Aquaculture Reports 46 (2026) 103261

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Implantation of aromatase inhibitor fadrozole and 17β-estradiol antagonistically affect gonad development in the protandrous barramundi (*Lates calcarifer*)

Quyen Q.T. Banh [a,1,2,3], Jarrod L. Guppy [a,b,c,d,*,2,4], Julian R. Wilson [a], Jose A. Domingos [a,e,5], Dean R. Jerry [a,d,e,6]

[a] Centre for Sustainable Tropical Fisheries and Aquaculture, College of Science and Engineering, James Cook University, Townsville, QLD 4811, Australia
[b] Aquaculture Breeding and Reproductive Technologies Lab, James Cook University, Townsville, QLD 4811, Australia
[c] Centre for Tropical Bioinformatics and Molecular Biology, James Cook University, Bebegu Yumba Campus, Townsville, Qld 4811, Australia
[d] ARC Research Hub for Supercharging Tropical Aquaculture through Genetic Solutions, James Cook University, Townsville, Qld 4811, Australia
[e] Tropical Futures Institute, James Cook University, Singapore, Singapore

ARTICLE INFO

Keywords:
Barramundi
Fadrozole
Aromatase inhibitor
17β-estradiol
cyp19a1a
Sex differentiation
Sex-change

ABSTRACT

Barramundi, *Lates calcarifer*, are protandrous hermaphrodites, maturing and reproducing first as males before naturally changing sex into females several years later. This male-to-female development provides an excellent model for investigating the genetic mechanism underlying natural sex change in fish, and in aquaculture manipulating this process is key to control reproduction and enhance seedstock production. Exogenous hormones and aromatase inhibitors have been used to obtain control of sex in a range of fish. Fadrozole (FAD) is a non-steroidal inhibitor that reduces biosynthesis of estrogenic hormones, however its use has yet to be tested in barramundi. In this study, 15-month-old male barramundi (2102 ± 126 g) were divided into four treatments and either implanted with i) 17β-estradiol (E2) at 8 mg kg^{-1}, ii) FAD at 8 mg kg^{-1}, iii) 8 mg E2 and 8 mg FAD kg^{-1} together, or iv) implanted with cholesterol pellets (controls). After nine weeks, gonadal phenotype was examined histologically and the expression of sex-related genes were quantified by qPCR. All fish with E2 only implants had sex-changed into females, with significantly higher *cyp19a1a* and *foxl2* expression and lower *dmrt1*, *cyp11b* and *esr1* expression than control fish. All fish treated with FAD only remained male, and expression of male genes (*dmrt1* and *cyp11b*) were significantly upregulated, whilst the female-biased gene foxl2 was downregulated. Combined FAD and E2 treatment resulted in 42 % of fish remaining male, 42 % becoming female and 16 % that were transitional, with each individuals gene expression patterns reflecting their gonadal phenotype. These results show FAD can impede the feminizing effects of E2 in some individuals, indicating potential for holding barramundi as male for longer before they sex-change. These findings highlight utility of E2 and FAD as tools for modulating sex change in barramundi and inform strategies for improved reproductive control in aquaculture breeding programs.

* Correspondence to: Centre for Sustainable Tropical Fisheries and Aquaculture, College of Science and Engineering, James Cook University, 1 Angus Smith Dr, Douglas, QLD 4811, Australia.

E-mail addresses: quyenquyen.banhthi@gmail.com (Q.Q.T. Banh), jarrod.guppy@jcu.edu.au (J.L. Guppy), julian.wilson@my.jcu.edu.au (J.R. Wilson), jose.domingos1@jcu.edu.au (J.A. Domingos), dean.jerry@jcu.edu.au (D.R. Jerry).

[1] Current address: Fly Farm Australia Ltd, 75 MacArthur St, Alexandra Hills QLD 4161 Australia
[2] These two authors contributed equally to this work.
[3] ORCID:0000–0002-2636–3534
[4] ORCID:0000–0003-1864–9644
[5] ORCID:0000–0001-8914–8666
[6] ORCID:0000–0003-3735–1798

https://doi.org/10.1016/j.aqrep.2025.103261
Received 12 August 2025; Received in revised form 29 October 2025; Accepted 26 November 2025
Available online 8 December 2025

1. Introduction

Having control over the sex and subsequent reproduction of aquaculture species is essential for their efficient propagation and selective breeding. Barramundi (*Lates calcarifer*) are a mass-spawning, protandrous hermaphrodite fish, where individuals first mature and reproduce as male (~2 years) and then naturally sex change into female several years later (~4 years) (Davis, 1982). This natural sex change process presents significant challenges to the management of broodstock in barramundi hatcheries, as male broodfish of high-quality unpredictably change sex to female, requiring reorganisation of broodstock spawning groups and ongoing recruitment of new male fish for broodstock (Terence et al., 2021; Guppy et al., 2022). In addition, in the context of selective breeding, this sequential sex change of barramundi results in one generation difference between sexes, with females being 2–3 years older than males. Inter-generational breeding thereby reduces the possible annualized rate of genetic progress that could otherwise be made through a single-generation selection program (Robinson and Jerry, 2009). Therefore, the development of methods to control the processes of sex differentiation and gonad development of barramundi is of great importance to seedstock production and selective breeding of the species (Budd et al., 2015).

The gonadal development of fish, including barramundi, has been found to be highly plastic in nature and the phenotypic sex of individuals can be altered by exposure to exogenous hormones and endocrine-disrupting chemicals (EDC) (Piferrer, 2001; Devlin and Nagahama, 2002). In previous studies involving barramundi, exposure of undifferentiated larvae (30–160 days post hatch) and 6-month-old male juveniles to exogenous 17β-estradiol (E2) induced precocious male-to-female sex change (Banh et al., 2020, 2021). Furthermore, expression of sex-biased genes was altered by this E2 administration, with the male-related genes, doublesex and mab-3-related transcription factor 1 (*dmrt1*), cytochrome P450 11β-hydroxylase gene (*cyp11b*) and estrogen receptor 1 (*esr1*), significantly down-regulated at 9 weeks post-E2 implant. Conversely, female-biased genes, including the forkhead box protein L2 (*foxl2*) and cytochrome P450 aromatase (*cyp19a1a*) were significantly upregulated (Banh et al., 2020, 2021). These results suggest that in barramundi, the elevated levels of E2 provided by E2 implants initiates a positive feedback loop of the female-bias genetic pathway, and results in the upregulation of *foxl2* which may promote *cyp19a1a* expression (Banh et al., 2021). In vertebrates, *Cyp19a1a* plays a pivotal role within the process of female gonad development and genetic pathways by encoding for the enzyme aromatase, which when expressed, acts to catalyse the conversion of testosterone to estradiol (Guiguen et al., 2010). Further research has confirmed that even after exogenous E2 treatment ceases, a stable physiological environment is reached in barramundi whereby endogenous E2 hormone production increases and provides sufficient ongoing support for continued female maintenance, ovarian development, vitellogenesis and spawning (Guppy et al., 2022).

In vertebrates, including fish, the activity of aromatase has been observed to be influenced by a number of environmental and social factors, along with endocrine disruptors including steroidal and nonsteroidal aromatase inhibitors (Piferrer and Blázquez, 2005). The ability of nonsteroidal aromatase inhibitors to reduce E2 production has been studied across a range of fields, including human cancer research, developmental biology and applied animal breeding (Dowsett et al., 1994; Shetty et al., 1995; Moudgal et al., 1996; Babiak et al., 2012; Doering et al., 2021). Of the nonsteroidal aromatase inhibitors available, Fadrozole (FAD) has been commonly used as it selectively inhibits estrogen production by binding reversibly to the enzyme aromatase (Steele et al., 1987). Specifically, its action is based on noncovalent, reversible interaction with the heme portion of aromatase and occupation of its substrate-binding site (Miller et al., 2008). Treatment with FAD has been effective at reducing estrogen biosynthesis in breast cancer patients (Dowsett et al., 1994) and also resulted in decreased E2 production in other mammals (Shetty et al., 1995; Moudgal et al., 1996). Complete functional masculinization due to FAD exposure has been reported in chickens (*Gallus gallus*) (Elbrecht and Smith, 1992; Abinawanto et al., 1996), turtles (Dorizzi et al., 1994; Richard-Mercier et al., 1995), lizards (Wennstrom and Crews, 1995) and some fish species (Piferrer et al., 1994b; Kitano et al., 2000; Kwon et al., 2000; Babiak et al., 2012; Hur et al., 2012; Luzio et al., 2016). However, there is still a poor understanding of how FAD influences gonad development and sex in hermaphroditic species where sex-change is part of their normal life history (Mondal et al., 2025), and in the case of barramundi, whether inhibiting aromatase with FAD disrupts the role E2 plays in upregulation of the feminising gene network. Studying the expression of sex-related genes in the gonads of FAD-implanted fish would contribute to our understanding of the genetic mechanisms underlying the important process of sex change in barramundi.

In the context of barramundi aquaculture, it is necessary to test if FAD supports the maintenance of genetic pathways and the male gonadal phenotype. However, undertaking such research has been difficult to achieve due to the impractical nature of obtaining large numbers of simultaneously sex-changing barramundi for experiments. To address this, E2 implants have been utilized in previous research to reliably transition males to female precociously for breeding purposes and to serve as a study model for sex-change in barramundi (Banh et al., 2021; Guppy et al., 2022). E2 implants are known to induce in barramundi male-female sex-change within 9 weeks of implantation, with gonadal development, gene expression, methylation and spawning performance of E2-induced fish confirmed to mimic those of natural females (Domingos et al., 2018; Banh et al., 2021; Budd et al., 2022; Guppy et al., 2022).

In this study, the effect of FAD treatment, when applied both with and without exogenous E2, on the gonadal phenotype of 15-month-old male barramundi was examined. Additionally, the underlying genetic pathways, including expression of female (*cyp19a1a* and *foxl2*) and male (*dmrt1*, *cyp11b*, *esr1*) biased genes, were analysed to further understand the processes of sex differentiation and sex change in barramundi, and inform on-going efforts to obtain control over sex-change in this commercially important aquaculture species.

2. Materials and methods

2.1. Experimental design

All laboratory procedures were approved by the Animal Ethics Committee of James Cook University (Approval #A2014) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Fifteen-month-old male barramundi (2102 ± 126 g body weight (BW)) purchased from a commercial freshwater farm were kept in three 2500 L tanks in a 13,500 L freshwater recirculating aquaculture system (RAS) and acclimated under ambient photoperiod (~14 L:10 D) and temperature conditions (28–30 °C). The RAS was equipped with two cartridges of activated carbon (8–10 kg activated carbon/cartridge replaced fortnightly; Acticarb GC1200, Activated carbon Technologies Pty Ltd., Australia) to absorb any potential hormones released into the system.

After a week of acclimatization, fish were anesthetized with AQUI-S (Aqui-S New Zealand Ltd), individually PIT tagged and had their BW (g) recorded. Implants were prepared as described previously (Banh et al., 2021) and contained either 17β-estradiol (E2, Sigma-Aldrich, E8875) (E2) and/or Fadrozole hydrochloride (FAD, Sigma-Aldrich, F3806) depending on the treatment. Briefly, E2 or FAD stock were diluted in 80 % ethanol before being thoroughly mixed with cholesterol (C8867, Sigma-Aldrich) and coconut oil (5 % w/w). The mixture was allowed to dry at room temperature in a fume hood 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 E2 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, pellets were pressed from the mould to form a compacted cylindrical pellet. Hormonal pellets were stored at 4 °C until implantation.

The experiment consisted of three treatments and a control, with 12 individuals in each treatment group. The control fish were implanted with a cholesterol pellet without the addition of E2 or FAD. The three treatment groups were implanted with either i) E2 at 8 mg kg^{-1} (n = 12), ii) FAD 8 mg kg^{-1} (n = 12), or iii) combination of 8 mg E2 and 8 mg FAD kg^{-1} (n = 12). Each fish was implanted with three pellets into the left dorsal musculature using a RalGun pellet injector (Syndel Laboratories Ltd.). Four fishes from each group were held in each tank to account for any unexpected potential tank effects on gonadal development. All fish were recovered from implantation successfully and survived until completion of the experiment.

Initial sampling to confirm the gonadal status of fish was conducted on five randomly chosen fish one day prior to beginning the experiment. Final sampling (n = 12 for each group) was conducted at 9 weeks after implantation to align with when the feminising effects of E2 implantation on gonadal phenotype were shown by previous studies (Banh et al., 2021; Guppy et al., 2022). For the gonad sampling, both the left and right gonads of each fish were subsampled into separate pieces for various analyses. Three small pieces (~1 cm long) at the anterior, middle and posterior region of each gonad were preserved separately for histological analysis. The remaining portion of gonad tissue for each fish was preserved in RNAlater™ stabilization solution (ThermoFisher Scientific) at 4 °C overnight before being stored at −20 °C until RNA extraction. Liver and kidney tissues were dissected and 0.5 cm^2 pieces were preserved for histological analysis.

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. Embedded samples were transversely sectioned at a thickness of 5 μm and stained with hematoxylin – eosin. Between 10 and 20 slides were obtained from each sample to ensure all three portions (anterior, middle and posterior) of both left and right gonads were assessed thoroughly. The slides were examined using an Olympus CelSens Microscope Digital Camera System (Olympus, Japan).

Testicular and ovarian development were categorized according to Guiguen et al. (1994). Specifically, immature and testicular tissue were classified from stage M0 (immature with no visible differentiated germ cells); M1 (predominance of spermatogonia); M2 (mostly spermatocytes and spermatids); M3 (containing predominantly spermatozoa); and M4 (post-spawning, i.e. when testicular lobules are devoid of spermatozoa). Transitional stages include stages from T1 to T2 corresponding to the degeneration of testicular tissue without and with ovarian tissue; from T3 to T4 equivalent to only ovarian tissue distributed less and more than 50 % of histological section. For female stages, stage F1 contains oogonia and previtellogenic oocytes; stage F2 and F3 are when less and more than 50 % of the cross-section contains vitellogenic oocytes, respectively. Lastly, stage F4 ovary is characterized by atretic oocytes. For female histological samples the 10 largest oocytes were measured from photos using Image J. Histopathology of liver and kidney tissues examined to assess the health condition of hormone treated fish.

2.3. Gene expression analysis by qPCR

In gonad tissue the expression of five genes, *dmrt1*, *cyp11b*, *esr1*, *cyp19a1a* and *foxl2*, were studied at 9 weeks after implantation. RNA extraction, DNAse treatment, cDNA synthesis and quality control were conducted as described previously (Banh et al., 2017).

2.3.1. RNA extraction

Briefly, total RNA was extracted using Trizol® RNA Isolation Reagents (Thermo Fisher Scientific). The RNA obtained was quantified using the NanoDrop 1800 (Nanodrop Technologies, USA) spectrophotometer at a wavelength of 260 nm. All the samples were treated with TURBO DNA-free™ kit (Invitrogen™, USA). The DNAse-treated RNA was then cleaned with an ammonium acetate precipitation protocol (Osterburg et al., 1975). RNA samples were quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, USA). All samples had absorbance ratios (260/280 nm and 260/230 nm) greater than 1.9, indicating high-purity RNA. Assessment of RNA quality was also performed by electrophoresis on 1.5 % agarose gel (in 1x TBE made with DEPC treated water) with GelGreen™ (Biotium Inc., USA) post-staining. RNA samples that showed no smear and two clear bands (28S:18S) were included for subsequent cDNA synthesis.

2.3.2. qPCR analysis

cDNA was produced with the Tetro cDNA synthesis kit (Bioline) according to the manufacturer specification. A total of 1 μg of DNAse-treated RNA template 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 (200 u/μl) and DEPC treated water to a total 20 μl. All the cDNA synthesis of the RNA sample run alongside multiple 'no reverse transcriptase' (NRT) as the negative controls. All tubes (including the real cDNA syntheses and NRT) were then placed in a C1000 Thermal Cycler (Bio-Rad) using the following cycling conditions: 45 °C for 30 min, 25 °C for 10 min followed by 45 °C for 30 min then terminated reaction by incubating at 85 °C for 5 min, before chilling on ice. The resulting single-stranded cDNA then was stored at −20 °C.

qPCR was 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/or E2 implantation. The primer sequences used to amplify the reference and target genes are shown in Table 1. Noting primer sequences used here for *cyp11b*, have previously been used in studies noted to amplifying cDNA for *cyp11c* (Ravi et al., 2014), but it has since been found cDNA sequences used for primer development were from *cyp11b* and as such instead amplify *cyp11b* mRNA (Banh et al., 2021). qPCR reactions were set up in triplicate using SsoFast™Evagreen® master mix (Bio-Rad) and run on a Corbett Rotor-Gene 6000 thermocycler (Qiagen). Each sample contained 5 μl of 1:500 diluted cDNA templates, 7.5 μl of SsoFast™Evagreen® master mix, 0.6 μl of 0.2 μM

Table 1
Primer sequences used for qPCR to study the expression of the genes *dmrt1*, *cyp11b*, *esr1*, *cyp19a1a* and *foxl2* in the gonads of barramundi implanted with Fadrozole and 17β-Estradiol.

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

forward and reverse primers, and 1.3 μl of water. All the reagents were placed into the 100-well ring (Qiagen) by an automatic pipetting system (Corbett Robotics, Qiagen). Cycling conditions were 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 58 °C (*dmrt1*, *cyp11b, esr1, cyp19a1a, and foxl2*) 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. 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, $R^2 \geq 0.99$).

For each individual gene, two 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, qPCR product specificity for each gene was also confirmed by Sanger sequencing (Australian Genomic Research Facility). qPCR data of pre-implant individuals was not obtained for *cyp11b* due to technical error during the qPCR analysis process. Insufficient cDNA remained for re-analysis and as such was excluded from comparison.

2.4. Statistical analysis

Statistical analyses were performed using the SPSS software package (IBM SPSS Statistics 23). The C_T value for each target gene for each sample was obtained from the mean of the triplicate qPCR measurements. The relative transcript abundance of target genes was normalized to the reference gene *ubq* according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Normality and homogeneity of variance were tested using the Kolmogorov-Smirnov and Levene test, respectively. Normalized C_T values that did not meet the criteria of either of these tests were log-transformed with outliers removed. Normalized data of *cyp19a1a* and *esr1* expression conformed to parametric assumptions and one-way analysis of variance (ANOVA) and Post-hoc Tukey's test were used to test for differences across treatments. As normalized C_T values of the gene *dmrt1*, *foxl2* and *cyp11b* did not meet parametric assumptions, non-parametric Kruskal-Wallis tests were performed, followed by Mann-Whitney U tests for pairwise comparisons. Further comparison was undertaken for gene expression between male and female individuals of the E2 + FAD treatment with Mann-Whitney *U* test to validate the relationship between gene expression and phenotype in this treatment. Differences between treatments were regarded as statistically significant when $P < 0.05$.

3. Results

3.1. Altered sex ratio of barramundi induced by administration of 17β-estradiol and Fadrozole

Sampling of gonad tissue was conducted for histological analysis one day before implantation of the pellets (n = 5) and again for final sampling 9 weeks after the implantation (n = 12 for each treatment). The proportion of gonadal phenotypes present in the barramundi at these two sampling points are shown in Fig. 1.

All fish sampled immediately before implantation (n = 5) were confirmed to be males with stage M3 testes that contained primarily spermatozoa (Fig. 2A). At the final sampling, 9 weeks after implantation, 92 % (11/12) of the control fish retained stage M3 testes (Fig. 2D), while one fish exhibited early signs of testicular degeneration, i.e. less spermatozoa and appearance of spermatocytes and fibrous tissue (Fig. 2E). According to the classification of Guiguen et al. (1994), this fish was tentatively classified as transitional stage T1 and may have begun undergoing sex change. However, in line with classification as T1 there was no ovarian tissue observed in the gonad (e.g. T2) and may have been a sexually immature or regressed male.

After 9 weeks as expected 100 % (12/12) of the fish in the 8 mg E2 kg^{-1} treatment were completely feminised (Fig. 2H). Their ovaries were filled with pre-vitellogenic oocytes (PO) with a mean diameter of the largest 10 oocytes 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 E2 treated gonad. Ovarian cavity was observed in all the fish of this treatment (Fig. 2H).

Testicular tissue was observed in gonads of 100 % (12/12) of the fish that received the FAD implants (8 mg FAD kg^{-1}) and contained extensive cystic compartments that were filled with mostly spermatozoa and some spermatids (Fig. 2K). The size of these cysts and the interstitial tissues among those cysts were visually observed to be larger in FAD-implanted fish when compared to the normal males of the control group (Fig. 2A, D, K).

In the treatment of fish combining E2 and FAD (8 mg E2 and 8 mg FAD kg^{-1}), three types of gonad phenotypes were observed, with 42 % (5/12) identified as F1 stage females (Fig. 2 P), 42 % (5/12) as M3 males (containing mostly spermatozoa; Fig. 2 Q) and 17 % (2/12) at stage T1 and T3 transitional (Fig. 2 O and N). There were no discernible histological differences in the ovary and testicular tissues of female and male fish from the combined E2 + FAD treatment compared to either the E2-

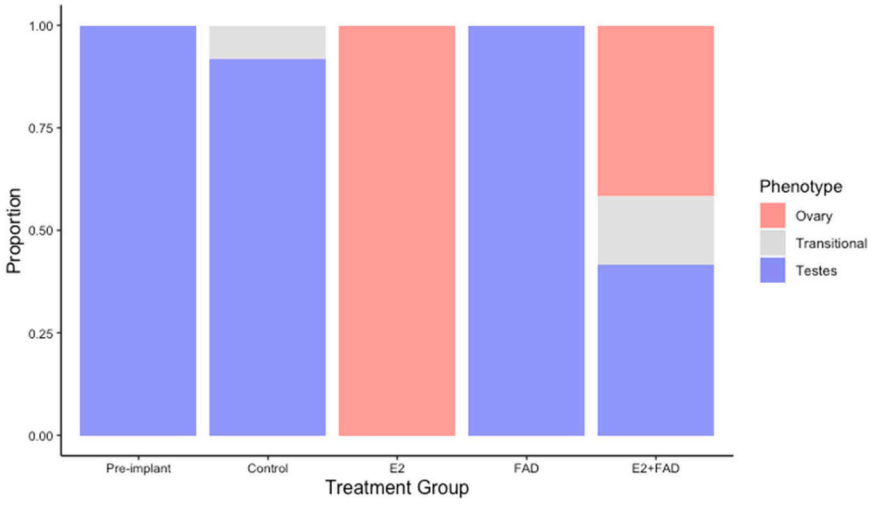

Fig. 1. The proportion of barramundi (*Lates calcarifer*) that exhibited various gonadal phenotypes (testis, transitional stage and feminized gonad with previtellogenic oocytes) before (pre) implantation and 9 weeks after implantation with cholesterol pellets containing either Estradiol 2 (E2), Fadrozole (FAD), both E2 + FAD in combination, or Control (no E2 or FAD).

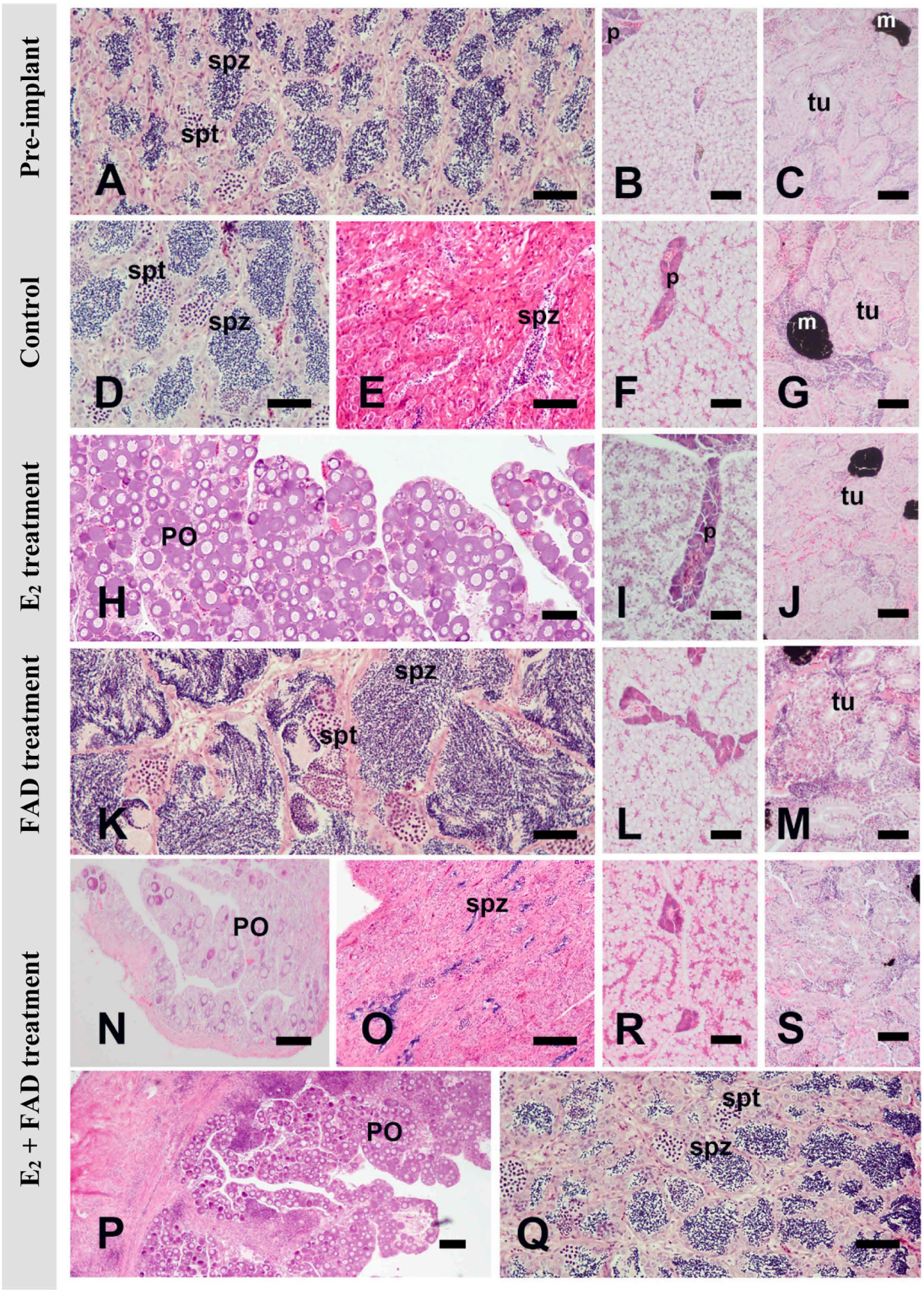

Fig. 2. Histological images of gonad, liver and kidney tissues of barramundi implanted with E2 (8 mg kg^{-1} BW; H, I and J), FAD (8 mg kg^{-1} BW; **K**, **L** and **M**) and a combination of E2 and FAD (E2 + FAD, 8 mg of E2 and 8 mg of FAD kg^{-1} BW**; N**, **O**, **P**, **Q**, **R** and **S**) at 9 weeks post-implantation, as well as pre-implant (**A**, **B**, and **C)** and control barramundi **(D**, **E**, **F** and **G)**. Scale bars indicate 20 μm for all panels except **H**, where the scale bar indicates 50 μm. **A)** Testis of pre-implant fish at M3 stage. **B)** Liver of pre-implant fish. **C)** Kidney of pre-implant fish. **D)** Testis of control fish at M3 stage. **E)** One (out of 12) fish in the control group had the gonadal tissue at transitional stage (T1). **F)** Liver of control fish. **G)** Kidney of control fish. **H)** Ovary of fish in the E2 treatment. **I)** Liver of fish in the E2 treatment. **J)** Kidney of fish in the treatment E2 treatment. **K)** Testis of fish in the FAD treatment at M3 stage. **L)** Liver of fish in the FAD treatment. **M)** Kidney of fish in the FAD treatment. **N)** Gonad of 1 (out of 12 fish) in the E2 + FAD treatment at T3 stage. **O)** Gonad of 1 (out of 12 fish) in the E2 + FAD treatment at T1 stage. **P)** Ovary of fish in the E2 + FAD treatment at F1 stage. **Q)** Testis of fish in the E2 + FAD treatment at M3 stage **R)** Liver of fish in the E2 + FAD treatment. **S)** Kidney of fish in the E2 + FAD treatment. Abbreviations: fi, fibrotic tissue; m, macrophage; p, pancreatic cells; PO, previtellogenic oocytes; spg, spermatogonia; spc, spermatocytes; spt, spermatids; spz, spermatozoa; tu, tubule.

female and/or control male fish, respectively (Fig. 2).

Histological analysis of liver and kidney tissues was used to examine for any detrimental side-effects of E2 and FAD exposure. In the E2 treatment fish, 100 % (12/12) of fish had livers demonstrating low-level hyperemia (excess of blood in the vessels supplying an organ; Fig. 2 I). The liver sections of all the remaining samples of other treatments, including the control, showed uniform hepatocytes with distinct nuclei and nucleoli, an abundance of cytoplasmic lipid and vacuolization, which are typical for healthy farmed fish (Fig. 2 B, F, L, R). Kidney tissues showed a normal appearance of nephrons and mesonephric tubules (Fig. 2 C, G, J, M and S).

3.2. Fadrozole and 17β-estradiol treatments caused alteration of gene expression profiles in barramundi gonads

The expression of the sex-related genes (*dmrt1*, *esr1*, *cy11b*, *cyp19a1a*, and *foxl2*) in the gonadal tissues of barramundi sampled at the beginning of the experiment (pre-implant) and at 9 weeks after the implantation (control and three treatments) displayed significant changes across time and treatment (Fig. 3).

The expression of *dmrt1*, *cyp11b* and *cyp19a1a* was not significantly different between the initial samples and the control fish at the final sampling ($P > 0.05$; Fig. 3A, C, D)). Meanwhile, *esr1* and *foxl2* were significantly increased in the untreated control fish compared to the pre-implant fish ($P < 0.05$; Fig. 3B, E).

The expression of the female-biased gene, *cyp19a1a*, was significantly upregulated in the fish exposed to E2 and E2 + 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 ($P > 0.05$). *Cyp19a1a* expression in the E2 + FAD treatment was significantly higher than in the gonad of FAD-implanted fish ($P < 0.05$; Fig. 3 D). Likewise, *foxl2,* was significantly affected in both the fish exposed to E2 or FAD, but was significantly up- or down-regulated by E2 or FAD implantation, respectively ($P < 0.05$). Although the *foxl2* expression in the combined E2 + FAD treatment was five-fold higher than the control, this difference was not statistically significant ($P > 0.05$), with large intra-treatment variation in expression observed. Meanwhile, *foxl2* expression was significantly higher in the combined FAD+E2 treatment than in the fish exposed to only FAD ($P < 0.05$; Fig. 3E).

At 9 weeks after implantation, *dmrt1* expression was significantly up-regulated in the FAD treatment ($P < 0.05$). In contrast, exposing barramundi to E2 significantly down-regulated expression of *dmrt1* in their gonads ($P < 0.05$). Expression of *dmrt1* mRNA in the gonads of fish implanted with the combined E2 + FAD was not significantly different from control fish. Among treatments, E2-exposed fish displayed the lowest *dmrt1* expression ($P < 0.05$; Fig. 3A). Exposure to either E2 only, or the combined E2 + FAD treatment, induced down-regulation of *cyp11b* compared to control and FAD treatments ($P < 0.05$). However, there was no significant difference in the *cyp11b* expression between E2 and E2 + FAD treatments ($P > 0.05$). Similarly, *cyp11b* expression in the FAD treatment did not significantly differ from the control fish ($P > 0.05$; Fig. 3C).

After 9 weeks, fish with E2 (only) showed down-regulation in the expression of *esr1* (approx. 8-fold decrease) ($P < 0.05$) compared to all other treatments, including the E2 + FAD treatment ($P < 0.05$). The *esr1* expression in the FAD fish and the combined E2 + FAD fish were not significantly different from the controls ($P > 0.05$). However, E2 + FAD fish had significantly lower expression of *esr1* than FAD fish ($P < 0.05$; Fig. 3B).

For the fish subjected to the combined E2 + FAD treatment, which had 42 % males and 42 % females, gene expression levels for each animal were ultimately associated to their gonadal phenotype for all genes ($P < 0.05$). In the E2 + FAD treatment, when testes were observed expression levels were similar to those of either males of control and FAD only fish, while when ovaries were observed expression levels were

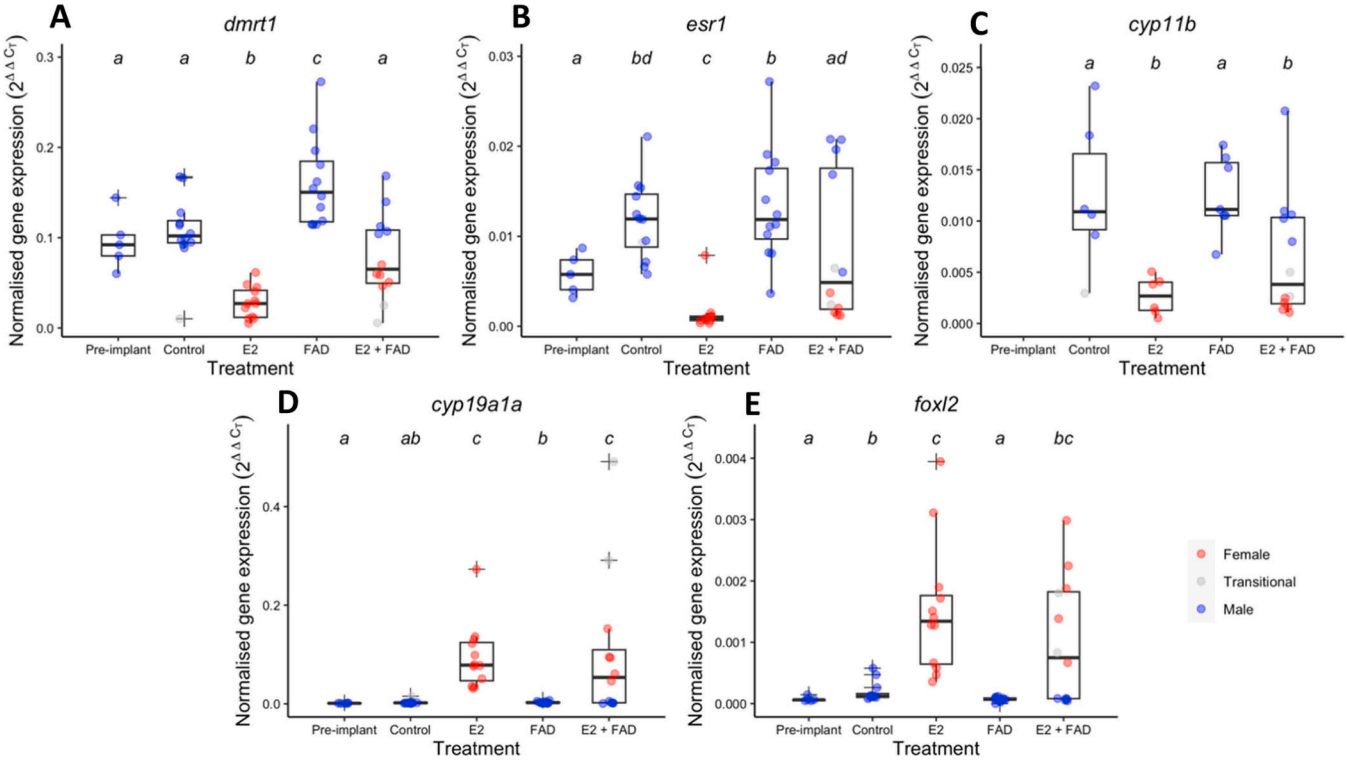

Fig. 3. Relative gene expression of different sex-related genes measured by RT- qPCR in barramundi gonads implanted with only E2, only FAD or combination of E2 + FAD sampled at 9 weeks post-implant, compared to control (no E2 or FAD) and pre-implant barramundi. **A)** *dmrt1*, **B)** *esr1,* **C)** *cyp11b,* **D)** cyp19a1a and **E)** *foxl2*. The relative expression of each target gene was normalised based on the abundance of the reference gene *ubq* according to Livak and Schmittgen (2001). Different letters represent statistical differences ($P < 0.05$) between treatments ($n = 12$ per treatment). Outliers are indicated by cross symbols.

similar to E2 females (Fig. 3).

4. Discussion

The unpredictable timing of sex-change in barramundi broodstock, presents several challenges to the efficient operation of breeding programs for this commercially important species (Budd et al., 2015; Banh et al., 2021; Fine-Idan et al., 2024). Consequently, developing methods to control sex is of critical importance to improve routine commercial production and ensure efficient operation of selective breeding programs (Robinson et al., 2014; Banh et al., 2021; Terence et al., 2021). One potential strategy involves the use of endocrine-manipulating compounds such as the aromatase inhibitor, Fadrozole (FAD) to prevent male to female sex change from occurring and encourage continued testicular development in male broodstock (Mondal et al., 2025). As such, in this study, the effect of FAD on barramundi sexual development and its potential application in achieving control over sex change in broodstock populations was evaluated. Results of this study are particularly relevant in areas where early sex change of the species has been observed. In Singapore, 81 % of farmed populations are reportedly females or transitional at 2.4 year of age, with animals changing sex at half the body weight as previously described in other regions such as Australia, western Papua and French Polynesia (Terence et al., 2021). The low numbers of reproductive males in these broodstock populations challenges the implementation of commercial selective breeding programs for the species. In such cases, the use of FAD implants in half of the fish selected before two years of age may assist to ensure sufficient males are available for breeding.

To investigate approaches that prevent sex change from occurring in a sequential hermaphrodite, a large, consistent group of transitional fish is required (Goikoetxea et al., 2021). However, such experimental groups are not easily available within natural or farmed populations of barramundi (due to large size, age and unpredictable occurrence of sex-changing animals), and therefore alternate experimental approaches are required to undertake these studies. In the current study, E2 implants were utilised to produce model precociously transitioning fish, following previous research where E2 implantation has been shown to reliably induce feminisation of male barramundi (Banh et al., 2021; Guppy et al., 2022). Here complete feminisation of E2 only implanted barramundi was observed 9 weeks after implantation in direct agreement with the previous trials in barramundi using the same E2 dose (8 mg kg^{-1} BW, (Banh et al., 2021; Guppy et al., 2022)). The use of E2-implanted barramundi as a model in the study of female and male sex differentiation is supported by the consistent success of sex change seen across repeated studies, as well as subsequent confirmation of the spawning success of E2-induced females (Guppy et al., 2022; Fine-Idan et al., 2024). Expanding upon the established utility of E2-induced feminisation for studying sex differentiation, the current study further explores the antagonistic effects of combining E2 with the aromatase inhibitor FAD to investigate their potential for controlling sex change in barramundi.

The use of FAD to prevent sex change and to increase the proportion of males in a population has been explored in several other fish species (Babiak et al., 2012; Budd et al., 2015). However, only a limited number of studies have studied the effect of a combined application of multiple endocrine-disrupting chemicals on teleost sex differentiation (Santos et al., 2006; Micael et al., 2007; Luzio et al., 2015) with a range of treatment methods (immersion, feeding, implantation) used. In our study, through applying a combined implantation of E_2 and FAD at equal doses (8 mg kg^{-1}) in barramundi, FAD was able to neutralize the feminizing effect of E2 treatment in some individuals, maintaining the testicular phenotype in ~42 % of the fish. However equal proportion of the E2 + FAD-treated fish were also observed to have undergone male-to-female sex change, while a further 18 % of individuals were at different stages of transition. The observation of both the distinct male and female sexual phenotypes in the E2 + FAD treatment provides support for the antagonistic relationship in barramundi between the aromatase inhibitors FAD and estrogens. The varied final phenotype highlights the complexity and plasticity of sex differentiation in barramundi, showing differential responses between individuals. In particular, the varied phenotype may have arisen due to several factors, potentially reflecting; inter-individual sensitivity to either E2 or FAD, differences in the initial developmental stage at treatment, genetic variation between individuals or other uncontrolled physiological factors (Segner et al., 2003; Filby et al., 2007; Brazzola et al., 2014). Further examination of these factors, alongside different doses of each endocrine disruptor, is required to fully understand the cause of differential phenotypes. To date, no other studies using combined implantation are available for comparison. Instead, the combined application of endocrine disruptors has been most commonly undertaken through immersion and/ feeding (Santos et al., 2006; Luzio et al., 2015). These studies have focused on early life stages, such as undifferentiated larvae, and have used environmentally relevant concentrations to examine ecotoxicological effects of endocrine disruptors, rather than their utility in controlling sex change. In the study of zebrafish, the combined application of 17α-ethynylestradiol (EE2; EE2 3.5 ng L^{-1}) through immersion and feeding of aromatase inhibitor tributyltin (25 ng g^{-1} or 100 ng g^{-1}) resulted in ~80 % and ~95 % feminisation respectively (Santos et al., 2006). Similarly, the combined treatment of zebrafish (*Danio rerio*) of EE2 (4 ng L^{-1}) and FAD (50 μg FAD L^{-1}) immersion in another study, induced masculinisation in 90 % of individuals (Luzio et al., 2015). When compared, it could be assumed that FAD is more effective at mitigating the feminisation of estrogen than tributyltin and therefore a better option for sex control; however, equally it must be considered that the contrasting results of aromatase inhibitor compounds may also result from unoptimized doses of the aromatase inhibitor compounds compared to the EE2 dose applied. In future studies, multiple doses of aromatase inhibitors should be assessed to more comprehensively examine their potential use for sex control, and also ensure multiple antagonistic doses are tested to understand dose-specific effects.

In this study FAD implants were also applied to male barramundi without the simultaneous use of exogenous E2, to assess the impact of FAD treatment alone on normal male development. As expected from previous research on other species including chinook salmon (*Oncorhynchus tshawytscha*; (Piferrer et al., 1994a)), Japanese flounder (*Paralichthys olivaceus*; (Kitano et al., 2000)), medaka (*Oryzias latipes*; (Paul-Prasanth et al., 2013), and zebrafish (*Danio rerio;* (Takatsu et al., 2013)), 100 % of barramundi implanted with FAD had testicular gonad tissue at the conclusion of the experiment. Similar effects were observed in Nile tilapia (*Oreochromis niloticus*), where oral treatment with FAD (500 mg kg^{-1}) resulted in complete phenotypic masculinisation of genetically female (XX) fry (Kwon et al., 2002). FAD also effectively induced female-to-male sex changes in several 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), coral goby *Gobiodon erythrospilus (*Kroon et al., 2005*)*, honeycomb grouper (Bhandari et al., 2004a, 2004b), white grouper (*Epinephelus aeneus*; (Evliyaoğlu et al., 2019)), red-spot grouper (*Epinephelus akaara* (Li et al., 2006)). Beyond the effect on the gonadal phenotype, FAD treatment has been seen to influence genes involved in sex differentiation and reproduction processes more broadly.

In this study FAD treatment resulted in higher expression of the pro-male gene *dmrt1* in treated fish than in control male individuals. Overexpression of *dmrt1* has been observed in a number of studies of aromatase inhibitors (Smith et al., 2003; Wang Qi et al., 2012; Wang et al., 2017; Yan et al., 2021). Likewise, in previous studies, the inhibition of aromatase by FAD has prevented endogenous E2 synthesis (Schroeder et al., 2017), which removes the inhibitory feedback effect that E2 has on *dmrt1* expression (Fernandino et al., 2009; Herpin and Schartl, 2011; Wang et al., 2017). Importantly, *dmrt1* also acts as a transcription factor that directly impedes aromatase gene expression (Guiguen et al., 2010; Herpin and Schartl, 2011). When these two regulatory relationships are

combined, they could form a continuous self-stabilising feedback loop that acts to maintain male development and repress feminising pathways. This hypothesis is supported by low expression levels of aromatase and *foxl2* seen in this study, however, long-term male or female related gene expression patterns were not able to be documented. If the over-expression of *dmrt1* in barramundi continues to be maintained on a long-term basis by natural regulatory genetic/endocrine feedback loops rather than by the presence of FAD circulating, this would indicate a single dose enables long-term suppression of aromatase and supports a persistent and self-sustained testicular phenotype. Similar effects have been observed in the protandrous black porgy (*Acanthopagrus schlegelii*), where oral administration of aromatase inhibitors (FAD and 1,4, 6-androstatriene-3,17-dione, each at 10 mg kg^{-1}) suppressed aromatase activity and inhibited the natural sex change of males to females (Lee et al., 2002). However, these results were varied depending on treatment during spawning or non-spawning season and also involved ongoing treatment with FAD for 9 months with no assessment after treatment ceased. In comparison, in earlier studies of black porgy, FAD injection was not found to be effective at suppressing gonadal aromatase expression (Lee et al., 2001). Effect of FAD treatment type, treatment over an extended treatment window, and post treatment period is required to fully explore the potential application of FAD for long-term sex-control of male barramundi.

While *dmrt1* expression was elevated in FAD treated fish, over-expression of masculinising genes was not consistently observed. In contrast, *cyp11b* expression was not higher than controls. *Cy11b* produces the enzyme, 11β-hydroxylase, which catalyzes the conversion of testosterone to precursors of the potent masculinising hormone 11-KT (Socorro et al., 2007). As production of the androgenic enzyme *cy11b* was stable in FAD-treated fish it suggests that androgen production itself may not be elevated despite the absence of aromatase/E2 activity caused by FAD implants. In this case, further regulatory feedback mechanisms may be acting to achieve stable 11β-hydroxylase production and therefore prevent the overexpression 11-KT which would act to down-regulate spermatogenesis promoting gonadotropins, FSH and LH (Yamaguchi et al., 2003). However, when androgenic hormone production was assessed more directly in Black porgy (Lee et al., 2001, 2002), elevated plasma levels of 11-KT were observed after FAD treatment, alongside the promotion of spermatogenesis and increased spermiation. In future research, the long-term effect of a single FAD dose on transcriptome-wide gene expression and regulation (e.g. epigenetics) and androgen/estrogen expression should be explored to develop a complete, network-level, understanding of FAD's ability to retain male gonad development, spermatogenesis, and spermiation in barramundi.

As observed in this study, FAD treatment has been confirmed to suppress *cyp19a1a* expression in Japanese flounder fed 0.1–1 mg kg^{-1} (Kitano et al., 2000; Yamaguchi and Kitano, 2023), zebrafish fed 0.5 mg kg^{-1} (Fenske and Segner, 2004), and Nile tilapia fed 0.5 mg kg^{-1} (Dai et al., 2024), among others. In contrast, the upregulation of *cyp19a1a* has been reported in zebrafish exposed to 100 μg L^{-1} FAD for 4 days (Villeneuve et al., 2009b), medaka exposed to 10 and 100 μg L^{-1}FAD for 7 days (Tompsett et al., 2009), Murray River rainbowfish (*Melanotaenia fluviatilis*) exposed to 50 μg L^{-1} of FAD (Shanthanagouda et al., 2014), fathead minnows exposed to 3 or 30 μg L^{-1} FAD (Villeneuve et al., 2009a) and adult western mosquito fish exposed to 2 or 30 L^{-1} of FAD (Doering et al., 2021). These previous studies have utilised contrasting concentrations of FAD, with some studies utilising immersion in low concentrations that mimic the levels (ug – ng L^{-1}) currently seen in environmental pollution (Doering et al., 2019a), while those used in an aquaculture context apply much higher doses through the direct implant of larger fish or feeding larvae during the labile period (eg. 1–10 mg per kg BW or 1–700 mg per kg feed; (Kitano et al., 2000; Kwon et al., 2000; Kwon et al., 2002; Bhandari et al., 2004a; Bhandari et al., 2004b; Hur et al., 2012)). Low-level FAD exposure may only partially prevent E2 production and as such may act paradoxically by stimulating a positive-feedback response that increases overall *cyp19a1a* expression to compensate for the decreased enzymatic activity of the aromatase protein (Villeneuve et al., 2006, 2009a; Doering et al., 2019a, 2019b, 2021). In contrast, when higher doses of aromatase inhibitors are applied to control sex-change in aquaculture, this positive feedback mechanism may be completely blocked, and as no E2 production is able to occur, and *cyp19a1a* is further downregulated by increased male-bias gene expression (Yamaguchi and Kitano, 2023; Dai et al., 2024).

It is notable that in some fish species aromatase has important testicular, metabolic and immune functions and as a result may be required for normal male development, albeit at lower levels than in females (Afonso et al., 2000; Böhne et al., 2013; Takatsu et al., 2013; Ravi et al., 2014). Although sperm quality was not assessed in this study, the gonads of FAD-treated fish showed an advanced degree of spermiation compared to controls, with enlargement of the seminiferous tubules, accompanied by an abundant accumulation of sperm in their lumina. Similar outcomes have been observed in other studies assessing FAD exposure (Afonso et al., 2000; Ankley et al., 2002; Takatsu et al., 2013) and suggests an important regulatory factor of normal male development has also been impacted by FAD treatment. In zebrafish, FAD treatment has also resulted in the production of large quantities of sperm, however, sperm heads were enlarged and did not have tails, preventing fertilisation of eggs (Takatsu et al., 2013). Once aromatase inhibitor treatment was ceased, normal sperm morphology and production were observed after 8 weeks, indicating E2 may be required for normal development (Takatsu et al., 2013). In contrast, no impact on sperm quality was observed in *cyp19a1a* knockout zebrafish (Tang et al., 2017), with this study instead suggesting that E2 may not be necessary to maintain male fertility in zebrafish. Considered together, the contrasting results of these two studies on zebrafish (Takatsu et al., 2013; Tang et al., 2017) could also suggest FAD has further biological interaction beyond preventing E2 production, such as with isoforms of *cyp11b* (Brixius-Anderko and Scott, 2019). Full exploration of the impact of FAD on sperm quality has been limited in aquaculture species, however, neomale Atlantic salmon produced by FAD treatment were found to have normal testicular morphology and fertility compared to normal males (Lee, 2005). With the different results observed within and between species, it is critical that further studies thoroughly assess the reproductive competency of individuals to ensure their utility for breeding.

Broader consideration of the physiological impacts of sex control treatments on other tissues is also important. E2 is well understood to interact with the liver of fish, driving hepatic synthesis of vitellogenin and due to this positive effect on reproductive maturation (Gupta et al., 2021), has often been the focus of sex control studies. However, further physiological interactions and histological changes to livers can also occur after E2 exposure, but are less commonly studied in sex control studies. Excessive or prolonged E2 exposure can impair liver function by disrupting lipid metabolism, inducing oxidative stress and overstimulating protein synthesis. These effects can lead to varying degrees of hepatocellular damage, inflammation and metabolic dysfunction (Thilagam et al., 2010). Here we observed low-level hyperaemia in the liver of individuals provided implants with only E2, however this was not observed in other treatments including those were E2 and FAD were implanted simultaneously. The effect of E2 only treatment observed here agrees with previous studies using the same approaches including those with the study on histological effects available for comparison. These aspects should be assessed as varied dosages of FAD and other aromatase inhibitors are tested.

When the results of this study are considered together, it is clear FAD can influence sex in barramundi; however, further research is required to comprehensively determine an optimised approach for application of FAD in achieving sex control. The incomplete suppression of sex change observed in this study could be related to several factors, and optimisation of dose, treatment schedule, and the assessment of other aromatase inhibitors should be explored and their effectiveness compared.

Further to this, given precocious females can now be produced, future research should examine the utility of aromatase inhibitors for reverting E2-induced females back into functional males to provide broodstock managers complete control of sex in their populations. Importantly, whether aromatase inhibitors are employed to block the natural sex change in barramundi or revert sex-changed individuals, the reproductive competency of broodstock and the quality offspring produced should be examined to confirm the commercial utility of these approaches.

5. Conclusions

In conclusion, administration of the aromatase inhibitor Fadrozole at 8 mg kg^{-1} was shown to promote male-biased genetic pathways, including an over-stimulation of the key male gene *dmrt1* and supported ongoing maintenance of testicular tissues. Fadrozole also suppressed the potent feminising effects of E2 in some barramundi, but was not effective at preventing E2 induced sex-change in all fish. Further research is required to fully examine the utility of aromatase inhibitors for obtaining control over sex in barramundi.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to acknowledge the Australian Research Council for funding this research with the ARC Linkage Project LP130100007. The scholarships for PhD students Quyen Banh and Jarrod Guppy were granted by the Australian Government (Australian Postgraduate Award Scholarships). Further support has been provided to Jarrod Guppy by the ARC Early Career Industry Fellowship (IE230100648). We gratefully acknowledge Kien Nguyen and Julie Goldsbury for their assistance in sampling and lab work, and JCU Marine and Aquaculture staff for assistance with experimental facilities.

Data availability

Data will be made available on request.

References

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 (2), 241–246. https://doi.org/10.1006/gcen.1996.0065.

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 (1–2), 175–187. https://doi.org/10.1016/S0044-8486(00)00335-5.

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 (1), 121–130. https://doi.org/10.1093/toxsci/67.1.121.

Babiak, J., Babiak, I., van Nes, S., Harboe, T., Haugen, T., Norberg, B., 2012. Induced sex reversal using an aromatase inhibitor, Fadrozole, in Atlantic halibut (*Hippoglossus hippoglossus L.*). Aquaculture 324-325, 276–280. https://doi.org/10.1016/j.aquaculture.2011.11.001.

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.

Banh, Q.Q.T., Domingos, J.A., Pinto, R.C.C., Nguyen, K.T., Jerry, D.R., 2020. 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). Aquac. Res. 52 (4), 1414–1430. https://doi.org/10.1111/are.14996.

Banh, Q.Q.T., Guppy, J.L., Domingos, J.A., Budd, A.M., Pinto, R.C.C., Marc, A.F., Jerry, D.R., 2021. Induction of precocious females in the protandrous barramundi (*Lates calcarifer*) through implants containing 17β-estradiol - effects on gonadal morphology, gene expression and DNA methylation of key sex genes. Aquaculture 539, 736601. https://doi.org/10.1016/j.aquaculture.2021.736601.

Bhandari, R.K., Higa, M., Nakamura, S., Nakamura, M., 2004a. Aromatase inhibitor induces complete sex change in the protogynous honeycomb grouper (*Epinephelus merra*). Mol. Reprod. Dev. 67 (3), 303–307. https://doi.org/10.1002/mrd.20027.

Bhandari, R.K., Komuro, H., Higa, M., Nakamura, M., 2004b. Sex inversion of sexually immature honeycomb grouper (*Epinephelus merra*) by aromatase inhibitor. Zool. Sci. 21 (3), 305–310.

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 (10), 2268–2285. https://doi.org/10.1093/molbev/mst124.

Brazzola, G., Chèvre, N., Wedekind, C., 2014. Additive genetic variation for tolerance to estrogen pollution in natural populations of Alpine whitefish (*oregonus sp., Salmonidae*). Evolut. Appl. 7 (9), 1084–1093. https://doi.org/10.1111/eva.12216.

Brixius-Anderko, S., Scott, E.E., 2019. Structure of human cortisol-producing cytochrome P450 11B1 bound to the breast cancer drug fadrozole provides insights for drug design. J. Biol. Chem. 294 (2), 453–460. https://doi.org/10.1074/jbc.RA118.006214.

Budd, A.M., Banh, Q.Q., Domingos, J.A., Jerry, D.R., 2015. Sex control in fish: approaches, challenges and opportunities for aquaculture. J. Mar. Sci. Eng. 3 (2), 329–355.

Budd, A.M., Guppy, J.L., Thépot, V., Domingos, J.A., Jerry, D.R., 2022. Delayed effect of low rearing temperature on gonadal DNA methylation in juvenile barramundi (*Lates calcarifer*). Aquaculture 560, 738541. https://doi.org/10.1016/j.aquaculture.2022.738541.

Dai, S., Li, M., Yuan, J., Wei, X., Ma, E., Wang, D., Li, M., 2024. dmrt1 Is responsible for androgen-induced masculinization in Nile Tilapia. Genes (Basel) 15 (9). https://doi.org/10.3390/genes15091238.

Davis, T., 1982. Maturity and sexuality in barramundi, *Lates calcarifer* (Bloch), in the Northern Territory and south-eastern Gulf of Carpentaria. Mar. Freshw. Res. 33 (3), 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. https://doi.org/10.1007/s10126-010-9277-z.

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. https://doi.org/10.1016/S0044-8486(02)00057-1.

Doering, J.A., Villeneuve, D.L., Poole, S.T., Blackwell, B.R., Jensen, K.M., Kahl, M.D., Kittelson, A.R., Feifarek, D.J., Tilton, C.B., LaLone, C.A., 2019b. Quantitative response-response relationships linking aromatase inhibition to decreased fecundity are conserved across three fishes with asynchronous oocyte development. Environ. Sci. Technol. 53 (17), 10470–10478.

Doering, J.A., Villeneuve, D.L., Fay, K.A., Randolph, E.C., Jensen, K.M., Kahl, M.D., LaLone, C.A., Ankley, G.T., 2019a. Differential sensitivity to in vitro inhibition of cytochrome P450 aromatase (CYP19) activity among 18 freshwater fishes. Toxicol. Sci. 170 (2), 394–403.

Doering, J.A., Villeneuve, D.L., Tilton, C.B., Kittelson, A.R., Blackwell, B.R., Kahl, M.D., Jensen, K.M., Poole, S.T., Cavallin, J.E., Cole, A.R., Dean, K.N., LaLone, C.A., Ankley, G.T., 2021. Assessing effects of aromatase inhibition on fishes with group-synchronous oocyte development using western mosquitofish (*Gambusia affinis*) as a model. Aquat. Toxicol. 232, 105741. https://doi.org/10.1016/j.aquatox.2020.105741.

Domingos, J.A., Budd, A.M., Banh, Q.Q.T., 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), e0204182. https://doi.org/10.1371/journal.pone.0204182.

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), 1–8. https://doi.org/10.1046/j.1432-0436.1994.5810001.x.

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 (10), 1453–1458.

Elbrecht, A., Smith, R.G., 1992. Aromatase enzyme activity and sex determination in chickens. Science 255. https://doi.org/10.1126/science.1734525.

Evliyaoğlu, E., Eroldoğan, O.T., Yılmaz, H.A., Genç, M.A., Genç, E., Duncan, N., Aktaş, M., Güroy, D., 2019. Artificial sex reversal of white grouper (*Epinephelus aeneus*) utilizing aromatase inhibitor (Fadrozole). Aquac. Res. 50 (5), 1539–1546. https://doi.org/10.1111/are.14030.

Fenske, M., Segner, H., 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (*Danio rerio*). Aquat. Toxicol. 67 (2), 105–126. https://doi.org/10.1016/j.aquatox.2003.10.008.

Fernandino, J., Hattori, R., Shinoda, T., Kimura, H., Strobl-Mazzulla, P., Strüssmann, C., Somoza, G., 2009. 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. https://doi.org/10.1016/j.aquatox.2006.12.003.

Fine-Idan, A., Levavi-Sivan, B., Aizen, J., 2024. Induction of precocious females in the protandrous barramundi (*Lates calcarifer*) with long-acting estradiol implants. Aquac. Int. 32 (4), 4581–4596. https://doi.org/10.1007/s10499-024-01391-w.

Goikoetxea, A., Muncaster, S., Todd, E.V., Lokman, P.M., Robertson, H.A., De Farias, E. M.C.E., Damsteegt, E.L., Gemmell, N.J., 2021. A new experimental model for the

investigation of sequential hermaphroditism. Sci. Rep. 11 (1), 22881. https://doi.org/10.1038/s41598-021-02063-y.
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. Fishes 39. https://doi.org/10.1007/bf00005126.
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. https://doi.org/10.1016/j.ygcen.2009.03.002.
Guppy, J.L., Marc, A.F., Jerry, D.R., 2022. Maturation and spawning performance of hormonally-induced precocious female barramundi (*Lates calcarifer*) and implications of their use in selective breeding. Aquaculture 552, 737991. https://doi.org/10.1016/j.aquaculture.2022.737991.
Gupta, G., Kumar, M., Rani, S., Mohanta, B., 2021. Vitellogenesis and their endocrine control in fishes. In: Sundaray, J.K., Rather, M.A., Kumar, S., Agarwal, D. (Eds.), Recent updates in molecular Endocrinology and Reproductive Physiology of Fish: An Imperative step in Aquaculture. Springer Singapore, Singapore, pp. 23–34.
Herpin, A., Schartl, M., 2011. Dmrt1 genes at the crossroads: a widespread and central class of sexual development factors in fish. FEBS J. 278 (7), 1010–1019. https://doi.org/10.1111/j.1742-4658.2011.08030.x.
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 (1), 149–150. https://doi.org/10.1023/B:FISH.0000030505.28138.d1.
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. Anim. Cells Syst. 16 (2), 127–134. https://doi.org/10.1080/19768354.2011.607515.
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), 1–5. https://doi.org/10.1002/(SICI)1098-2795(200005)56:1;2-3.
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 (2), 273–283.
Kroon, F.J., Munday, P.L., Westcott, D.A., Hobbs, J.-P.A., Liley, N.R., 2005. Aromatase pathway mediates sex change in each direction. Proc. R. Soc. B Biol. Sci. 272 (1570), 1399–1405. https://doi.org/10.1098/rspb.2005.3097.
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 (1), 46–53. https://doi.org/10.1002/1097-010X(20000615)287:1;2-X.
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 (3), 625–636. https://doi.org/10.1111/j.1095-8649.2002.tb01689.x.
Lee, P., 2005. University of Tasmania. Control Sex. Invers. Female Atl. Salmon (Salmo salar).
Lee, Y.H., Du, J.L., Yen, F.P., Lee, C.Y., Dufour, S., Huang, J.D., Sun, L.T., Chang, C.F., 2001. Regulation of plasma gonadotropin II secretion by sex steroids, aromatase inhibitors, and antiestrogens in the protandrous black porgy, *Acanthopagrus schlegeli* Bleeker. Comp. Biochem Physiol. B Biochem Mol. Biol. 129 (2-3), 399–406. https://doi.org/10.1016/s1096-4959(01)00337-2.
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 (6), 1749–1754. https://doi.org/10.1095/biolreprod66.6.1749.
Li, G.-L., Liu, X.-C., Zhang, Y., Lin, H.-R., 2006. Gonadal development, aromatase activity and P450 aromatase gene expression during sex inversion of protogynous red-spotted grouper *Epinephelus akaara* (Temminck and Schlegel) after implantation of the aromatase inhibitor, fadrozole. Aquac. Res. 37 (5), 484–491. https://doi.org/10.1111/j.1365-2109.2005.01453.x.
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. https://doi.org/10.1006/meth.2001.1262.
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. https://doi.org/10.1016/j.aquatox.2015.07.015.
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 (ement C), 90–105. https://doi.org/10.1016/j.aquatox.2016.03.014.
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 (8), 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? Oncologist 13 (8), 829–837. https://doi.org/10.1634/theoncologist.2008-0055.
Mondal, R.C., Guppy, J.L., Villacis-Escobar, M.A., Jerry, D.R., 2025. Beyond the binary: controlling natural sex-change in hermaphroditic fishes. Rev. Aquac. 17 (4), e70066. https://doi.org/10.1111/raq.70066.
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.
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. https://doi.org/10.1042/bj1470367.
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.
Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture 197 (1–4), 229–281. https://doi.org/10.1016/S0044-8486(01)00589-0.
Piferrer, F., Blázquez, M., 2005. Aromatase distribution and regulation in fish. Fish. Physiol. Biochem. 31 (2-3), 215–226.
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 (3), 255–262. https://doi.org/10.1002/jez.1402700304.
Piferrer, F., Zanuy, S., Carrillo, M., Solar, I.I., Devlin, R.H., Donaldson, E.M., 1994a. Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males. J. Exp. Zool. 270 (3), 255–262. https://doi.org/10.1002/jez.1402700304.
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), 5.
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 (3), 314–326. https://doi.org/10.1006/gcen.1995.1162.
Robinson, N., Loughnan, S., Calogeras, C., 2014. Dev. a Barramundi Sel. Breed. Entity II.
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, p. 58. Project No. 2008/758.
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. Pharm. 142 (1-2), 151–155.
Schroeder, A.L., Ankley, G.T., Habib, T., Garcia-Reyero, N., Escalon, B.L., Jensen, K.M., Kahl, M.D., Durhan, E.J., Makynen, E.A., Cavallin, J.E., Martinovic-Weigelt, D., Perkins, E.J., Villeneuve, D.L., 2017. Rapid effects of the aromatase inhibitor fadrozole on steroid production and gene expression in the ovary of female fathead minnows (*Pimephales promelas*). Gen. Comp. Endocrinol. 252, 79–87. https://doi.org/10.1016/j.ygcen.2017.07.022.
Segner, H., Navas, J.M., Schäfers, C., Wenzel, A., 2003. Potencies of estrogenic compounds in in vitro screening assays and in life cycle tests with zebrafish in vivo. Ecotoxicol. Environ. Saf. 54 (3), 315–322. https://doi.org/10.1016/S0147-6513(02)00040-4.
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 (2), 270–280.
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 (3), 347–353. https://doi.org/10.1016/0960-0760(95)00192-1.
Smith, C.A., Katz, M., Sinclair, A.H., 2003. DMRT1 Is upregulated in the gonads during female-to-male sex reversal in ZW chicken embryos. Biol. Reprod. 68 (2), 560–570. https://doi.org/10.1095/biolreprod.102.007294.
Socorro, S., Martins, R.S., Deloffre, L., Mylonas, C.C., Canario, A.V.M., 2007. A cDNA for European sea bass (*Dicentrachus labrax*) 11β-hydroxylase: Gene expression during the thermosensitive period and gonadogenesis. Gen. Comp. Endocrinol. 150 (1), 164–173. https://doi.org/10.1016/j.ygcen.2006.07.018.
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 (1), 147–161. https://doi.org/10.1016/0039-128X(83)90068-5.
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. https://doi.org/10.1038/srep03400.
Tang, H., Chen, Y., Liu, Y., Yin, Y., Li, G., Guo, Y., Liu, X., Lin, H., 2017. New insights into the role of estrogens in male fertility based on findings in aromatase-deficient zebrafish. Endocrinology 158 (9), 3042–3054. https://doi.org/10.1210/en.2017-00156.
Terence, C., Domingos, J.A., Uichanco, J.A., Monteil, Y., Monteil, L., De Braux, E., Banh, Q.Q.T., Gibson-Kueh, S., Jerry, D.R., 2021. Early sex change of barramundi *Lates calcarifer* farmed in Singapore. Aquac. Rep. 20, 100712. https://doi.org/10.1016/j.aqrep.2021.100712.
Thilagam, H., Gopalakrishnan, S., Qu, H.-D., Bo, J., Wang, K.-J., 2010. 17β estradiol induced ROS generation, DNA damage and enzymatic responses in the hepatic tissue of Japanese sea bass. Ecotoxicology 19 (7), 1258–1267. https://doi.org/10.1007/s10646-010-0510-3.

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. Saf. 72 (4), 1257–1264.

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 (3), 353–368. https://doi.org/10.1016/j.aquatox.2005.10.016.

Villeneuve, D.L., Mueller, N.D., Martinović, D., Makynen, E.A., Kahl, M.D., Jensen, K.M., Durhan, E.J., Cavallin, J.E., Bencic, D., Ankley, G.T., 2009a. Direct effects, compensation, and recovery in female fathead minnows exposed to a model aromatase inhibitor. Environ. Health Perspect. 117 (4), 624–631. https://doi.org/10.1289/ehp.11891.

Villeneuve, D.L., Wang, R.L., Bencic, D.C., Biales, A.D., Martinović, D., Lazorchak, J.M., Toth, G., Ankley, G.T., 2009b. 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 (8), 1767–1782.

Wang, F., Jia, Y.-F., Wang, P., Du, Q.-Y., Chang, Z.-J., 2017. Effects of letrozole on gonad differentiation of carp (*Cyprinus carpio*). Pak. J. Zool. 49 (6), 1969–1981.

Wang Qi, W.Q., Guo ZhengLong, G.Z., Li YanShen, L.Y., Yun Dan, Y.D., Huang Bo, H.B., Zhou ZhongLiang, Z.Z., 2012. Effects of letrozole on gonadal differentiation and related gene expression in gonad of *Takifugu obscurus*. Chinese. J. Zool. 47 (5), 16–23.

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 (3), 316–322. https://doi.org/10.1006/gcen.1995.1115.

Yamaguchi, S., Gen, K., Okuzawa, K., Kumakura, N., Kagawa, H., Matsuyama, M., 2003. Regulation of gonadotropin subunit genes expression by 11-ketotestosterone during early spermatogenesis in male red seabream, *Pagrus major*. Fish. Physiol. Biochem. 28 (1), 111–112. https://doi.org/10.1023/B:FISH.0000030494.13499.06.

Yamaguchi, T., Kitano, T., 2023. Amh/Amhr2 signaling causes masculinization by inhibiting estrogen synthesis during gonadal sex differentiation in Japanese flounder (*Paralichthys olivaceus*). Int. J. Mol. Sci. 24 (3), 2480.

Yan, H., Shen, X., Jiang, J., Zhang, L., Yuan, Z., Wu, Y., Liu, Q., Liu, Y., 2021. Gene expression of *Takifugu rubripes* gonads during AI- or MT-induced masculinization and E2-induced feminization. Endocrinology 162 (10). https://doi.org/10.1210/endocr/bqab068.