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Title: 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.

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Abstract

Sex control is vital for the efficient breeding of aquaculture species. Barramundi (*Lates calcarifer*) is a protandrous sequential hermaphrodite that naturally sex change from male to female over its lifespan. The induction of precocious female barramundi will permit breeding of males and females of the same-generation individuals in selective breeding programs, increasing the rates of genetic gain, while reducing infrastructure costs. Accordingly, the efficacy of two dosages of 17β-estradiol (E_2) delivered via implants to induce the precocious female barramundi were evaluated. Six-month-old male barramundi (405 ± 50 g body weight (BW)) were given a single cholesterol-based pellet implant containing either 0 mg E_2 kg^{-1} BW (untreated control), 4 mg E_2 kg^{-1} BW ('low dose'), or 8 mg E_2 kg^{-1} BW ('high dose'). Changes in gonadal morphology and liver condition of implanted males, along with RT-qPCR and bisulfite amplicon sequencing to quantify expression profiles and DNA methylation of key male-female sex-related genes were then examined after 9 weeks post-implantation. Results showed that at 9 weeks post-implantation, in the 'high dose' E_2 treatment group, 78% (7/9) of fish sex-changed completely to female, signified by gonads containing oocytes (20-30 μm) and no observed residual sperm. Comparably, 44% (4/9) of fish in the 'low dose' E_2 treatment group had sex-changed, while remaining fish showed complete testicular regression with gonads containing only undifferentiated germ cells. In the 'high dose' E_2 treatment, upregulation of female-biased genes (*cyp19a1a* and *foxl2*) and downregulation of male-biased genes (*dmrt1*, *cyp11b* and *esr1*) were observed. Increased gene expression was accompanied by decreased DNA methylation in *cyp19a1a*, but no significant changes in DNA methylation of *foxl2* or *esr1* were observed. The success of artificially-induced sex change in barramundi provides an important tool that is critical to improving selective breeding of this species.

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1. Introduction

Barramundi is an important tropical farmed finfish in Southeast Asia, India, China, United States of America, Middle East and Australia (FAO, 2018). Like most aquaculture species, however, barramundi production is primarily based on genetically unimproved stocks (Domingos et al., 2013; Jerry, 2013). While commercial-scale improvement programs for barramundi are underway, their operation has proven to be challenging due to difficulties in maintaining a desired sex ratio in breeding stock.

Barramundi are protandrous sequential hermaphrodites, whereby individuals mature first as male (~2 years old) and naturally undergo sex change to female at a later age (~4-6 years old; (Davis, 1982; Guiguen et al., 1994). This sequential sex change dictates that male fish require long-term housing for several years after harvest until they undergo sex change to female, which results in substantial maintenance and infrastructure costs for hatcheries. Once sex change occurs, females are usually one generation older than males in the breeding cohort, halving the annualized rate of genetic progress that could otherwise be made through a single-generation selection program (Robinson and Jerry, 2009). As such, the development of methods to obtain precocious females, which can be bred with males of the same generation, will enable single-generation selection and eliminate current impediments (Robinson et al., 2010).

Controlled feminization has been successfully achieved in a diverse range of teleost species by taking advantage of the significant plasticity of phenotypic sex (Budd et al., 2015; Devlin and Nagahama, 2002). The most commonly used approach is to administer exogenous steroids to override the sex of the fish species, which would otherwise be determined by genetic, social or environmental factors (Piferrer, 2001). Exposure to 17 β-estradiol (E_2) has been shown to be highly effective in inducing feminization in fishes of a number of taxonomic families, namely, Cyprinidae, Anabantidae, Poecilidae, Ictaluridae, Salmonidae and Cichlidae (Pandian and Sheela, 1995; Piferrer, 2001). Sex manipulation is most efficient when fish are exposed to hormone during the labile period, a highly-sensitive sex development period when fish gonads are undifferentiated; however, this does not preclude the manipulation of sex outside this period as sex plasticity of some teleost species remains after sexual differentiation (Piferrer, 2001; Takatsu et al., 2013). For instance, feminization of testes was observed in juvenile Chum salmon (*Oncorhynchus masou*), for which sex is distinguishable by 25 day post-hatch (dph), by exposing individuals to 1 µL E_2 L^{-1} from 34-100 dph (Nakamura, 1984). Similarly, feminization of juvenile common snook (*Centropomus undecimalis*) was achieved in 90% of juveniles using feed supplemented with 100 mg kg^{-1} E_2 for 45 days (Carvalho et al., 2014) and in 100% of 3 year old male fish implanted with exogenous E_2 (Passini et al., 2016). Recently, feminization of juvenile barramundi was achieved using commercial pellets supplemented by 20 mg E_2 kg^{-1} feed from 30 – 120 dph (Banh et al., 2020). Although successful, feminization was only observed in 33-50% of treated individuals at 160 days and 12 month post-hatch, respectively.

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

The effect of estrogen across specific tissues is mediated by positive and negative feedback interactions with estrogen receptors, *esr1* and *esr2* (Chesnis et al., 2007). In the protandrous gilt-head seabream (*Sparus aurata*), expression of *esr1* is highly specific to the testis, while *esr2* is present in most tissues, but more abundantly in ovary, testis, liver, intestine and kidney (Socorro et al., 2000). Similar male-specific expression of *esr1* has been reported in medaka (*Oryzias latipes*) (Chakraborty et al., 2011), zebrafish (Menuet et al., 2002), Atlantic croaker (*Micropogonias undulatus*) (Hawkins et al., 2000) and barramundi (Ravi et al., 2014). Conversely, *esr1* expression is less in testicular tissue than in ovarian tissue of the protandrous black porgy (*Acanthopagrus schlegelii*) (Chakraborty et al., 2011; Lee et al., 2001), protogynous orange-spotted grouper (*Epinephelus coioides*) (Chen et al., 2011) and differs throughout gonadal development and gametogenesis. For instance, expression of *esr1* is low during early ovarian development (pre-vitellogenic stage), but increases in the matured ovary of goldfish (*Carassius auratus*) (Choi and Habibi, 2003), rainbow trout (*Oncorhynchus mykiss*) (Nagler et al., 2000) and eel (*Anguilla* sp.) (Lafont et al., 2016). As the expression of *esr1* has been shown to be species-specific, examining its regulation in barramundi induced to sex change may help to clarify its role in the sex changing process of this particular species.

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The activation and suppression of many genes involved in sex differentiation is underpinned by epigenetic mechanisms, the most well studied of which is DNA methylation (reviewed in Piferrer (2013)). In vertebrates, DNA methylation involves the physical attachment of a methyl group to the DNA, occurring almost exclusively at CpG dinucleotides; i.e. where cytosine's (C's) are phosphate-bonded to guanidine's (G's). The methylation of CpG sites often leads to decreased accessibility of the target gene to transcriptional machinery and causes decreases in gene expression (Gardiner-Garden and Frommer, 1987). In cases where sex is epigenetically regulated, this inverse relationship between methylation and expression implies that DNA methylation in ovarian tissue will be low for female-related genes and expression levels will be high, whereas DNA methylation will be high for male-related genes and expression levels will be low; the opposite pattern is implied for testicular tissue (Piferrer et al., 2019). Differences in DNA methylation have been associated with the sex biased expression of genes in many commercially important fish species such as European seabass (*Dicentrarchus labrax*), half-smooth tongue sole (*Cynoglossus semilaevis*) and Nile tilapia (*Oreochromis niloticus*), particularly in response to temperature-induced changes in sexual phenotype (Navarro-Martín et al., 2011; Shao et al., 2014; Wang et al., 2019). The relationship between hormone-induced changes in sexual phenotype, gene expression and DNA methylation in fish, however, has been the subject of far fewer studies and, as a result, is unclear (see Navarro-Martín et al. (2011) and Fan et al. (2017) for examples).

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

2. Material and methods

2.1. Experimental design

All the experiments conducted for this study were approved by the Animal Ethics Committee of James Cook University (Approval No. A2014).

2.1.1. E_2 'dosage-range-finding' trial

An E_2 hormone implantation 'dosage-range-finding' trial was initially conducted on 18 male barramundi (6.5 ± 0.8 kg BW) individually tagged with passive integrated responder (PIT) tags. The fish were stocked in three 2,500 L tanks (n = 6 fish/tank) connected to a 13,500 L closed freshwater recirculation system. All experimental systems were equipped with two cartridges of activated carbon (8-10 kg activated carbon /cartridge) to absorb potential hormones in the water. Activated carbon (Acticarb GC1200, Activated Carbon Technologies Pty Ltd., Australia) was replaced every 2 weeks. Two fish in each of the three tanks received cholesterol pellets, containing either 0 mg E_2 kg^{-1} body weight (BW) (n = 6), 10 mg E_2 kg^{-1} BW (n = 6) or 20 mg E_2 kg^{-1} BW (n = 6), implanted into the peritoneal cavity. Control fish were implanted a 'dummy' cholesterol pellet that did not contain hormone. Preparation of E_2 hormonal pellets and the implantation procedure is described in *Section 2.3*.

Prior to implantation, three fish from the same cohort of fish as those subsequently implanted were randomly chosen and sacrificed to sample gonad tissue for histological analysis and confirm that fish used at the start of the trial were all males. All fish had testis at stage M3, containing mostly spermatids and spermatozoa, as defined in Guiguen et al. (1994). At days 15 to 20 post-implantation, mortality was observed in four fish treated with 20 mg E_2 kg^{1} BW and three fish treated with 10 mg E_2 kg^{-1} BW, suggesting both E_2 dosage rates were too high.

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Gonads and livers from deceased animals were collected and subjected to histological examination. After consideration for the health of remaining fish, they were sacrificed 20 days post-implantation and the gonads and livers collected for histology. The gonads of E_2 treated fish, including those observed as mortalities, contained early stage oocytes (previtellogenic oocytes). The livers of the treated fish were detrimentally compromised, showing necrosis and thickened arteriole walls containing deposits (i.e. arterial hyalnosis) (see Fig.4.C for example). These pathological findings have been previously reported in studies on the effects of estrogenic compounds in fish (Herman and Kincaid, 1988; Weber et al., 2004; Zha et al., 2007). As results of the preliminary trial indicated that E_2 overdose had occurred, the primary experiment dosages were lowered (described below). This trial also proved that exogenous E_2 could induce sex change of barramundi in a freshwater environment.

2.1.2. The primary E_2 implantation experiment

In this primary experiment, male barramundi (405 ± 50 g BW, 317.1 ± 13.3 mm total length - TL) were obtained from a commercial supplier, where they were cultured in freshwater before being transferred into the acclimation system. Fish were acclimated for one week in a 13,500 L recirculating freshwater system, subsequently anesthetized with AQUI-S (Aqui-S New Zealand Ltd, New Zealand), individually PIT tagged, BW (g) and TL (mm) recorded, and then stocked into the experimental system. The experimental system consisted of nine 600 L fiberglass conical tanks receiving recirculated water at 5 L min^{-1} from a 5,000 L sump. Fish were reared in freshwater at 28-31 ^{o}C, with a stable photoperiod 12 h light and 12 h dark. Water quality parameters were checked daily and maintained within acceptable limits for barramundi (TAN < 1 mg/L; NO_2^- < 2 mg/L; pH 7.8-8.0) (Schipp et al., 2007). Fish were fed a commercial barramundi diet (Ridley Corporation) twice daily at 3% BW per day. The rearing system was equipped with two cartridges of activated carbon (8-10 kg activated carbon /cartridge) to absorb any hormone leaching into the water. Activated carbon (Acticarb GC1200, Activated Carbon Technologies Pty Ltd., Australia) in filters was replaced every 2 weeks. Water supplied to experimental tanks was sampled every week using 17 β-Estradiol ELISA kits (quantitative analysis ranges from 0.05 μg/L to1 μg/L; Ecologiena for Environmental Pollutants, Tokiwa Chemical Industries Co., Ltd., Japan) to detect residuals of E_2. No E_2 was detected in the water supplied to experimental units.

The experiment to induce feminization of barramundi consisted of two hormonal dosage rates, 4 mg E_2 kg^{-1} BW ('low dose') and 8 mg E_2 kg^{-1} BW ('high dose'), and a control group ('untreated control': 0 mg E_2 kg^{-1} BW). Eighteen individuals were randomly assigned to each group. Estrogen-exposed fish in the 'low dose' treatment group received cholesterol pellet implants containing 1.6 mg E_2 that achieved an average effective dose of 4 mg E_2 kg^{-1} BW. Likewise, fish in the 'high dose' treatment group received a cholesterol pellet with 3.2 mg E_2 that achieved an average effective dose of 8 mg kg^{-1} BW. Controls also received a cholesterol implant that contained no E_2 hormone. Following implantation (procedure described below), two fish from each treatment group were placed in each of the nine tanks to eliminate any unexpected potential tank effects on gonadal development.

2.2. Samplings

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

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Fish were euthanized by immersion in AQUI-S solution until reaching stage IV anesthesia (Coyle et al., 2004), followed by subsequent cervical dislocation. Gonad and liver tissues were collected for histological examination (morphology and condition) at all samplings. At 9 weeks post-implantation, gonad tissues were sampled for histology, gene expression and DNA methylation analyses as indicated in Fig. 1. For nucleic acid (DNA and RNA) isolation, sampled tissues were cut into small pieces (less than 2 mm), preserved in RNAlater^{TM} Stabilization Solution (Thermo Fisher Scientific), and incubated at 4 ^{o}C overnight prior to storage at -20 ^{o}C. Tissue preservation for histological analyses are mentioned in detail in the *Section 2.4*.

2.3. Hormone pellet preparation and implantation procedures

Standardized hormone pellet implants (2.3 mm diameter,15 mm long and 20 mg each), containing either 0, 1.6 and 3.2 mg E_2, were prepared according to Lee et al. (1986), with some modifications. Briefly, E_2 powder was dissolved in 80% ethanol and thoroughly mixed with cholesterol (C8867, Sigma-Aldrich) and coconut oil (5% w/w). The E_2-cholesterol-coconut oil mixture was dried in a fume hood at room temperature until a paste-like consistency was achieved. Holes (2.3 mm in diameter) drilled into a sheet of 15 mm plastic acted as a mold for pellet size and shape, while a similar flat plastic sheet acted as a base. The mixture was compressed into the mold and compacted by hand with the flat end of a 2.3 mm drill bit. Once the mold was full, a sharp strike of the drill bit with a hammer expelled a compacted cylindrical pellet. Hormonal pellets were stored at 4 ^{o}C until implantation. Before the procedure, fish were anaesthetized with AQUI-S (Aqui-S New Zealand Ltd, New Zealand). Hormone pellets according to the experimental treatments were inserted into the left dorsal musculature of each fish with a RalGun pellet injector (Syndel Laboratories Ltd.). All fish successfully recovered from implantation and survived until completion of the experiment.

2.4. Histological analysis

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

Testicular and ovarian development was categorized according to Guiguen et al. (1994). Specifically, gonads classified as stage M0 were immature with no visible differentiated germ cells; M1 gonads exhibited predominance of spermatogonia; M2 gonads were filled with mostly spermatocytes and spermatids; M3 gonads contained predominantly spermatozoa; M4, also known as post-spawning, testicular lobules are devoid of spermatozoa. Transitional gonads that are classed as stages T1 and T2 corresponds to the degeneration of testicular tissue without and with ovarian tissue respectively. T3 and T4 stages are identified by the presence of ovarian tissue that is distributed less or more than 50% in the histological section, respectively. Female ovarian development stages were classed as: stage F1 when ovaries contained gonia and previtellogenic oocytes; stage F2 and F3 when less or more than 50% of the cross-section contained vitellogenic oocytes, respectively; stage F4 when oocytes were atretic. Histopathology of liver tissues was also conducted to assess the health condition of hormone-treated fish.

2.5. Nucleic acid extraction and cDNA synthesis

RNA extraction, DNAase treatment, cDNA synthesis and quality control procedures were conducted as described previously in Banh et al. (2017). Briefly, total RNA was extracted from approximately 50 mg 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, USA). For DNAse treatment, 4 µg RNA extracts were processed for each sample using a TURBO DNA-free™ kit (Invitrogen™, USA) as instructed in the manufacturer's protocol. DNAse-treated RNA was then treated with an ammonium acetate precipitation protocol for cleaning (Osterburg et al., 1975). 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 1.90 to 2.02. 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 RNA bands (28S:18S) were included for subsequent cDNA synthesis.

cDNA was synthesized using a Tetro cDNA synthesis kit (Bioline, USA). Specifically, 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 ^{o}C for 10 min, followed by 45 ^{o}C for 30 min, before a final termination cycle by incubating at 85 ^{o}C for 5 min. The cDNA was then stored at -20 ^{o}C until RT-qPCR.

Genomic DNA (gDNA) was extracted following the CTAB protocol (Doyle and Doyle, 1987) with an extended, overnight digestion with Proteinase-K and the addition of a

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phenol:chloroform:isoamyl alcohol (25:24:1) step to assist with the digestion and subsequent removal of proteins. For RNAse treatment, RNase A (Thermo Fisher Scientific) was added to a final concentration of 100 µg/ml and incubated at 37 ^{o}C for 30 min. Extracted DNA was then bead cleaned using Sera-Mag SpeedBeads (GE Healthcare) to remove highly degraded fragments. The gDNA yield and purity of all samples was assessed with a NanoDrop 1800 spectrophotometer (Nanodrop Technologies, USA) and integrity was assessed by visualisation on a 0.8 % agarose gel. The gDNA was then stored at -20 ^{o}C until DNA methylation analysis.

2.6. Gene expression and DNA methylation analysis

The gonadal expression of five genes, *dmrt1*, *cyp11b*, *esr1*, *cyp19a1a* and *foxl2*, and DNA methylation of three genes, *esr1*, *cyp19a1a* and *foxl2*, were studied in barramundi on completion of the 9-week treatment period.

RT-qPCR was optimized and performed to compare the level of target gene mRNA expression in the gonads of nine fish from each treatment. Primers from *dmrt1*, *cyp11b*, *esr1*, *cyp19a1a* and *foxl2* and the validated reference gene *ubq* (ubiquitin) were derived from previous studies (De Santis et al., 2011; Domingos et al., 2018; Ravi et al., 2014) (Table 1). Reaction efficiencies (E) for each gene were validated using standard curves prepared from serially diluted *c*DNA (E = 0.98-1.03, $R^2 \geq 0.99$).

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

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

To analyze DNA methylation patterns in *dmrt1, esr1, cyp19a1a* and *foxl2*, bisulfite amplicon sequencing was performed (BSAS; Masser et al. (2013)). Approximately 500 ng of extracted gDNA was subject to bisulphite treatment using EZ DNA Methylation-Gold™ (Zymo Research) following the manufacturer's instructions. Primers were derived from previous studies (Table 1) (Budd, 2020; Domingos et al., 2018) and PCR amplification was carried out using Platinum® Taq DNA Polymerase (Thermo Fisher Scientific) following the manufacturer's instructions with an annealing temperature of 57.5 °C. PCR products were purified using Sera-Mag SpeedBeads as described above and quantified using QuantiFluor (Promega) fluorometric nucleic acid quantitation on an EnSpire Multimode plate reader (PerkinElmer). Library preparation was adapted from a 16s metagenomic sequencing library preparation protocol (Illumina, 2013) with the modifications and analysis performed as described in Budd (2020). *Dmrt1* reactions were observed to amplify poorly during PCR amplification, generating an insufficient number of sequence reads for further analysis, and as such were excluded.

2.7. Statistical analysis

For RT-qPCR, statistical analyses were performed using the SPSS software package (IBM SPSS Statistics 23). All samples were run in triplicate. 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 outliers removed. One-way analysis of variance (ANOVA) and Post-hoc Tukey's test were used to determine differences among hormonal treatments on gene expression of *dmrt1*, *cyp19a1a* and *foxl2*. Normalized C_T values of the gene *cyp11b* and *esr1* that did not present heterogeneous variances after transformation were analyzed using a non-parametric Kruskal-Wallis test, followed by the Mann-Whitney U test for pairwise comparisons. Differences were regarded as statistically different at $P < 0.05$. RT-qPCR visualizations were generated in RStudio v1.2.1335 (Allaire, 2012) with packages ggplot2 v3.1.0 (Wickham, 2016).

For BSAS data, all statistical analyses were carried out using RStudio v1.2.1335 (Allaire, 2012). To overcome heterogeneity of variance issues associated with proportional data generated by BSAS, methylation values were subject to logit transformation using the *logit* function from *car* v3.0-9 (Fox et al., 2012). Subsequent analyses of variance and multiple pair-wise comparisons using Tukey's Honest Significant Difference with Bonferroni corrections were carried out using the following functions from *stats* v3.4.2 and *multcomp* v1.4-8 in the following format: *glht*(*aov*(methylation ~ treatment)), *linfct* = *mcp*(*Treatment* = “Tukey”), *test* = *adjusted*(“Bonferonni”) (Hothorn et al., 2016). Box plots were drawn using ggplot2 v3.1.0 (Wickham, 2016).

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3. Results

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

Sampling of gonads for histological analysis was conducted one-day prior to implantation of the hormone pellets (initial sampling), 4 weeks post-implantation and 9 weeks post-implantation (final sampling). Gonadal phenotype percentages of barramundi at these three time points are shown in Fig. 2.

The phenotypic sex of fish (5/5) on initial sampling was confirmed as being male, as testes contained primarily spermatocytes and spermatids and were classed as stage M2. Likewise, in all subsequent sampling events, E_2-untreated control fish possessed testes at stages M2 to M3. Specifically, at 4 weeks post-implantation two control fish had stage M3 testes, identified as lobules containing large amounts of spermatozoa; the remaining 10 fish had stage M2 testes (Fig. 3A and 3B). At final sampling, 44% (4/9) of untreated control fish had stage M3 testes and the remaining 56% (5/9) of fish had stage M2 testes (Fig. 3C and 3D).

Four weeks after the administration of E_2, barramundi, in both 'low dose' and 'high dose', treatment groups possessed morphological changes to their gonads. All the individuals in the 'low dose' treatment showed incomplete suppression of spermatogenesis, classified as transitional stage T1, where gonads contained few lobules with spermatogonia distributed along the periphery of the gonadal lamellae and residual spermatocytes (Fig. 3F). In 33% (3/9) of gonads, fibrous connective tissue was detected with some dispersed lobules of spermatogonia (Fig. 3E).

Complete testicular regression was observed in 100% (9/9) of 'high dose' E_2-treated fish at 4 weeks post-implantation. Furthermore, 56% (5/9) of fish had early stage oocytes (perinucleolar and previtellogenic oocytes) dispersed throughout the gonad (Fig. 3J). Phenotypically, the gonads of the remaining 44% (4/9) 'high dose' E_2-treated fish consisted of clusters of gonia restricted to lamella-like structures and dispersed oocytes (chromatin-nucleolus stage and perinucleolar oocytes) (Fig. 3I). Additionally, there was a high prevalence of vascularity (blood capillary formation) and basophilic cells in ‘high dose’ E_2-treated fish.

At final sampling, 78% (7/9) of individuals in the ‘high dose’ group showed complete feminization. Gonadal cross-sections contained exclusively previtellogenic oocytes (20-30 µm diameter) and there was no evidence of residual sperm or testicular tissues (Fig. 3L). Elevated vascularity was also apparent. Comparatively, individuals in the ‘low dose’ group did not ubiquitously show sex inversion; however, 44% (4/9) of gonads of fish in this group contained mostly gonia and some dispersed perinucleolar oocytes (Fig. 3I). The remaining 56% (5/9) and 22 % (2/9) of ‘low dose’ and ‘high dose’E_2-treated fish, respectively, had transitional gonads staged T2, which contained mainly spermatogonia with some dispersed perinucleolar oocytes.

Histological analysis of the anterior, middle and posterior regions of both the left and right gonads revealed morphological similarities in all untreated control fish and notable differences in the ‘low’ and ‘high’ dose E_2-treated fish. Four weeks post-implantation, the gonads of 100% (9/9) of ‘low dose’ and 33% (3/9) of ‘high dose’ E_2-treated fish exhibited complete fibrosis of the anterior region (Fig. 3E). Nine weeks post-implantation, complete fibrosis of the anterior gonadal regions was observed in 67% (6/9) of ‘low dose’ and 33% (3/9) of ‘high dose’ E_2-treated fish. Otherwise, within both ‘low’ and ‘high’ dose treatments, there were no discernible difference between the middle and posterior gonadal regions. Partially or fully formed ovarian lumen were not observed in individuals from ‘low’ or ‘high’ dose treatments, however tissue invagination was common.

To examine the potential side effects of exogenous E_2 administration, liver tissues were histologically examined. Livers of untreated control fish were typical of healthy farmed fish (i.e. with the presence of cytoplasmic lipid and vacuolization). Liver sections showed uniform hepatocytes with distinct nuclei and nucleoli, abundance of cytoplasmic lipid and vacuolization (Fig. 4D and 4G). No differences were observed between the untreated control and the 'low dose' treatment at both samplings (4 and 9 weeks post-hormone-implantation). In the 'high dose' E_2-treated group, at 4 weeks post-treatment, 67% (6/9) fish had livers with hyperemia (excess of blood in the vessels supplying an organ) (Fig. 4F). At the final sampling, all sampled livers showed no significant tissue damage regardless of the E_2 dosage (Fig. 4H and 4I).

3.2. E_2 altered gene expression profiles within barramundi gonads

The expression of the five genes (*dmrt1, cyp11b, esr1, foxl2* and *cyp19a1a*), studied by RT-qPCR, is shown in Fig. 5. No significant differences were observed in gene expression of target sex genes between fish sampled before commencement of the trial and in untreated control fish at final sampling. Conversely, regardless of the dose of exogenous hormone, the expression of all targeted sex-related genes was significantly affected by E_2 implantation ($P < 0.05$).

Specifically, E_2 significantly downregulated the mRNA expression of the known male-biased genes (*dmrt1*, *cyp11b* and *esr1*) in barramundi gonads at final sampling ($P < .05$). Gonadal expression of *dmrt1* and *esr1* was significantly higher (~two-folds) in untreated control fish than in the gonads of fish in both E_2 treatments. The expression of *dmrt1* and *cyp11b* in gonads of fish in the 'low' and 'high' dose E_2 treatment groups did not differ significantly. 'High dose' E_2 implants resulted in *cyp11b* expression levels below detectable limits of RT-qPCR, suggesting complete suppression of the gene. Meanwhile, 'low dose' E_2 partially suppressed *cyp11b* expression, resulting in only one-third-fold expression compared to the control ($P < .05$).

Significantly, the mRNA expression of female-related genes, *cyp19a1a* and *foxl2,* were upregulated in the fish with both ‘low’ and ‘high’ dose E_2 implants when compared to the control ($P < .05$). Expression of *cyp19a1a* was relatively low in all initial samples and untreated control fish at final sampling. In E_2-treated fish, *cyp19a1a* expression showed a dose-dependent response to E_2; ‘high’ E_2 dose significantly upregulated *cyp19a1a* expression by approximately three-fold compared to the ‘low’ E_2 dose. Similar gene expression patterns were observed for *foxl2.* E_2 implantation induced upregulation of *foxl2* expression by three-fold and five-fold in the ‘low’ and ‘high’ E_2 dosage groups, respectively, when compared to untreated control fish ($P < .05$).

3.3. E_2 altered DNA methylation levels within barramundi gonads

The DNA methylation levels of three genes (*esr1, foxl2* and *cyp19a1a*), studied by BSAS, are shown in Fig. 6. No significant differences in DNA methylation levels between untreated control fish and E2 implanted fish were observed for *esr* and *foxl2*. Conversely, regardless of the dose of exogenous hormone, DNA methylation of *cyp19a1a* was significantly affected by E_2 implantation ($P < 0.05$). Specifically, treatment with E_2 was associated with significant decreases in known female-biased gene *cyp19a1a* DNA methylation. DNA methylation of *cyp19a1a* was > 80% for control fish, compared to ~70 % for treated fish (Fig. 6C). DNA methylation levels in *foxl2* were very low for fish in control and both treatments (< 2 %) (Fig. 6B). DNA methylation levels in *esr1* were ~50% for all fish, with no significant differences among treatment groups (Fig. 6A).

4. Discussion

The production of precocious females is critical for the implementation of high-gain selective breeding programs in barramundi. In the present study, gonadal female sex change was achieved using exogenous estradiol pellet implantation in 78% of 'high' (8 mg E_2 kg BW^{-1}) and 44% of 'low' (4 mg E_2 kg BW^{-1}) dose hormone-treated barramundi. Microscopically, the ovaries of fish considered to have undergone sex change consisted of developing lamellae and early stage oocytes (chromatin-nucleolus and pre-vitellogenic stages). Individuals sampled prior to hormone implantation and from the control group were confirmed as male by the presence of stage M2 to M3 testes. When compared to a previous trial of orally delivered E_2 (Banh et al., 2020), this experiment obtained a higher feminization ratio with shorter treatment duration and preparation of exogenous estradiol pellets was less laborious.

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

In the present study, gonadal sex change was not observed in 100% of individuals in either treatment group at 9 weeks post-implantation; however, all E_2-treated fish showed complete suppression of testicular tissue. Similarly, morphological changes to testicular tissue that inhibit spermatogenesis, such as testicular atrophy, testis involution, spermatogenesis regression and loss of functional maturity, have been documented in fathead minnows (Panter et al., 1998) and abnormal gonadal phenotypes (involute testes, small ovaries and ovaries lacking germ cells) were induced in estuarine killifish (*Fundulus heteroclitus*) (Urushitani et al., 2002) as a result of early exposure to exogenous E_2. Injection of two year old male summer flounder (*Paralichthys dentatus*) with 1.0 and 10 mg E_2 kg BW^{-1} suppressed testicular development and resulted in regression of spermatogenic cells to primary spermatogonia (Zaroogian et al., 2001). Male barramundi, like other Perciformes, have paired and elongated testes that join caudally into a single spermatic duct. Each of the two testes are further defined by displaying a lobular arrangement of germinal tissue, with each lobule arranged radially around the testicular lumen. Through the process of sex change, barramundi ovaries form from the invagination of transitional testes followed by the progressive enclosure of invaginated spaces by connective tissues to form the ovarian cavity rather than from reformation of the sperm duct itself (Guiguen et al., 1994). Additionally, in 'high dose' E_2-treated fish, high prevalence of blood capillary formation and basophilic cells was observed, which is commonly considered as a precursor of gonads entering sex transition (Chaves-Pozo et al., 2009; Chaves-Pozo et al., 2003, Liarte et al., 2007). Fibrosis of the anterior region of 'low dose' and 'high dose' E_2-treated fish was observed however the exact drivers of which are unclear. It is possible that in response to the rapid loss of spermatogenic tissue that occurs during induction of sex change, the fibrous tissue initially allows the retention of overall organ integrity, and the fibrous tissue is infiltrated by oocytes later (Guiguen et al., 1994). Future research addressing the spawning potential of E_2 implants females should assess the implications of fibrous tissue and whether spawning performance is detrimentally impacted.

Significant differences in gene expression profiles of E_2-treated and untreated control fish showed that implantation of exogenous estrogen suppressed expression of male-related genes, *dmrt1*, *cyp11b* and *esr1*, and increased expression of female-related genes, *cyp19a1a* and *foxl2*. Among the known genes specific to ovarian differentiation (Yao, 2005), *foxl2* is highly conserved across divergent taxonomic groups from fish to humans (Baron et al., 2005; Crespo et al., 2013). In the present study, *foxl2* was minimally expressed in untreated control

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fish when compared to the 'low' and 'high' dose E_2-treated groups (three- and five-times less, respectively); conversely, administration of exogenous E_2 resulted in the upregulation of *foxl2* during early-stage ovarian development and feminization of E_2 treated barramundi. This suggests that the ovarian-specific role of *foxl2* has remained conserved in barramundi. Similar sexually dimorphic expression of *foxl2* has been reported in mammals, reptiles (Baron et al., 2005; Loffler et al., 2003; Oshima et al., 2008), and teleost species, including medaka (Nakamoto et al., 2006), rainbow trout (Baron et al., 2004), European seabass (Crespo et al., 2013) and Chinese rare minnow *Gobiocypris rarus* (Jiang et al., 2011). Differential expression has also been observed in other hermaphrodites, including protogynous rice field eels (*Monopterus albus*) (Zhang et al., 2010b), protandrous black porgy (Wu et al., 2010) and the rudimentary hermaphrodite, sparid sharpsnout seabream (*Diplodus puntazzo*) (Manousaki et al., 2014).

In accordance with *foxl2* expression, transcript levels of *cyp19a1a*, a key gene in estrogen synthesis and ovarian differentiation in various teleosts (Kitano et al., 1999; Leet et al., 2011), were detected at very low levels in untreated male barramundi. Conversely, treatment with exogenous E_2 upregulated *cyp19a1a* expression in the gonadal tissue of both 'low' and 'high' dose E_2-treated fish when compared to the controls. Higher expression of *cyp19a1a* was seen in the 'high dose' E_2-treated fish compared to the 'low dose' (three-fold increase); however, this difference was not statistically significant. The results from our study suggest the incidence of a positive feedback loop, in which estrogen-induced upregulation of *foxl2* resulted in increased expression of *cyp19a1a* and, in turn, increased the irreversible conversion of endogenous androgens to estrogens (Guiguen et al., 2010; Kazeto et al., 2004; Luckenbach et al., 2009; Piferrer, 2011). This process resulted in the accumulation of endogenous estrogen and lowered endogenous androgens, which is believed to be the determinant for sex differentiation (Piferrer, 2001) and, as a consequence, led to ovarian differentiation in E_2-treated barramundi. Our previous work involving feeding E_2 to barramundi juveniles supports this theory, as significantly dimorphic expression of *cyp19a1a* induced by dietary estrogen was maintained (~6 months) after the cessation of hormone treatment (Banh et al., 2020). Positive correlation in *foxl2* and *cyp19a1a* expression has also been observed in the gonads of goat (*Capra hircus*) (Pannetier et al., 2006; Pannetier et al., 2005), chicken (Govoroun et al., 2004), African catfish (*Clarias gariepinus*) (Sridevi et al., 2012; Sridevi and Senthilkumaran, 2011), rainbow trout (Baron et al., 2004; Vizziano et al., 2007), medaka (Nakamoto et al., 2006), Nile tilapia (Wang et al., 2007) and black porgy (Wu et al., 2008). *Foxl2* upregulates the transcription of *cyp19a1* genes, either directly or indirectly, by interacting with Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) or fushi tarazu factor 1 (FTZ-F1) (Sridevi et al., 2012; Wang et al., 2007; Yamaguchi et al., 2007). Further research investigating the expression of these genes is needed to clarify the mechanism by which *foxl2* regulates *cyp19a1a* in the protandrous barramundi.

In confirmation of an antagonistic cascade, our results demonstrated that E_2 administration downregulated the expression of known male-related genes, *dmrt1*, *cyp11b* and *esr1*. *Dmrt1* is a key regulator of male sexual development (Ferguson-Smith, 2007; Matson and Zarkower, 2012; Smith et al., 2009) and has been reported as dominantly or exclusively expressed in testes of humans, chicken, reptile and frog (Kettlewell et al., 2000; Raymond et al., 1999; Shibata et al., 2002; Smith et al., 1999; Smith and Sinclair, 2004) and teleost fishes (Berbejillo et al., 2012; Fernandino et al., 2008; Johnsen et al., 2010; Marchand et al., 2000). Suppression of *dmrt1* expression due to exposure to estrogenic compounds has also been recorded in reptiles (Murdock and Wibbels, 2006) and other teleosts (Fernandino et al., 2008; Filby et al., 2007; Kobayashi et al., 2008; Marchand et al., 2000; Schulz et al., 2007). In barramundi, *dmrt1* and *cyp11b* were found to be upregulated during early testicular differentiation (Banh et al., 2017). Male specific expression of *cyp11b*, one of the key steroidogenic enzymes, which catalyzes biosynthesis of the potent androgen 11-ketotestosterone (Kime, 1993; Kusakabe et al., 2002), was documented in Nile tilapia (Zhang et al., 2010a), sparid sharpsnout seabream (Manousaki et al., 2014), bluehead wrasse (*Thalassoma bifasciatum*) (Liu et al., 2015) and barramundi (Ravi et al., 2014). Sexually dimorphic patterns of *esr1* (i.e. higher expression in testis than ovary) were also reported in Nile tilapia (Ijiri et al., 2008; Tao et al., 2013), rainbow trout (Baron et al., 2008; Delalande et al., 2015), European sea bass (Blázquez et al., 2008), and barramundi (Ravi et al., 2014); yet, the opposite expression of *esr1* in other species (Davis et al., 2008; Lynn et al., 2008), and the regulation of other possible estrogen receptor subtypes, such as *esr2* in teleosts (Nelson and Habibi, 2013), suggests there needs to be further study on the mechanism of estrogen hormones and their receptors in sex change/differentiation of barramundi.

Administration of exogenous E_2 has the potential to alter the ratio of endogenous estrogens and androgens (Akhavan et al., 2015; Bjerregaard et al., 2008; Falahatkar et al., 2014). In rice field eel (Yuan et al., 2011), rainbow trout (Depiereux et al., 2014), common snook (Passini et al., 2016) and black porgy (Chang et al., 1995), a decrease in plasma 11-ketotestosterone in the presence of exogenous E_2 directed sex change and gonadal restructuring (e.g. testicular inhibition). Furthermore, E_2 potentially triggered feminizing feedback mechanisms that operate naturally in females by activating *foxl2* and *cyp19a1a* and, as such, interacted with the ovarian development pathway. The gene expression profile seen here in barramundi supports the genetic pathways proposed by previous studies, in which the female regulatory gene network would suppress the opposing transcriptional network (*dmrt1*, *cyp11b* and *esr1*) (Capel, 2017; Lamm et al., 2015; Liu et al., 2015; Ravi et al., 2014; Todd et al., 2016). In turn, the downregulation of *dmrt1* resulted in the suppression of *cyp11b* (Kobayashi et al., 2013; Wang et al., 2010). While experimental gene-knockdown has not yet been undertaken in barramundi, *foxl2*-deficient XX tilapia exhibited oocyte degeneration or complete sex reversal (Li et al., 2013). Furthermore, significant upregulation of *dmrt1* and *cyp11b* and downregulation of *cyp19a1a* highlights the role of *foxl2* in supporting the feminizing gene pathway.

Changes in gonadal morphology and gene expression following E_2 administration were also accompanied by changes in DNA methylation. Specifically, significant increases in steroidogenic enzyme encoding *cyp19a1a*, but not nuclear receptor *esr1* or transcription factor *foxl2*, were observed. This result does not conform to the predominant pattern that epigenetic regulation of steroidogenic enzymes largely occurs indirectly, through epigenetically induced changes in the expression of transcription factors and nuclear receptors (Martinez-Arguelles and Papadopoulos, 2010; Zhang and Ho, 2011). In fish, however, a recent meta-analysis found that DNA methylation in ovaries was significantly different between the sexes for *cyp19a1a* (10 species), but not *foxl2* (3 species) (Piferrer et al., 2019). Furthermore, the analyses showed that, *cyp19a1a* demonstrates consistent, sex-specific patterns of DNA methylation that are inversely correlated with *cyp19a1a* gene expression (Piferrer et al., 2019). The results of the present study are consistent with the findings of Piferrer et al. (2019), revealing no significant differences in DNA methylation for *foxl2*, but significant decreases in DNA methylation accompanied by increases in gene expression of *cyp19a1a* in E_2 treated and thus feminized fish. This result is also consistent with previous research on barramundi, where methylation of *cyp19a1a*, but not *foxl2, i*s significantly different in adult male and female barramundi (Domingos et al., 2018). While the expression of *esr1* is known to be epigenetically mediated in humans (Yoshida et al., 2000) and DNA methylation in *esr1* is significantly different in adult male and female barramundi (Budd, 2020), no significant changes in methylation of *esr1* were observed in response to the E_2 treatment applied here. Given the variation of *esr1* expression patterns seen across taxa (Budd, 2020), the functional role of *esr1* in barramundi is still not definitive.

Further fine scale characterization studies across cell types and various conditions are required.

The effect of E_2 treatment on DNA methylation levels in the gonads of teleost fish is largely unclear. For example, in European seabass, treatment with E_2 resulted in a significant increase in the frequency of female fish (2.5 – 90%), but no significant differences in DNA methylation of *cyp19a1a* were detected (Navarro-Martín et al., 2011). Conversely, in olive flounder, E_2 treatment was shown to result in not only an increase in female fish, but was also associated with high levels of *cyp19a1a* expression and significant changes in *cyp19a1a* DNA methylation (Fan et al., 2017). The differences in DNA methylation and gene expression between E_2 treated fish and controls, however, was variable throughout the treatment period (Fan et al., 2017). In the present study, both the expression and DNA methylation of *cyp19a1a* were significantly different from the controls under both low and high dose treatments. Whether changes in DNA methylation are a cause or a consequence of changes in gene expression is unclear, but it is likely that both situations occur (Piferrer et al., 2019). Repeated measurements of gene expression and DNA methylation throughout the treatment period and gonadal differentiation process in barramundi and other fish species would offer increased insight into the relationship between E_2 treatment, DNA methylation and gene expression.

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

5. Conclusions

In summary, this study demonstrated that young male barramundi (~6 month post-hatching, ~400 g BW) can be safely sex-changed using E_2 implants. At 9 weeks post-implantation, the feminised rate achieved with a single implant (8 mg E_2 kg^{-1} BW) was 78%. Exogenous E_2 administration influenced barramundi sex-determining networks by inducing upregulation of female (*cyp19a1a* and *foxl2*) and suppression of male genetic pathways (*dmrt1, cyp11b* and *esr1*). The E_2 induced upregulation of *cyp19a1a* was accompanied by significant decreases in *cyp19a1a* DNA methylation. Moreover, considering the adverse effects on health and survival of fish, E_2 implantation at a maximum dosage of 8 mg kg^{-1} BW is recommended. This result opens the potential for more efficient breeding systems to be applied in barramundi selection programs, which will most importantly include the mating of same-generation males and females. Further studies are required to assess the breeding potential of E_2-induced, precociously sex-changed females.

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Figure 1. Gonad sampling designation for histological and genetic analyses of barramundi implanted with different dosages of E_2

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

Figure 2. Proportion of barramundi that exhibited various gonadal phenotypes (testis, transitional stage and feminised gonad with previtellogenic oocytes) before implant and after 4 and 9 weeks of implantation with E_2 at concentrations of 4 mg kg^{-1} BW or 8 mg kg^{-1} BW

Figure 3. Transverse sections of barramundi gonads implanted with E_2 at concentrations of 4 mg kg^{-1} BW or 8 mg kg^{-1} BW at 4 weeks and 9 weeks post-implantation

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

Figure 4. Histological images of livers of barramundi implanted with E_2 at different concentrations

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

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implanted with 4 mg E_2 kg^{-1} BW at 4 weeks post-implantation. Scale bar 20 µm. **F)** Liver of the barramundi implanted with 8 mg E_2 kg^{-1} BW at 4 weeks post-implantation. Scale bar 20 µm. **G)** Liver of the control barramundi implanted with 'dummy" cholesterol pellets at 9 weeks post-implantation. Scale bar 20 µm. **H)** Liver of the barramundi implanted with 4 mg E_2 kg^{-1} BW at 9 weeks post-implantation. Scale bar 20 µm. **I)** Liver of the barramundi implanted with 8 mg E_2 kg^{-1} BW at 9 weeks post-implantation. Scale bar 20 µm. Abbreviations: n, necrosis; h, hyaline.

Figure 5. Relative gene expression of different sex-related genes measured by RT-qPCR in gonads of barramundi implanted with E_2 sampled at 9 weeks post-implantation

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

Figure 6. Methylation measured by BSAS of different sex-related genes in gonads of barramundi implanted with E_2 sampled at 9 weeks post-implantation

(A) *esr1*, **(B)** *foxl2* and **(C)** *cyp19a1a*. Different letters represent statistical differences ($P < 0.05$) between treatments (n = 8 for each value). Outlier values indicated by cross.

Table 1. Primer sequences used for RT-qPCR and BSAS to study the expression and DNA methylation of the genes *dmrt1*, *cyp11b* (RT-qPCR only), *cyp19a1a*, *esr1* and *foxl2* (both analyses) in the gonads of barramundi implanted with different dosages of E_2

Target gene	Accession	Nucleotide sequences (5'-3')[1]	References
RT-qPCR primers			
dmrt1	KR232516.1	GTGACTCTGACTGGCCCAGAG CAGCAGGTCGGACGTTCC	Ravi et al. (2014)
cyp11b	KF444447	ACACCGGGGTTCTGGGCCAG CACCGCTGTCGTGTCGACCC	Ravi et al. (2014)
esr1	KF444452	CTGCTCCAGGGTGCTGAGCC TGGCCCAGGCATCATGTGG	Ravi et al. (2014)
cyp19a1a	KR492506.1	CACTGTTGTAGGTGAGAGACA CTGTAGCCGTCTATGATGTCA	Domingos et al. (2018)
foxl2	KF444454	CAACCGCCCACCCCGATGTC CTGGGGAGCGCCATGCTCTG	Ravi et al. (2014)
ubq	XM_018704769	ACGCACACTGTCTGACTAC TGTCGCAGTTGTATTTCTGG	De Santis et al. (2011)
BSAS primers			
dmrt1	KR232516.1	FO-AAATTAAGTGTAGTAGAGTGATGTTAT RO-AAACACTAACAATCCCTCCAATTAC	Budd (2020)
esr1	KR492509.1	FO-	Budd (2020)

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		TGTGTTGTGATGTTGTTTAGGTAGAG RO- TTCCAAAAAATCCACAATAACTACC	
cyp19a1a	KR492506.1	FO-TGGTTGTTTATAAAGGGGAAGTTT RO- CCAACAACAAACAAACAAATAACATA	Domingos et al. (2018)
foxl2	KR492507.1	FO- AAAGGGTTGGGTTTATTGATTTATAA RO- ATCCAAATACCAACAAACAAAACTT	Domingos et al. (2018)

[1]*FO (5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and RO (5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) are Illumina's forward overhang (FO) and reverse overhang (RO) adapter sequences added to locus-specific primer sequences*

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Declaration of interests

☒ 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.

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Highlights

- Estradiol 2 (E_2) implantation in barramundi, at dosage rate of 8 mg kg^{-1} body weight (BW) safely induced complete gonadal sex change in 78 % of individuals after 9 weeks of treatment.
- 'Low' dose E_2 treatment (4 mg kg^{-1} BW) completely suppressed testicular development and induced feminization in 44 % of individuals.
- E_2 hormone treatments induced upregulation of female-biased genes (*cyp19a1a* and *foxl2*) and downregulation of male-biased genes (*dmrt1*, *cyp11b* and *esr1*) in both 'high' and 'low' E_2-treated barramundi.
- Increased gene expression was accompanied by decreased DNA methylation in *cyp19a1a*, but no significant changes in DNA methylation of *foxl2* or *esr1* were observed.
- Survival rates were 100% for all treatment groups, with no significant damage observed in liver at nine weeks post-estrogen implantation.

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