.

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*(6), 316-324.

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*(2), 219-231. doi:https://doi.org/10.1016/j.aquatox.2006.12.003

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*(2–3), 93-109. doi:http://dx.doi.org/10.1016/S0166-445X(98)00060-5

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*(4), 267-278. doi:http://dx.doi.org/10.1016/0044-8486(79)90083-8

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*(2), 167-177. doi:10.1111/j.1749-7345.2004.tb01072.x

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*(11), 2587-2594. doi:10.1002/etc.5620181128

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*. doi:10.1006/bbrc.2000.2840

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*. doi:10.1007/bf00005126

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*. doi:10.1016/j.bbrc.2005.03.066

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. doi:http://dx.doi.org/10.1016/j.aquaculture.2010.09.015

Hendry, C. I., Martin-Robichaud, D. J., & Benfey, T. J. (2003). Hormonal sex reversal of Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture, 219*(1–4), 769-781. doi:http://dx.doi.org/10.1016/S0044-8486(02)00344-7

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*(4), 417-429. doi:https://doi.org/10.1016/S0166-445X(02)00207-2

Hu, S., Zhang, H., Shen, G., Yuan, Z., Xu, T., & Ji, R. (2017). Effects of 17β-estradiol and 17α-ethinylestradiol on the embryonic development of the clearhead icefish (Protosalanx hyalocranius). *Chemosphere, 176*, 18-24. doi:https://doi.org/10.1016/j.chemosphere.2017.02.094

Ijiri, S., Kaneko, H., Kobayashi, T., Wang, D. S., Sakai, F., Paul-Prasanth, B., . . . 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*. doi:10.1095/biolreprod.107.064246

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*(3), 197-205.

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*(5), 358-370. doi:10.1002/mrd.22170

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*(3), 241-252. doi:http://dx.doi.org/10.1016/0044-8486(79)90015-2

Kang, I. J., Yokota, H., Oshima, Y., Tsuruda, Y., Yamaguchi, T., Maeda, M., . . . Honjo, T. (2002). Effect of 17β-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). *Chemosphere, 47*(1), 71-80. doi:http://dx.doi.org/10.1016/S0045-6535(01)00205-3

Kavumpurath, S., & Pandian, T. (1993). Production of a YY female guppy, Poecilia reticulata, by endocrine sex reversal and progeny testing. *Aquaculture, 118*(3-4), 183-189.

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*(1), 25-34. doi:http://dx.doi.org/10.1016/j.aquatox.2004.04.008

Kettlewell, J. R., Raymond, C. S., & Zarkower, D. (2000). Temperature-dependent expression of turtle *dmrt1* prior to sexual differentiation. *genesis, 26*(3), 174-178. doi:10.1002/(SICI)1526-968X(200003)26:3<174::AID-GENE2>3.0.CO;2-J

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*.

Kitano, T., Yoshinaga, N., Shiraishi, E., Koyanagi, T., & Abe, S. I. (2007). Tamoxifen induces masculinization of genetic females and regulates P450 aromatase and Müllerian inhibiting substance mRNA expression in Japanese flounder (Paralichthys olivaceus). *Molecular Reproduction and Development, 74*(9), 1171-1177.

Klinge, C. M. (2000). Estrogen receptor interaction with co-activators and co-repressors. *Steroids, 65*(5), 227-251.

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*(3-4), 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*(1), 297-306.

Kobayashi, T., & Nagahama, Y. (2009). Molecular aspects of gonadal differentiation in a teleost fish, the Nile tilapia. *Sex. Dev., 3*(2-3), 108-117.

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*(3), 628-632.

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*(4), 359-370. doi:10.1002/mrd.1042

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*(2), 113-132.

Länge, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., . . . 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*(6), 1216-1227. doi:10.1002/etc.5620200610

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), 1-15. doi:10.1186/1477-7827-5-20

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*(4), 402-408. doi:http://dx.doi.org/10.1006/meth.2001.1262

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*.

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*.

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*(10), 1627-1633.

Marchand, O., Govoroun, M., D'Cotta, H., McMeel, O., Lareyre, J.-J., Bernot, A., . . . Guiguen, Y. (2000). *Dmrt1* expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. *Biochim. Biophys. Acta, 1493*(1–2), 180-187. doi:http://dx.doi.org/10.1016/S0167-4781(00)00186-X

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*(1), 163-176. doi:10.1007/s10577-011-9264-x

Nakamura, M., Bhandari, R. K., & Higa, M. (2003). The role estrogens play in sex differentiation and sex changes of fish. *Fish Physiol. Biochem., 28*(1-4), 113-117. doi:10.1023/B:FISH.0000030495.99487.17

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*.

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

Nilsson, S., Mäkelä, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., . . . Gustafsson, J.-Å. (2001). Mechanisms of estrogen action. *Physiol. Rev., 81*(4), 1535-1565.

Oropesa, A. L., Jiménez, B., Gil, M. C., Osswald, J., Fallola, C., Pula, H. J., . . . Gómez, L. (2014). Histological alterations in the structure of the testis in tench (*Tinca tinca*) after exposure to 17 alpha-ethynylestradiol. *Environ.Toxicol., 29*(10), 1182-1192. doi:doi:10.1002/tox.21850

Osterburg, H. H., Allen, J. K., & Finch, C. E. (1975). The use of ammonium acetate in the precipitation of ribonucleic acid. *Biochem. J., 147*(2), 367-368. doi:10.1042/bj1470367

Panter, G. H., Hutchinson, T. H., Hurd, K. S., Bamforth, J., Stanley, R. D., Duffell, 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*(3), 279-290. doi:http://dx.doi.org/10.1016/j.aquatox.2006.01.004

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*(4), 1090-1099. doi:10.1111/are.12565

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*(5), 941-946. doi:10.1002/etc.1789

Piferrer, F. (2001). Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture, 197*(1–4), 229-281. doi:http://dx.doi.org/10.1016/S0044-8486(01)00589-0

Piferrer, F. (2011). Hormonal control of reproduction and growth - endocrine control of sex differentiation in Fish In A. P. Farrell (Ed.), *Encyclopedia of Fish Physiology: From Genome to Environment* (pp. 1490-1499). San Diego: Academic Press.

Piferrer, F., Benfey, T. J., & Donaldson, E. M. (1994). Production of female triploid coho salmon (*Oncorhynchus kisutch*) by pressure shock and direct estrogen treatment. *Aquat. Living Resour., 7*(2), 127-131.

Piferrer, F., & Blázquez, M. (2005). Aromatase distribution and regulation in fish. *Fish Physiol. Biochem., 31*, 215-226.

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*(2–3), 251-262. doi:http://dx.doi.org/10.1016/0044-8486(89)90207-X

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*(2), 183-193. doi:http://dx.doi.org/10.1016/0044-8486(92)90202-V

Piferrer, F., & Guiguen, Y. (2008). Fish Gonadogenesis. Part II: Molecular Biology and Genomics of Sex Differentiation. *Reviews in Fisheries Science, 16*(sup1), 35-55. doi:10.1080/10641260802324644

Raghuveer, K., Garhwal, R., Wang, D. S., Bogerd, J., Kirubagaran, R., Rasheeda, M. K., . . . 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*(2), 143. doi:10.1007/s10695-006-0016-3

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), 1-14. doi:10.1186/1477-7827-12-5

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. doi:http://doi.org/10.1016/j.cbpc.2014.04.003

Robinson, N. A., & Jerry, D. R. (2009). *Development of a genetic management and improvement strategy for Australian cultured barramundi*. Retrieved from

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*(9), 768-779. doi:10.1080/15287390701236470

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

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*(4), 363-373. doi:http://dx.doi.org/10.1016/S0166-445X(00)00090-4

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*(17), 6305-6310. doi:10.1021/es070785+

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*(8), 1692-1698. doi:10.1002/etc.5620210822

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), 1-21. doi:10.1186/1297-9686-46-26

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*(3), 513-523. doi:10.1677/joe-07-0295

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

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*(2), 125-137. doi:https://doi.org/10.1016/j.cbpb.2013.03.011

Strüssmann, C. A., & Nakamura, M. (2002). Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. *Fish Physiol. Biochem., 26*(1), 13-29. doi:10.1023/a:1023343023556

Sun, P., You, F., Liu, M., Wu, Z., Wen, A., Li, J., . . . Zhang, P. (2010). Steroid sex hormone dynamics during estradiol-17β induced gonadal differentiation in *Paralichthys olivaceus* (Teleostei). *Chin. J. Oceanol. Limnol., 28*(2), 254-259. doi:10.1007/s00343-010-9011-8

Todd, E. V., Liu, H., Muncaster, S., & Gemmell, N. J. (2016). Bending genders: The biology of natural sex change in fish. *Sex. Dev., 10*(5-6), 223-241.

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*(2), 183-195. doi:https://doi.org/10.1016/j.aquatox.2003.09.004

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*(5), 277-288. doi:10.1677/jme-08-0039

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*(3), 792-797. doi:10.1897/02-570

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*(4), 431-446. doi:http://dx.doi.org/10.1016/S0166-445X(02)00208-4

Wen, A. Y., You, F., Sun, P., Li, J., Xu, D. D., Wu, Z. H., . . . 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*(1), 193-205. doi:10.1111/jfb.12277

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*(6), 1111-1120.

Yamamoto, T.-O. (1969). Sex Differentiation. In W. S. Hoar & D. J. Randall (Eds.), *Fish physiology* (Vol. 3, pp. 117-175). New York: Academic Press.

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*(5), 1545-1553. doi:10.1095/biolreprod.102.010843

Zillioux, E. J., Johnson, I. C., Kiparissis, Y., Metcalfe, C. D., Wheat, J. V., Ward, S. G., & Liu, H. (2001). The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: A partial life-cycle test with 17α-ethynylestradiol. *Environ Toxicol Chem, 20*(9), 1968-1978. doi:10.1002/etc.5620200915