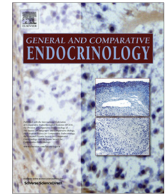
General and Comparative Endocrinology 192 (2013) 170–180

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

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



Involvement of pituitary gonadotropins, gonadal steroids and breeding season in sex change of protogynous dusky grouper, *Epinephelus marginatus* (Teleostei: Serranidae), induced by a non-steroidal aromatase inhibitor

Carlos Eduardo de O. Garcia [a,c], Bruno C. Araújo [a,c], Paulo H. Mello [a,c], Amanda de M. Narcizo [a], Jandyr A. Rodrigues-Filho [b,c], Andreone T. Medrado [a], Ricardo A. Zampieri [a], Lucile M. Floeter-Winter [a], Renata Guimarães Moreira [a,c,*]

[a] *Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Trav. 14, 321, São Paulo 05508-090, SP, Brazil*
[b] *Universidade Estadual Paulista "Júlio de Mesquita Filho", Centro de Aquicultura (CAUNESP), Via de Acesso Prof. Paulo Donato Castelane, s/n., Jaboticabal 14884-900, SP, Brazil*
[c] *Centro de Biologia Marinha da Universidade de São Paulo (CEBIMar/USP), Rodovia Manoel Hypólito do Rego, Km. 131,5, São Sebastião CEP 11600-000, SP, Brazil*

A R T I C L E I N F O

Article history:
Available online 19 June 2013

Keywords:
Artificial sex change
Aromatase inhibitor
Gonadal steroids
Follicle-stimulating hormone
Letrozole
Luteinizing hormone

A B S T R A C T

Two experiments were performed using the aromatase inhibitor (AI) letrozole (100 mg/kg) to promote sex change, from female-to-male, in protogynous dusky grouper. One experiment was performed during the breeding season (spring) and the other at the end of the breeding season (summer). During the spring, AI promoted sex change after 9 weeks and the sperm produced was able to fertilize grouper oocytes. During the summer, the sex change was incomplete; intersex individuals were present and sperm was not released by any of the animals. Sex changed gonads had a lamellar architecture; cysts of spermatocytes and spermatozoa in the lumen of the germinal compartment. In the spring, after 4 weeks, 11ketotestosterone (11KT) levels were higher in the AI than in control fish, and after 9 weeks, coincident with semen release, testosterone levels increased in the AI group, while 11KT returned to the initial levels. Estradiol (E_2) levels remained unchanged during the experimental period. Instead of decreasing throughout the period, as in control group, 17 α-OH progesterone levels did not change in the AI-treated fish, resulting in higher values after 9 weeks when compared with control fish. *fshβ* and *lhβ* gene expression in the AI animals were lower compared with control fish after 9 weeks. The use of AI was effective to obtain functional males during the breeding season. The increase in androgens, modulated by gonadotropins, triggered the sex change, enabling the development of male germ cells, whereas a decrease in E_2 levels was not required to change sex in dusky grouper.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Teleosts show an exceptional range of reproductive strategies regarding the expression of their sexuality (Devlin and Nagahama, 2002). Many marine teleosts exhibit sequential hermaphroditism, wherein an individual changes from one sex to the other in adulthood. During this process, the gonads change from ovaries to testis (protogynous) or vice versa (protandrous); these changes are induced by social situations, behavioral events, or environmental cues (Norris, 2007; Mitcheson and Liu, 2008).

An alteration of social patterns associated with changes in the standard gonadal steroidogenesis occurs during the sex change of hermaphrodite teleosts and constitutes the physiological basis for the behavioral and sex change that occur during this natural process. Gonadal steroids, modulated by pituitary and brain hormones, play the main role in the proliferation, maintenance and maturation of male or female gonadal tissue and are crucial for the initiation and/or regulation of the sex change of sequential hermaphrodites (Bhandari et al., 2003; Frish, 2004).

In a variety of fish species, the administration of exogenous estrogens or androgens during sex differentiation to genetically male or female individuals, respectively, transforms the sexual phenotype to the opposite gender. These findings attest to the involvement of sex steroids in sex differentiation (Nakamura et al., 1998; Strüssmann and Nakamura, 2002; Singh, 2013) as a result of the effects of these steroids on germ cells (Strüssmann and Nakamura, 2002). Following the same reasoning, synthetic androgens have been used for a long time to artificially induce sex

* Corresponding author at: Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Trav. 14, 321, São Paulo 05508-090, SP, Brazil. Fax: +55 1130917568.
E-mail address: renatagm@ib.usp.br (R.G. Moreira).

0016-6480/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2013.06.012

change in protogynous groupers, but the time required for the entire process is usually quite long. The oral administration of 17α-methyltestosterone (MT) took 4 months to induce sex change in *Epinephelus fario* (Kuo et al., 1988), 7 months in *Epinephelus tauvina* (Chao and Chow, 1990) and 6 months in *Epinephelus marginatus* (Sanches et al., 2009). Implants of MT in juvenile specimens of *E. marginatus* promoted a complete sex change of the animals after 12 (Sarter et al., 2006) and 8 (Cabrita et al., 2009) weeks, respectively, from the beginning of the treatment. However, sex change achieved using MT is transient, and each year the same procedure must be performed to obtain functional males (Cabrita et al., 2009), while (Sarter et al., 2006) succeeded with a complete and permanent sex inversion using MT implant in the same species.

A nonsteroidal aromatase inhibitor (AI) and MT promoted sexual change, a decrease in estrogen levels and suppression of P450arom gene expression in the genetically female larvae of the gonochoristic teleost *Paralichthys olivaceus* (Kitano et al., 2000). Aromatase has been implicated in the sex differentiation of non-mammalian vertebrates (Piferrer et al., 2005) because it irreversibly converts androgens into estrogens; therefore, its activity determines the androgen-to-estrogen ratio in developing gonads (Navarro-Martín et al., 2009). In hermaphrodite teleosts, the administration of fadrozole, a second generation nonsteroidal AI, induced sex change in the protogynous goby, *Coryphopterus nicholsii* (Kroon and Liley, 2000) in 7 weeks. In the honeycomb grouper, *Epinephelus merra*, sex change also was obtained with fadrozole after 13 weeks during the non-breeding season (Bhandari et al., 2004a), 11 weeks during the early pre-breeding season (Bhandari et al., 2004b) and 3 weeks during the spawning season (Alam et al., 2006a) and in protogynous wrasse, *Halichoeres trimaculatus*, after just 5 days (Nozu et al., 2009).

In addition to the use of AI and synthetic androgens for promoting sex change in protogynous teleosts, some studies have shown that pituitary gonadotropins that modulate the synthesis of gonadal steroids in vertebrates (Norris, 2007) can also play a key role in this process (Zhang et al., 2007; Kobayashi et al., 2010; Murata et al., 2012). In the protandrous cinnamon clownfish, *Amphiprion melanopus,* the neurohormone gonadotropin-releasing hormone (GnRH) was also involved in the control of sex change (Kim et al., 2012). Therefore, considering the neuroendocrine control of reproduction and presumably sex change in teleosts and the seasonality that characterizes this process in fish, the efficacy of sex change, whether natural or induced, can also depend on the breeding season (Bhandari et al., 2004a,b; Alam et al., 2006a).

The dusky grouper, *E. marginatus*, is a protogynous hermaphrodite teleost characteristic of rocky bottoms. These fish mature first as females; later in adult life, the ovaries are replaced by testis, changing the fish into reproductive males (Andrade et al., 2003). In many tropical and temperate regions, overfishing and environmental degradation have been depleting wild dusky grouper populations (Rodrigues-Filho et al., 2009) and this species has been included on the Red List of Threatened Species of the International Union for the Conservation of Nature (IUCN) since 1996 Cornish and Harmelin-Vivien, 2004. In Brazil, more specifically in São Paulo State, this species was included on the list of fish that were "over-exploited or threatened by over-exploitation" (São Paulo, 2008).

Sex change in wild *E. marginatus* occurs only when they reach approximately 90 cm of total length, i.e., when the fish are 9–10 years old (Marino et al., 2001). The artificial induction of sex change can be a suitable solution to obtain males for aquaculture. However, considering that the use of MT takes six months to effectively induce sex change in this species (Sanches et al., 2009), we decided to use a third generation AI, letrozole, to artificially induce sex change in *E. marginatus* females. Recent evidence suggests the alteration in steroidogenesis that drives sex change in protogynous groupers can be influenced by pituitary gonadotropins and the breeding season. Therefore, we aimed to evaluate the involvement of gonadal steroids, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the sex change of dusky grouper and the influence of the breeding season on the efficiency of the process.

2. Materials and methods

2.1. Experimental animals

Sex change experiments with *E. marginatus* adult females were performed in captivity during two seasons of the year, spring and summer. In the south coast of Brazil, in spring, the ovaries reach the maximum development in wild populations, while the last months of the reproductive period occur in the summer (Andrade et al., 2003). For the spring experiment, 11 females were collected in the wild on the southeastern coast of Brazil and immediately transported to the Marine Biology Center of the University of São Paulo (CEBIMar-USP) (23° 49, 58'S–045° 25, 31 W) in São Sebastião, SP, Brazil. During the summer experiment, 17 females were collected in the same region and transported to the same facilities at CEBIMar-USP. To remove external parasites, all fish were treated with freshwater for 5 min before being placed into the fish tank.

Wild-caught live dusky grouper were obtained by fishing in compliance with the standards of the Technical Advisory Committee (CAT-SISBIO) established by Ordinance No. 236/2008 that is coordinated by the Ministry of Environment of Brazil. All experiments were performed in accordance with the Animal Ethics Committee of the Biosciences Institute of the University of São Paulo (Protocol No. 055/2008).

2.2. Experimental design and aromatase inhibitor implantation

After 20 days of acclimatization, fish were anesthetized with 4 g of benzocaine that was previously dissolved in ethanol (10 mL), placed into 40 L of seawater, and individually tagged (transponders, AnimallTAG®). Total length and total body weight were recorded, and approximately 2 mL of blood was collected from the caudal vein to determine plasma levels of sex steroid hormones in each animal.

The animals were then distributed into two experimental groups, the control group (C) and the aromatase inhibitor group (AI). In the spring, the main dusky grouper breeding season, fish ranging from 1900 to 2348 g and 48 to 54.5 cm (total length) were stocked in two separated circular tanks (10 m^3) at a density of 5 kg/m^3, distributed into the C group ($n = 5$) that received only the vehicle (fish oil) into the abdominal cavity and the experimental group (AI) ($n = 6$) that received the vehicle with AI, letrozole (100 mg/kg). Every 4 weeks (until semen detection), the fish were anesthetized with the same procedure previously described, blood was collected from the caudal vein and another similar dose of AI was applied following the same protocol described above. The seawater renewal rate was 100%/day, and the water temperature was monitored daily. Fish were fed *ad libitum* four times a week with sardines under a natural photoperiod and at ambient temperature. Fish oil (Henrifarm, lot. NRT014011, Netherlands) was used as a vehicle to administer the nonsteroidal aromatase inhibitor (AI), letrozole (lot: 080328, Venturepharm, Beijing, China).

In the summer, the end of dusky grouper breeding season, fish ranging from 1000 to 2250 g (total weight) and 37–51 cm (total length) were also stocked in two separated circular tanks (10 m^3) at the same density, 5 kg/m^3. The control group ($n = 8$) fish received only the vehicle (2 mL of fish oil) intraperitoneally, and the experimental group (AI) ($n = 9$) received the same amount of vehicle but with aromatase inhibitor (AI) also intraperitoneally at

a dose of 100 mg/kg of body weight to promote sexual change in *E. marginatus*.

2.3. Sampling procedures

At the end of the experiment, i.e., when the presence of semen was detected in fish from the AI group in the spring, the animals were anesthetized with the same procedure previously described. Blood was collected from the caudal vein using heparinized syringes, centrifuged at 2500*g* for 8 min, and the plasma was stored at −80 °C. The animals were killed by decapitation as recommended by the Animal Ethics Committee of the University of São Paulo (Protocol No. 055/2008), and the pituitary was collected and frozen with 500 μL of Trizol Reagent (Invitrogen Life Technologies). The gonads were removed, weighed to calculate the gonadosomatic index (GSI = [gonad weight/total body weight] × 100), fixed for a period of 20–24 h in Bouin's solution and transferred to ethanol (70 °GL) for histological analyses.

The fresh semen was evaluated in relation to volume, density and motility. To calculate the volume, semen was collected from each animal directly from the urogenital pore with an automatic micropipette by manually pressing the abdominal region; the volume was recorded. From this volume, 2 μL of fresh semen was placed on a glass slide, and under the microscope, the same volume of seawater was added. Sperm motility was visually calculated under an optical microscope with phase contrast (400× magnification), and an arbitrary scale from 0% to 100% was employed that considered all spermatozoa that presented movement in a single randomly chosen focal field with the light intensity unchanged. The sperm motility was estimated by the number of mobile sperm/total sperm present in the chosen field, multiplied times 100, and the result was presented as a percentage. To avoid subjective bias, all measurements were carried out by the same experimenter.

Another semen sample (2 μL) was collected and diluted in 2000 mL of saline formol (5%) to determine the density of cells in ten fields of the hematology Neubauer chamber (cell mL^{-1}) according to a modified protocols (Bombardelli et al., 2006; Mylonas et al., 1997). To measure spermatozoa density in the Neubauer chamber, the following formula was used:

$$\mathrm{CSPZ}(\mathrm{SPZ}\cdot\mathrm{mL}^{-1}) = \left(\sum \mathrm{SPZ}/10\,\mathrm{qc}\right) \times 25\,\mathrm{qt} \times \mathrm{dilution} \times 1000/\mathrm{chamber\ depth}\,(\mathrm{mm})$$

where $\sum$ SPZ = total number of spermatozoa counted, qc = number of squares counted (10), qt = total squares (25), chamber depth = 0.1 mm, and dilution = dilution factor of semen fixed (1000).

The remaining volume of semen was used for spermatozoa cryopreservation. Sperm was diluted 1:4 (sperm: extender) in 8% dimethylsulfoxide (DMSO) according to a modified protocol (Cabrita et al., 2009).

2.4. Spermatozoa viability

After 10 days of spermatozoa cryopreservation, oocytes from a wild female (4 kg) that received two 1000 UI/kg doses of human chorionic gonadotropin (hCG, Ovidrel, Serono) were stripped by abdominal massage, and 100 μL of thawed sperm was spread over these oocytes (58 g), mixed and activated with 100 mL of sterilized seawater. After 15 min of sperm contact with the oocytes, 1 L of sterilized seawater was added, and the eggs were allowed to stand until the complete separation of floating (viable) from non-floating (non-viable) eggs in a millimeter beaker had occurred. The percentage of buoyant eggs (buoyancy rate) was determined by calculating volumetrically the eggs that floated out of the total number of eggs. Simultaneously, seven aliquots of floating eggs were analyzed under a stereomicroscope (Leica DM 1000), and the number of eggs that entered into the subsequent phases of cell division was registered. Fertilization rate (%) was calculated by considering the number of eggs with cell divisions out of the total number of floating eggs.

2.5. Histological procedures

The gonads were dehydrated through a progressive ethanol series, infiltrated and embedded in paraplast, cross-sectioned at a thickness of 5 μm and stained with hematoxylin and eosin following the routine procedure for light microscopy. Sections were analyzed under light microscope (Leica DM 1000) to evaluate the sex change identifying the presence of female and/or male germ cells. Images were captured using a computerized system for image analysis (Camera Leica DFC 295 Software Leica Application Suite Professional-LAS, V.3).

2.6. Plasma levels of gonadal steroids

Plasma levels of estradiol-17β (E_2), testosterone (T), 17α-OH progesterone (17α-OHP) and 11ketotestosterone (11KT) were quantified by enzyme-linked immunosorbent assay (ELISA) with commercial kits (E_2, T and 17α-OHP: Interteck, Virginia, USA; 11KT: Cayman Chemical Company, Michigan, USA). The analyses were performed according to the guidelines of the manufacturer, and the absorbance measurements were performed in a microplate reader (Spectra Max 250 Molecular Devices). The detection limit of the assay was 10 pg/ml for E_2, 50 pg/ml for 17α-OHP, 50 pg/ml for T and 1.3 pg/ml for 11-KT. In our laboratory, grouper's plasma validation intra-assay and inter-assay variation were performed and the coefficients of variation (CVs) found for E_2 were 6.8% and 9.5%; for 17α-OHP were 7.4% and 15.1%; 9.8% and 19.1% for T and for 11KT the variations were 9.2% and 8.6%, respectively.

2.7. Gene expression of *fshβ*, *lhβ* and elongation factor *ef1a*

Each fish pituitary was thawed, and RNA was extracted using Trizol Reagent (Invitrogen). The pituitary RNA obtained was treated with DNase (Promega), and a new extraction of total RNA was performed using the Trizol LS Reagent (Invitrogen). The RNA pellet was dissolved in RNase-free water and quantified by its absorbance at OD_{260}/OD_{280}. To obtain the partial sequences of *fshβ* and *lhβ* from *E. marginatus*, an amplification protocol was utilized with degenerate primers for *fshβ* and *lhβ* (Parhar et al., 2003) at 9 temperatures associated with the oligonucleotides (52.7, 53.6, 54.7, 56.1, 57.7, 58.8, 60.1, 61.2, and 62.0 °C).

To obtain the partial sequences of the constitutive gene *ef1a*, two forward and reverse primers were designed from conserved regions of Serranidae species: BMC (*Epinephelus bruneus*, GenBank: JF430667.1; *Mycteroperca rosacea*, GenBank: JN574476.1; *Epinephelus coioides*, GenBank: HQ441076.1) and BSM (*E. bruneus*, *Epinephelus septemfasciatus*, GenBank: AB604609.1 and *M. rosacea*). An amplification protocol was performed at 6 temperatures associated with the oligonucleotides (55.0, 55.7, 57.8, 60.5, 63.1, and 65 °C). All the primers used for PCR are listed in Table 1.

The PCR products were separated on a 2% agarose gel, and the desired size of the bands was approximately 281 bp for *fshβ*, 260 bp for *lhβ* and 121 bp for *ef1a*. The PCR fragments of the expected size were ligated into the pGEM-T-Easy plasmid (Promega Co., Madison, WI) and transformed into competent *Escherichia coli* Sure cells (Invitrogen Life Technologies). Single colonies of cells that grew on SOB plates were selected, and the plasmid DNA was extracted by alkaline lyses using the QIAprep Spin Miniprep Kit (Qiagen, Germany) and digested with the restriction enzyme EcoRI

Table 1
Primers used to isolate the *Epinephelus marginatus fshβ*, *lhβ* and *ef1a* subunit cDNA for PCR analysis.

Gene	Sequence (5′⟶3′)
fshβ-F	ACCAACATCAGCATCMCCGT
fshβ-R	NCAGACAGCTGGGTATGT
lhβ-F	TCTSTGGAGAAGGADGGCTG
lhβ-R	CGAWGGTRCAGTCGGANGTG
ef1a-BMC-F	TCGGAGGTATTGGAACTG
ef1a-BMC-R	CCTCAGTGGTCAGGTTGC
ef1a-BSM-F	TGGTACCTCTCAGGCTGAC
ef1a-BSM-R	ACCAAGGGTGAAGGCCAG

F, sense primer; R, antisense primer. Codes used to degenerate primers (*fshβ* and *lhβ*): D = A + G + T; M = A + C; N = A + C + G + T; R = A + G; S = G + C; W = A + T.

(Fermentas Life Science) according to the manufacturers' instructions. The sizes of the inserts were checked by electrophoresis using a 1% agarose gel with 10% ethidium bromide and visualized in a UV transilluminator (Bio-Rad Gel-Doc-1000 Darkroom Documentation System).

Strands were sequenced with an ABI 3100 sequencer (Applied Biosystems) at the Institute of Chemistry, University of São Paulo, and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used according to the manufacturer's instructions. Nucleotide sequences were identified by the NCBI BLAST x program (GenBank, NCBI).

2.8. Real-time quantitative PCR (qPCR)

Once the partial sequences of *fshβ*, *lhβ* and *ef1a* were determined, internal and specific primers were designed to perform the real-time quantitative PCR (qRT-PCR) experiments. Quantification (in duplicate samples) and amplification were performed with 0.5 μL of Maxima® SYBR Green qPCR Master Mix (Fermentas Life Sciences), 0.75 μL of each primer [10 μM] (sense and antisense) and 3 μL of cDNA with water added to obtain a final volume of 25 μL. The samples were amplified using an Exicycler™96 (Bioneer) thermocycler with the following conditions: 70 °C (2 min), 95 °C (1 min), 50 cycles at 94 °C (30 s) and 60 °C (20 s). For the melting curve, the temperature increased from 60 °C to 94 °C at a rate of 0.5 °C/30 s. For normalization of the cDNA loading, all samples were run in parallel using the reference gene *ef1a*. This gene has been commonly used as a reference gene in teleost pituitary studies (Varsamos et al., 2006; Tam et al., 2011) and was shown to be the most stable reference gene in validation experiments (Olsvik et al., 2005; Infante et al., 2008). *The relative mRNA expressions of fshβ* and *lhβ* were determined by *q*RT-PCR, using the software Exicycler Diagnosis3. Copy numbers of the target genes were estimated by calculating the absolute amount of each target using standard curves performed containing 1–10^9 copies of the cloned target. The parameters of qPCR analysis data were: efficiency (E) of the reactions, slope (s) and R^2 of the standard curves. For the spring experiment samples, the parameters values were: *fshβ* $E = 105\%$, $s = -0.31$ and $R^2 = 0.99$; *lhβ* $E = 106\%$, $s = -0.32$ and $R^2 = 0.99$; and *ef1a* $E = 96\%$, $s = -0.29$ and $R^2 = 0.98$. For the summer experiment samples, the parameters values were: *fshβ* $E = 102\%$, $s = -0.31$ and $R^2 = 0.99$, *lhβ* $E = 111\%$, $s = -0.32$ and $R^2 = 0.99$; and *ef1a* $E = 93\%$, $s = -0.29$ and $R^2 = 0.99$. The primers used for *q*RT-PCR are listed in Table 2.

2.9. Statistics

The data are represented as the mean ± SEM (standard error of the mean). Comparisons between animals from the C and AI groups were performed by one-way analysis of variance (ANOVA), followed by the Holm-Sidak multiple comparison test. The plasma levels of steroids throughout the experimental period in each group (C or AI) from the same tagged animals were analyzed using one-way repeated measures ANOVA (RM ANOVA). The significance level adopted was 95% ($P < 0.05$). Statistical analyses were performed using the software SigmaStat for Windows version 3.5 (Systat Software, San Jose, CA, USA).

3. Results

3.1. Sex change and GSI

During the spring (average water temperature 22.4 ± 0.10 °C), all the animals from the AI group released semen when the abdominal region was manually pressed 9 weeks after the beginning of the experiment (i.e., with two doses of AI), which suggests that the female-to-male sex change occurred. The GSI of the animals from the AI group significantly decreased compared with the C group after the same period ($P = 0.042$) (Fig. 1).

During the summer (average water temperature 26.8 ± 0.11 °C), the fish treated with AI did not show remarkable features of the occurrence of sexual change, such as the release of semen 9 weeks after the beginning of the experiment. The calculated values for GSI were not significantly different between animals from AI and C groups ($P = 0.079$) (Fig. 1).

3.2. Spermatozoa viability

At the end of the spring experiment, the volume of semen collected from the males ranged from 20 to 275 μL, with the density ranging from 4.4×10^9 to 17.6×10^9/mL and with a motility of 90%.

The oocytes fertilized with the spermatozoa produced by sex change and after 10 days of cryopreservation resulted in eggs that presented a buoyancy rate of 70%, and the fertilization rate was 73.2 ± 10.35%.

3.3. Histological analyses of gonads

During the spring, typical female gonads were observed with oocytes that were in early stages of maturation, perinucleolar and also cortical alveolar oocytes (Fig. 2A and B) as a primary growth stage in the animals from the control group. The animals from the AI group showed a gonadal disorganization typical to hermaphrodite protogynous monandry species. The gonads retained characteristics from the previous female phase; the lamellar structure was maintained, while newly formed 'sperm sinuses' arose within the walls of the original ovarian lumen with a few number of degenerated oocytes after the sex change (Fig. 2C,). In the germinal compartment, cysts of male germ cells were developed in advanced stages of spermatogenesis, such as spermatocytes (Fig. 2C) and also ruptured cysts with released spermatozoa into the lumen (Fig. 2D). Sertoli cell was also identified (Fig. 2D).

During the summer oocytes were found with nucleoli that migrated to the periphery (primary growth perinucleolar) (Fig. 3A

Table 2
Primers used for qRT-PCR gene expression analysis.

Gene	Sequence (5′⟶3′)
fshβ-F1	AGTTTCTGGCCACAGGGTAG
fshβ-R1	GGGCTGAACAGAAAGTCTGC
lhβ-F1	GTCACCCAGTGGAAACAACC
lhβ-R1	GTACGTGCACACATGCTGGT
ef1a-BSM-F1	TGGTACCTCTCAGGCTGAC
ef1a-BSM-R1	ACCAAGGGTGAAGGCCAG

F1, sense primer; R1, antisense primer.

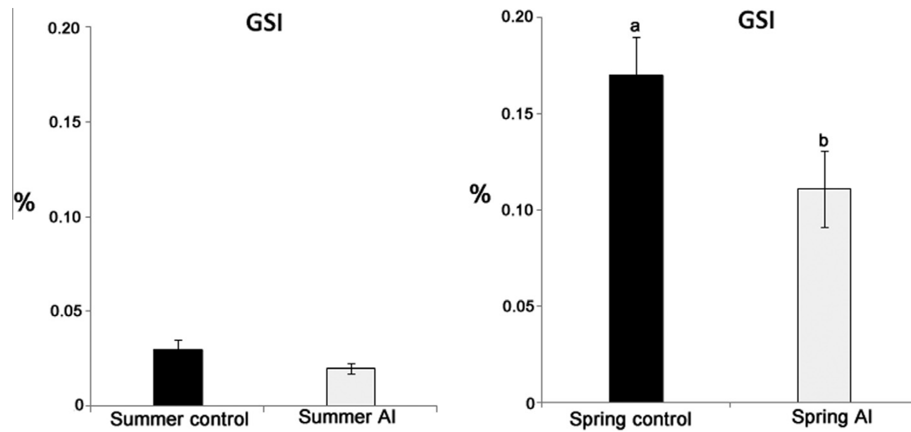


Fig. 1. Gonadosomatic index (GSI) of *Epinephelus marginatus* (spring and summer). [ab]Different letters indicate statistically significant difference.

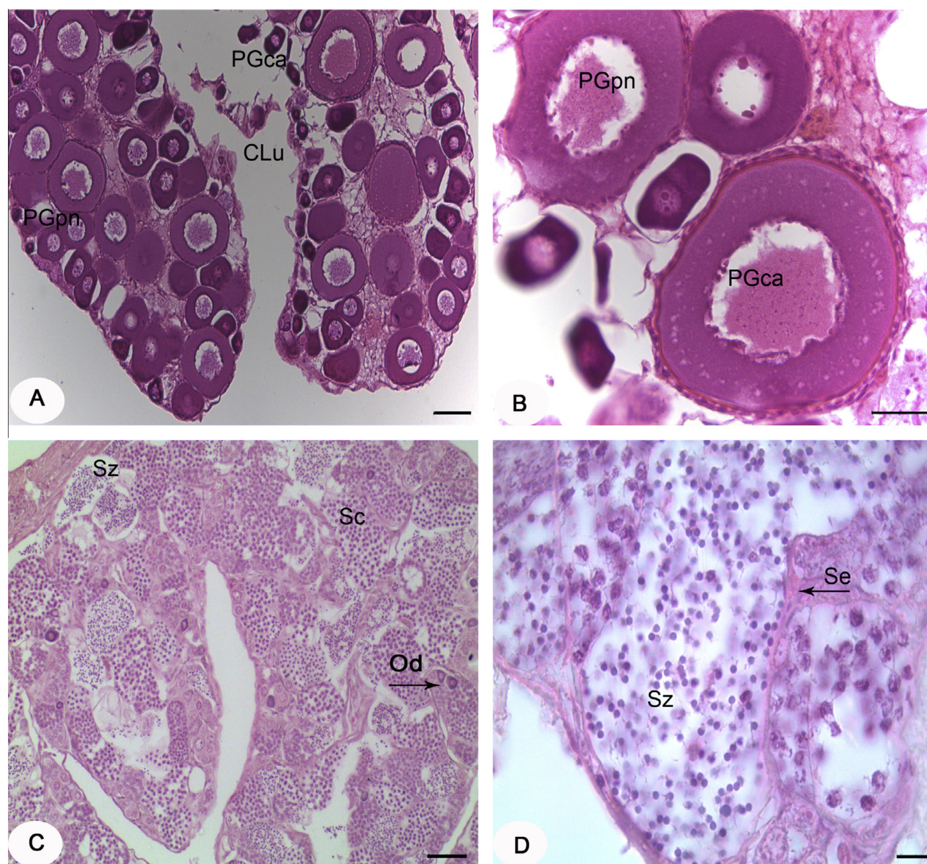


Fig. 2. *Epinephelus marginatus*. Spring experiment. (A) Ovary, control group, luminal cavity (CLu), primary growth perinucleolar oocyte (PGpn), cortical alveolar (PGca). (B) Ovary, control group, oocyte primary growth perinucleolar (PGpn), cortical alveolar (PGca). (C) Sex changed gonad, aromatase inhibitor group, spermatocytes cysts (Sc), cysts ruptured with the release of spermatozoa in the lumen (Sz), degenerated oocyte (Od). (D) Sex changed gonad, aromatase inhibitor group, spermatozoa (Sz), sertoli cell (Se). Stained with hematoxylin-eosin. (A) Bar 80 μm. (B) Bar 30 μm. (C) Bar 50 μm. (D) Bar 10 μm.

and B) and nests of oogonias were evident (Fig. 3A and B) in the control group. An intersex gonad stage (Fig. 3C and D) was observed in fish from the AI group during the summer experiment with a large number of degenerated oocytes (Fig. 3C and D), and the formation of cysts of male cells in early stages of development especially sites of development of spermatocytes (Fig. 3C and D).

3.4. Plasma levels of gonadal steroids

In the spring experiment, the plasma levels of 11KT in the animals from the AI ($P = 0.120$) and C ($P = 0.275$) groups did not change throughout the experimental period. However, 4 weeks after the beginning of the experiment, 11KT plasma levels in animals from the AI group were higher than in the C group ($P = 0.0332$). T levels did not change in the control fish ($P = 0.960$) and increased in animals from the AI group at the end of the experiment ($P = 0.00052$), when T levels were also higher than animals from the C group ($P = 0.00077$). E_2 levels did not change in animals from the AI group ($P = 0.380$) and increased in fish from the C group after 9 weeks ($P = 0.0018$). At this time, fish from the AI group presented lower E_2 levels than animals from the C group ($P = 0.044$). Plasma 17α-OHP levels did not change in animals from

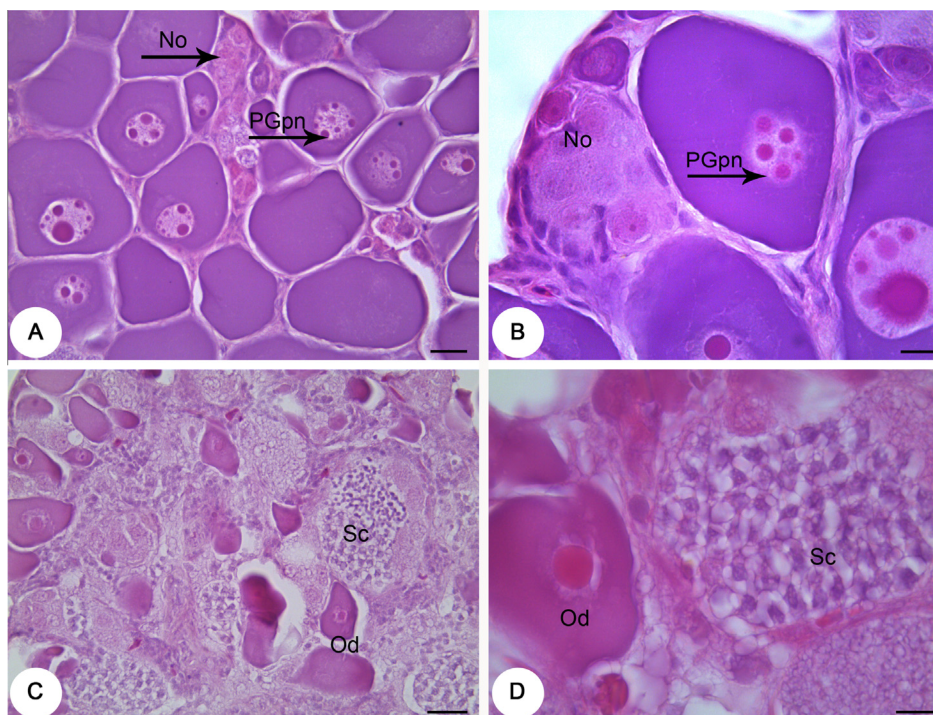


Fig. 3. *Epinephelus marginatus*. Summer experiment. (A) Ovary, control group, arrows showing the primary growth perinucleolar oocyte (PGpn) and nest of oogonias (No). (B) Ovary, control group, arrow showing the nucleolus of the primary growth perinucleolar oocyte (PGpn), nest of oogonias (No). (C) Intersex gonad, aromatase inhibitor group. Spermatocytes cyst (Sc), degenerated oocyte (Od) (D) intersex gonad, aromatase inhibitor group. Spermatocytes cyst (Sc), degenerated oocyte (Od). Stained with hematoxylin-eosin. (A) Bar 500 μm. (B) Bar 10 μm. (C) Bar 30 μm. (D) Bar 10 μm.

the AI group ($P = 0.115$), but decreased 4 weeks after the beginning of the experiment in the control fish; these low values were maintained until the end of the experiment. After 9 weeks, plasma levels of 17α-OHP were significantly higher is fish from the AI group compared to the C group ($P = 0.036$) (Fig. 4A–D).

In the summer experiment, plasma levels of 11KT did not change in the AI ($P = 0.053$) and C animals ($P = 0.15$) throughout the experimental period. Additionally, no variation was found in plasma T levels in the animals from the AI group ($P = 0.703$). However, in animals from the C group, T levels decreased after 4 weeks ($P = 0.007$) and returned to the initial levels ($P = 0.154$) at the end of the experiment. Four weeks after the beginning of the experiment, T levels were lower in the C than the AI fish ($P = 0.020$). In the beginning of this experiment, E_2 levels of the acclimated fish were higher in the plasma of the C than the AI fish ($P = 0.023$) and increased at the end of the experiment compared with the C animals that were acclimated and sampled after 4 weeks ($P = 0.006$). In the AI group, E_2 plasma levels increased after 4 weeks ($P = 0.010$) and returned to the acclimated levels at the end of the experiment ($P = 0.05$). Plasma levels of 17α-OHP decreased after 4 weeks ($P = 0.001$), and these low values were maintained until the end of the experiment in both groups. However, the animals from the AI group had higher 17α-OHP levels than the C fish ($P = 0.001$) at the end of the experiment (Fig. 5A–D).

3.5. Real-time quantitative PCR (qPCR)

In the spring experiment, *fshβ* and *lhβ* gene expression in fish from the AI group was lower than in the C group ($P = 0.029$) (Fig. 6A and B). Additionally, *lhβ* gene expression was higher than that of *fshβ* in animals from the AI group ($P = 0.049$) (Fig. 6D). This relationship was unchanged in animals from the C group ($P = 0.255$) (Fig. 6C).

In the summer experiment, no significant difference was observed in the expression levels of *fshβ* between the control and AI (P) groups ($P = 0.127$) (Fig. 7A). Also, no difference was observed in *lhβ* expression levels between both groups ($P = 0.287$) (Fig. 7B). Additionally, no significant difference was found when the expression levels of *fshβ* and *lhβ* in the control group ($P = 0.571$) (Fig. 7C) or in the AI group ($P = 0.635$) (Fig. 7D) were compared between them.

4. Discussion

4.1. Sex change using aromatase inhibitors and spermatozoa viability

In the present study, sex change of dusky grouper, *E. marginatus*, was successfully promoted in spring after 9 weeks using the third generation aromatase inhibitor (AI) letrozole. Gonadal steroids and the main pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), were also important in this process.

AI promoted sex change in *E. marginatus* during a shorter period than previously reported where oral administration of MT led to female-to-male sex change after 26 weeks (Sanches et al., 2009) and almost the same period (10 weeks) after MT implants (Cabrita et al., 2009). The efficiency of AI in promoting sex change after 9 weeks was morphologically demonstrated using gonad histology. The degeneration of the gonads from the first sex (ovaries) and the growth and maturation of gonadal tissue of the opposite sex (testis) reduced the gonadal weight, leading to a notable decrease in the GSI of animals from the AI group during the spring. It is important to highlight that the females used in both experiments had a mean total length above 45.2 ± 5.03 cm, which is approximately the mean length at first maturation (L_{50}) of 47.0 cm (Andrade et al., 2003); this factor could influence the presence of oocytes in early stages of development.

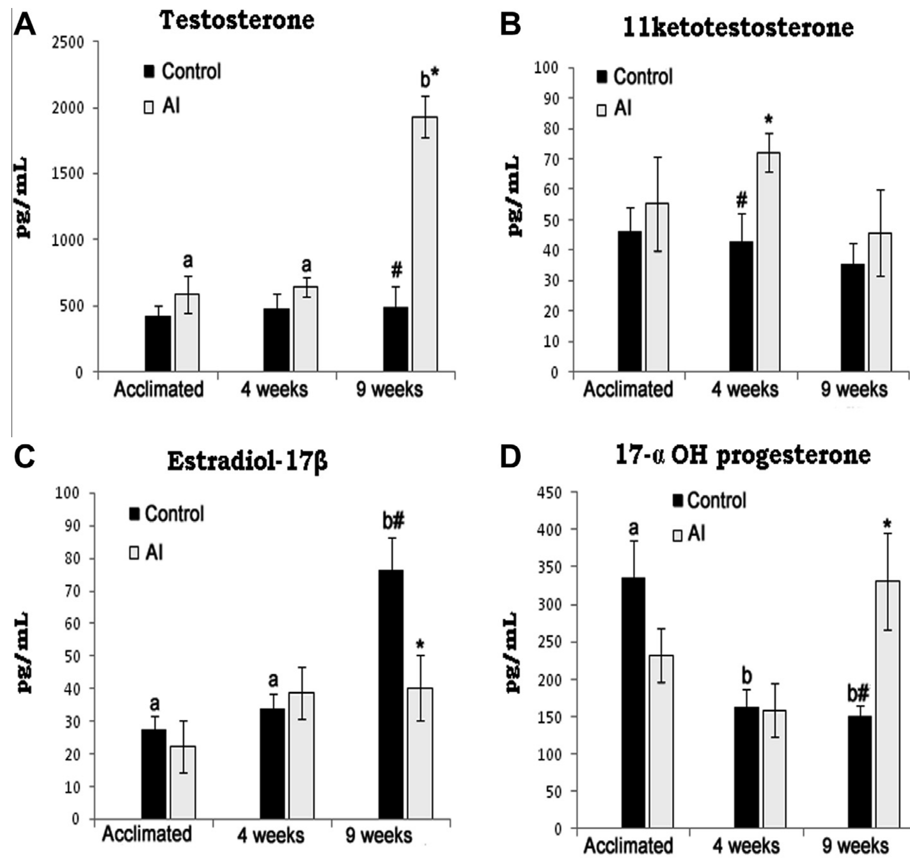


Fig. 4. *Epinephelus marginatus*. Spring experiment. (A) Plasma testosterone. (B) 11ketotestosterone. (C) Estradiol-17β. (D) 17-α OH progesterone. Acclimated; 4 weeks and 9 weeks after the beginning of the experiment. Control and AI groups. [ab]Different letters mean statistical differences between groups during the experimental period. [#*]Different symbols mean statistical differences between groups at the same week of the experiment (mean ± SEM).

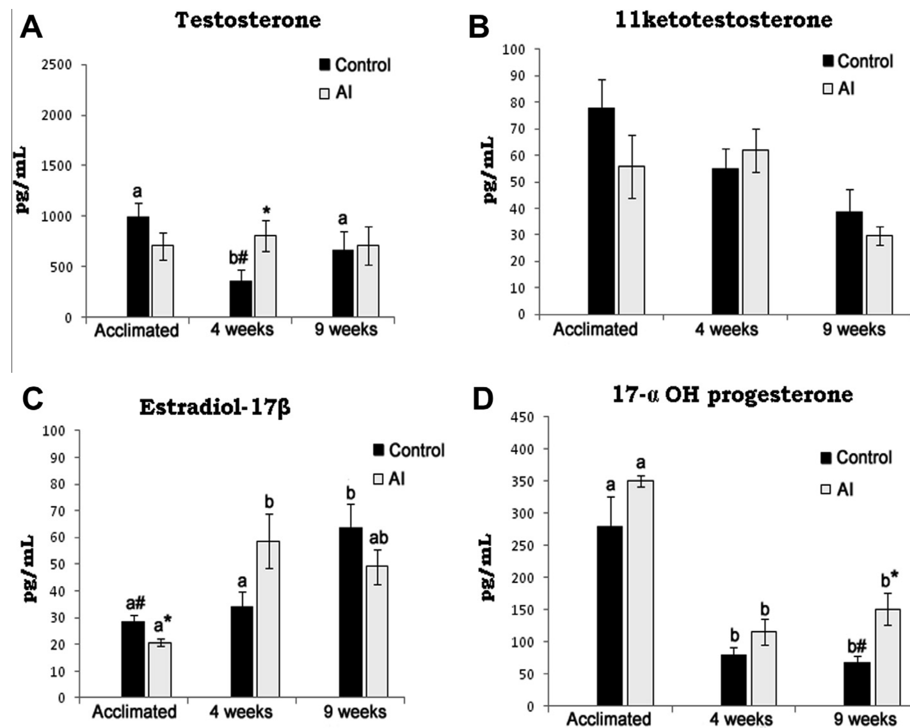


Fig. 5. *Epinephelus marginatus*. Summer experiment. (A) Plasma testosterone. (B) 11ketotestosterone. (C) Estradiol-17β. (D) 17- α OH progesterone. Acclimated; 4 weeks and 9 weeks after the beginning of the experiment. Control and aromatase inhibitor (AI) groups. [ab]Different letters mean statistical differences between groups during the experimental period. [#*]Different symbols mean statistical differences between groups at the same week of the experiment (mean ± SEM).

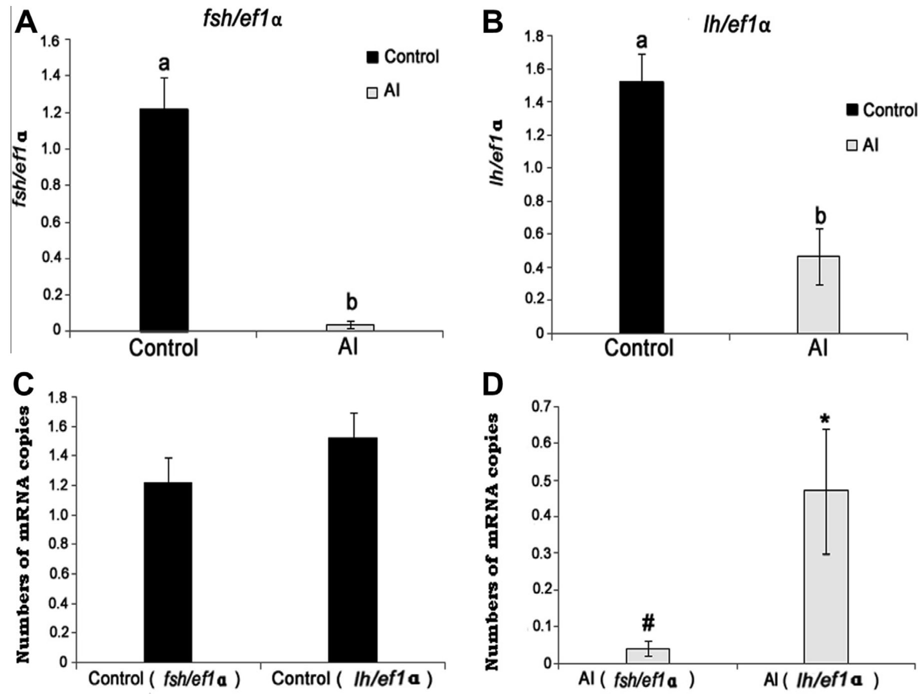


Fig. 6. *Epinephelus marginatus*. Spring experiment. Expression of β-gonadotropins genes normalized by the number of copies of mRNA *ef1α* gene. (A) Number of copies of *fshβ* mRNA in control and AI groups after 9 weeks. (B) Number of copies of *lhβ* mRNA in control and AI groups after 9 weeks. (C) Comparison between the number of copies of *fshβ* mRNA and *lhβ* mRNA copies in the control group. (D) Comparison between the number of copies of *fshβ* mRNA and *lhβ* mRNA copies in the AI group. [ab]Different letters mean statistical differences between control and AI group. [#*]Different symbols mean statistical differences between the number of copies of *fshβ* mRNA and *lhβ* mRNA.

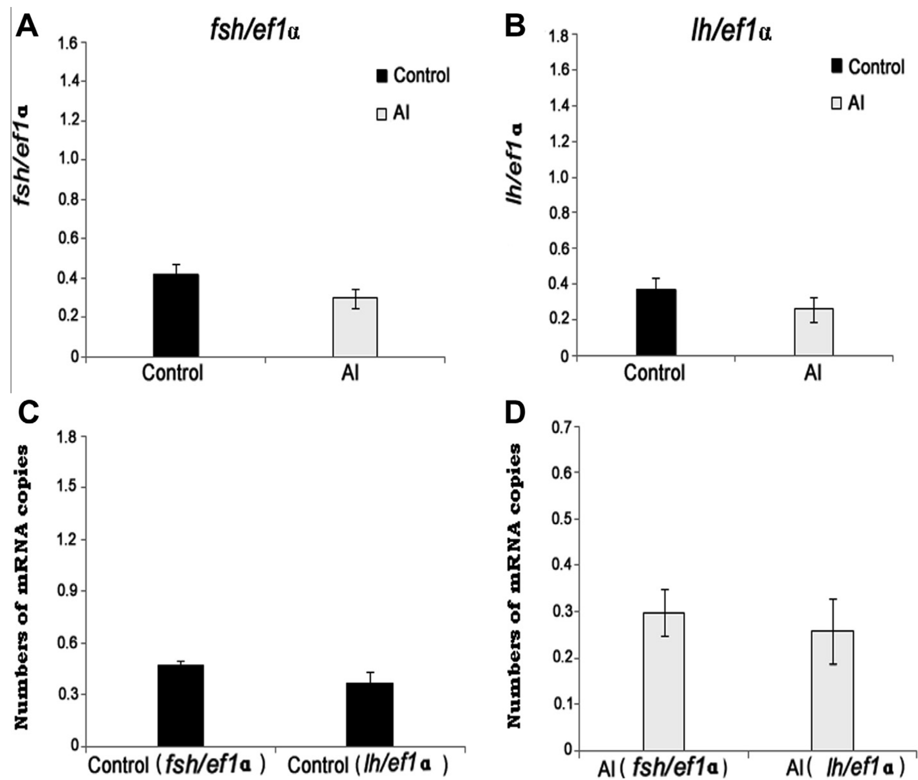


Fig. 7. *Epinephelus marginatus*. Summer experiment. Expression of β-gonadotropins genes normalized by the number of copies of mRNA *ef1α* gene. (A) Number of copies of *fshβ* mRNA in control and AI groups after 9 weeks. (B) Number of copies of *lhβ* mRNA in control and AI groups after 9 weeks. (C) Comparison between the number of copies of *fshβ* mRNA and *lhβ* mRNA copies in the control group. (D) Comparison between the number of copies of *fshβ* mRNA and *lhβ* mRNA copies in the AI group.

Males that resulted from AI treatment were functional males, producing sperm with 90% motility. After thawing, this sperm was able to fertilize dusky grouper oocytes at a fertilization rate of 73.2 ± 10.4%, which is similar to the fertilization rate (69.5 ± 17.7%) of fresh and cryopreserved sperm from sex-reversed dusky grouper studied by Cabrita et al. (2009). The motility and the

fertilization ability are important quality estimators of sperm (Bobe and Labbé, 2010). After 9 weeks of AI treatment, the volume of semen released by males was 118.20 ± 24.83 μL, within the range (5–400 μL) obtained by Cabrita et al. (2009) in *E. marginatus* treated with MT implants and lower than the average volume obtained for the same species (360 ± 26 μL) treated with MT for 180 days (Sanches et al., 2009). The sperm density obtained, $8.94 \pm 4.34 \times 10^9$ cells/mL, was in the same order of magnitude (10^9) of the values of $4.7–8.6 \times 10^9$ cells/mL (Spedicato and Boglione, 1999), $1.2–16.6 \times 10^9$ cells/mL (Cabrita et al., 2009) and 3.1×10^9 cells/mL (Sanches et al., 2009) obtained by administrating MT.

4.2. Sex change and plasma levels of gonadal steroids

Sex change in hermaphrodite fish may be influenced by the breeding season (Bhandari et al., 2004a,b; Alam et al., 2006a) and controlled by sex steroids as a result of a shift in the pattern of gonadal steroidogenesis (Frish, 2004). During the non-breeding season, steroidogenesis activity is usually low, but when fish enter into the breeding season, steroidogenesis becomes more active (Alam et al., 2006a). In the southern coast of Brazil, wild *E. marginatus* females mature in spring, and the period of spawning finishes in December (Andrade et al., 2003). This seasonality in the breeding season can explain the success in the sex change of animals in the spring experiment, i.e., during the main maturation phase of the dusky grouper when the steroidogenesis pathway mainly involves synthesis of androgens and estrogens, as seen for most fish species (Young et al., 2005).

The steroidogenic enzyme from the cytochrome P450 aromatase complex, P450arom, that converts T into E_2, plays an important role in ovarian differentiation (Strüssmann and Nakamura, 2002) and sex change (Frish, 2004; Nakamura et al., 2005). In the present study, the administration of the aromatase inhibitor (AI, letrozole) to *E. marginatus* females in spring increased the level of androgens, first 11KT (after 4 weeks) and later T levels (after 9 weeks, at the end of the sex change process). The increase of both androgens could trigger and promote sex change and gonadal restructuring. T appears to be important at the end of sex change; plasma levels at the end of the spring experiment were more than twice the levels measured at the end of the summer experiment. This profile suggests that the lower T levels in summer animals after 9 weeks of treatment were most likely not enough to support the morphological changes in the ovaries that would allow for the development of male germ cells. One might think that extra time and/or an extra dose of AI could be necessary for sex change in these animals during the summer. However, due to the higher temperature registered during this period (26.8 ± 0.11 °C), fish were likely stressed in the tanks as assumed by their erratic swimming. Therefore, we decided not to extend the experiment beyond the 9 weeks, a time period that was considered sufficient to promote sex change during the spring, to standardize experimental time.

11KT is a teleost-specific androgen with the main function of stimulating spermatogenesis from the proliferation of spermatogonia to spermiogenesis, i.e., the initiation of spermatogonial proliferation toward meiosis initiating spermatogenesis in gonochoristic fish species (Miura and Miura, 2003). The action of 11KT is mediated by some factors produced by the Sertoli cells, such as activin B and insulin-like growth factor 1 (IGF1) (Schulz et al., 2010). Despite being important in teleost spermatogenesis, some studies have been shown that 11KT is also involved in the control of oocyte growth (Lokman et al., 2002, 2007; Endo et al., 2008; Tosaka et al., 2010; Setiawan et al., 2012). In the protogynous honeycomb grouper, *E. merra*, 11KT produced in the tunica ovary near the vicinity of blood vessels may provide the stimulus for females to degenerate the oocytes and initiate sex change (Alam et al., 2006b). This role in stimulating the onset of spermatogenesis can explain the increase in 11KT plasma levels in animals from the AI group 4 weeks after the beginning of the spring experiment, a pattern that was not observed during the summer.

The pattern of steroids levels during sex change is quite variable in protogynous teleosts. During natural sex change 11KT plasma levels increase and E_2 decrease in *E. merra* (Bhandari et al., 2003; Alam et al., 2006b), *E. akaara* (Li et al., 2007) and also in the protogynous *C. nicholsii* (Kroon and Liley, 2000), and T levels increase in most studies to date, although not necessarily according to an homogenous pattern. However, when sex change is artificially induced, as in the present study, many experimental variables can affect the results, dose, experimental period, and origin of the induction agent, among others. Therefore, the profile of steroid levels is not always standardized. T levels increase during sex change when MT is administered to *E. fario* (Kuo et al., 1988) and *E. coiodes* (Yeh et al., 2003; Zhang et al., 2007). In addition, 11KT levels also increase in *E. coiodes* (Yeh et al., 2003) and *E. akaara* (Li et al., 2006) after MT treatment, but both androgens remain unchanged during sex change in *E. marginatus* (Sarter et al., 2006). However, when another Epinephelidae, *E. merra* is treated with bovine-FSH to induce sex change, 11KT plasma levels increase but E_2 levels remain unchanged (Kobayashi et al., 2010). E_2 plasma levels decrease in *E. fario* (Kuo et al., 1988) but do not vary in *E. coiodes* (Zhang et al., 2007; Yeh et al., 2003) and *E. akaara* (Li et al., 2006) under MT treatment. Thus, steroid levels diverge under induced sex change in protogynous teleost.

The administration of AI, alone or in combination with MT (Li et al., 2006), triggers an 11KT increase in *E. merra* (Bhandari et al., 2004a,b) and *E. akaara* (Li et al., 2006) plasma, as observed in the present study for *E. marginatus*. However, T and E_2 plasma levels do not always follow a homogenous pattern of variation across these studies. Previous research has shown that plasma E_2 levels decrease, but in AI dose-dependent way during the sex change period in *E. merra* (Bhandari et al., 2004a) and T plasma levels do not change. Unchanged E_2 levels during sex change using AI are reported in *E. akaara* after a treatment combining MT and AI (Li et al., 2006). In examining the mechanism of action of AI, we expected that letrozole would inhibit the conversion of T in E_2 and as a result, T levels would increase while E_2 levels would decrease. In fact, during the spring experiment, T levels increased almost 4 times in animals from the AI group at the end of the experiment compared with the acclimated values. At this time, E_2 levels in animals from the AI group were lower than the control animals but were not different from the initial values.

Thus, our data showed that in *E. marginatus*, using AI, the sex effectively changed without a reduction in E_2 levels. Aromatase gene is predominantly expressed in the ovaries (CYP19A) and in the brain (CYP19B) among other tissues. In the Japanese ricefish *Oryzias latipes* letrozole inhibits CYP19B but not CYP19A transcripts, that is upregulated in the ovaries as a compensatory response (Sun et al., 2011). According to (Sun et al., 2011) the different pattern in gene expression is mainly due to differences in the transcriptional control (in the promoter region) of these two genes. Thus, the AI dose (Bhandari et al., 2004a), the presence of genes that express aromatase in different tissues, and with different regulatory elements in the transcriptional control (Sun et al., 2011), may explain unchanged E_2 levels in AI-treated protogynous fish, as reported for *E. marginatus*, even with successful sex change.

Considering that E_2 is also an important male hormone in the early spermatogenetic cycle due to its role in the spermatogonial stem cell renewal, the maintenance of their levels seems to be important to trigger the development of male germ cells in the newly reorganized gonads (Miura and Miura, 2003; Schulz et al.,

2010). Additionally, estrogens have been reported to be of special importance during teleost protogynous sex change. In *E. coioides*, the characterization of estrogen receptors (ER) shows that the ER subtype β1, the main ER expressed in fish testes during the proliferation of spermatogonia, increases gradually in gonads during sex change, after MT implants (Chen et al., 2011).

The occurrence of spermiation and elevated levels of plasma 17-αOHP after 9 weeks of treatment during spring (and not during summer) clearly indicates that AI promoted complete sex change, allowing final gamete maturation in *E. marginatus*. Maturation of sperm is regulated by progestogens that modulate the pH increase in seminal plasma, increasing the cAMP content of the spermatozoa and consequently sperm motility in most teleosts studied to date (Miura and Miura, 2003). This mechanism has been described for the Atlantic croaker and is mediated by plasma membrane receptors (Thomas et al., 2005).

4.3. Real-time quantitative PCR (qPCR), *fshβ* and *lhβ* gene expression

In addition to gonadal steroids, the pituitary also regulates teleosts gametogenesis through the action of gonadotropic hormones, LH and FSH (Levavi-Sivan et al., 2010). The data on gene expression of *fshβ* and *lhβ* clearly showed that when AI treatment was effective in promoting female-to-male sex change, both gonadotropins were less expressed when compared with control animals after 9 weeks of treatment. High T levels at this time could modulate the decreased expression of *fshβ* at the final stage of sexual change, considering that T decreases *fshβ* gene expression in teleosts (Levavi-Sivan et al., 2010). The induction of sex change of precocious orange spotted grouper, *E. coioides,* with MT is also characterized by a decrease in the gene expression of *fshβ* (Zhang et al., 2007). However, in the honeycomb grouper, *E. merra*, the gene expression of *fshβ* is lower in females, increases in the earlier stage of sex change, but decreases in the late stage of sex change maintaining the same expression levels in males in association with testis development, while transcripts of *lhβ* do not change (Kobayashi et al., 2010).

These results indicate that gonadotropins control sex change in groupers, and support the hypothesis that sex change involves alteration of the brain-pituitary-gonads axis. However, the reported data of gonadotropin gene expression comes from different treatments in terms of MT administration (Zhang et al., 2007), synthetic gonadotropins (Kobayashi et al., 2010), or even analyses during natural sex change (Kobayashi et al., 2010). The influence of AI in *fshβ* and *lhβ* transcripts during teleost protogynous sex change, as presented in *E. marginatus* has not been reported yet in the literature.

Additionally, gene expression of *fshβ* in the animals that changed sex, i.e., the animals treated with AI during spring, was lower than the gene expression of *lhβ* at the end of the experiment; this finding was not observed in animals from control groups or in the AI animals during the summer. FSH plays an important regulatory role during the early phases of spermatogenesis, while LH is primarily involved in the final phase of maturation of spermatozoa. The mechanism that regulates the process of renewal of spermatogonia in fish seems to be dependent on the maintenance of levels of E_2 (Schulz et al., 2010), mainly regulated by FSH. At the end of the experimental period, during spring, plasma levels of 17α-OHP increased. This change supports the importance of increased *lhβ* gene expression levels compared with *fshβ* in the final stages of sexual change because LH is the main gonadotropin that stimulates the production of progestogens (Levavi-Sivan et al., 2010), the key sexual steroids involved in the control of spermatozoa motility (Miura and Miura, 2003). On the other hand, in *E. coiodes* (Zhang et al., 2007) and *E. merra* (Kobayashi et al., 2010) the gonadal morphology shows that the male germ cells did not reach the final spermatozoid stage in induced sex change treatments, as demonstrated here for *E. marginatus*. These authors also do not report milt release and progestogens levels at the end of the sex change period, which corroborates the absence of *lhβ* gene expression increase in these other Epinephelidae species.

5. Conclusions

We conclude that sex change in dusky grouper using the third generation AI letrozole successfully occurred in 9 weeks. The increase of both androgens (11KT and T) promoted sex change and gonadal restructuring, so that 11KT acts mainly in the beginning/middle of the process that triggers sex change, and T acts later, mainly sustaining male germ cells. Despite the mechanism of AI, a drop in E_2 levels was not essentially required in the AI protocol used in dusky grouper. Sex change seemed to be more effective during the maturation phase of the breeding cycle, and pituitary gonadotropins were important modulators of the sex change process due to the regulation of *fshβ* and *lhβ* gene expression by gonadal steroids.

Acknowledgments

This work was supported by a research grant from FAPESP (07/59122-7) and CNPq (558992/2009-9) and C.E. de O. Garcia had a CNPq fellowship (154673/2010-2). The authors would like to thank REDEMAR Alevinos that provided the facilities for a pilot trial; the fishermen who collected the wild dusky groupers; LAMEROA team for their help with sample collection and to CEBIMar/USP for providing the logistics and facilities for the experiment (Project # 722). A special thank to Dr. Renato M. Honji and MSc. Aline D. Gomes for their help with the histological micrographs. R.G. Moreira is a research fellow from the Brazilian CNPq (#306.026/2012-0).

References

Alam, M.A., Bhandari, R.K., Kobayashi, Y., Soyano, K., Nakamura, M., 2006a. Induction of sex change within two full moons during breeding season and spawning in grouper. Aquaculture 255, 532–535.

Alam, M.A., Bhandari, R.K., Kobayashi, Y., Soyano, K., Nakamura, S., Soyano, K., Nakamura, M., 2006b. Changes in androgen-producing cell size and circulating 11-ketotestosterone level during female–male sex change of honeycomb grouper *Epinephelus merra*. Mol. Reprod. Dev. 73, 206–214.

Andrade, A.B., Machado, L.F., Hostim-Silva, M., Barreiros, J.P., 2003. Reproductive biology of the dusky grouper *Epinephelus marginatus* (Lowe, 1834). Braz. Arch. Biol. Tech. 46, 373–381.

Bhandari, R.K., Komuro, H., Mikihiko, H., Massaru, N., 2004a. Sex inversion of sexually immature honeycomb grouper (*Epinephelus merra*) by aromatase inhibitor. Zool. Sci. 21, 305–310.

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

Bhandari, R.K., Komuro, H., Nakamura, S., Higa, M., Nakamura, M., 2003. Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper (*Epinephelus merra*). Zool. Sci. 20, 1399–1404.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165, 535–548.

Bombardelli, R.A., Mörschbächer, E.F., Campagnolo, R., 2006. Dose inseminante para fertilização artificial de ovócitos de jundiá *Rhamdia quelen* (Quoy & Gaimardm, 1824). Rev. Bras. Zootecn. 35 (4), 1251–1257.

Cabrita, E., Engrola, S., Conceição, L.E.C., Pousão-Ferreira, P., Dinis, M.T., 2009. Successful cryopreservation of sperm from sex-reversed dusky grouper, *Epinephelus marginatus*. Aquaculture 287, 152–157.

Chao, T.M., Chow, M., 1990. Effect of methyltestosterone on gonadal development of *Epinephelus tauvina* (Forskal). Singapore J. Primary Ind. 18, 1–14.

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

Cornish, A., Harmelin-Vivien, M., 2004. *Epinephelus marginatus.* In: IUCN 2011. IUCN Red List of Threatened Species. Version 2011.2. <http://www.iucnredlist.org> (accessed 03.02.12.)

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208, 191–364.

Endo, T., Todo, T., Lokman, P.M., Ijiri, S., Adachi, S., Yamauchi, K., 2008. *In vitro* induction of oil droplet accumulation into previtellogenic oocytes of Japanese eel, Anguilla japonica. Cybium 32, 239–240.

Frish, A., 2004. Sex change and gonadal steroids in sequentially-hermaphroditic teleost fish. Rev. Fish Biol. Fish 14, 481–499.

Infante, C., Matsuoka, M.P., Asensio, E., Cañavate, J.P., Reith, M., Manchado, M., 2008. Selection of housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR. BMC Mol. Biol. 9, 28–40.

Kim, N.N., Shin, H.S., Habibi, H.R., Lee, J., Choi, C.Y., 2012. Expression profiles of three types of GnRH during sex-change in the protandrous cinnamon clownfish, *Amphiprion melanopus*: Effects of exogenous GnRHs. Comp. Biochem. Physiol. B 161, 124–133.

Kitano, T., Takamune, K., Nagahama, Y., Abe, S.I., 2000. Aromatase inhibitor and 17alpha-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*). Mol. Reprod. Dev. 56, 1–5.

Kobayashi, Y., Alam, M.A., Horiguchi, R., Shimizu, A., Nakamura, M., 2010. Sexually dimorphic expression of gonadotropin subunits in the pituitary of protogynous honeycomb grouper (*Epinephelus merra*): Evidence that follicle-stimulating hormone (FSH) induces gonadal sex change. Biol. Reprod. 82, 1030–1036.

Kroon, F.J., Liley, N.R., 2000. The role of steroid hormones in protogynous sex change in the blackeye goby, *Coryphopterus nicholsii*. Gen. Comp. Endocrinol. 118, 273–283.

Kuo, C.M., Ting, Y.Y., Yeh, S.L., 1988. Induced sex reversal and spawning of blue-spotted grouper, *Epinephelus fario*. Aquaculture 74, 113–126.

Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412–437.

Li, G.L., Liu, X.C., Lin, H.R., 2007. Seasonal changes of serum sex steroids concentration and aromatase activity of gonad and brain in red-spotted grouper (*Epinephelus akaara*). Anim. Reprod. Sci. 99, 156–166.

Li, G.L., Liu, X.C., Lin, H.R., 2006. Effects of aromatizable and nonaromatizable androgens on the sex inversion of red-spotted grouper (*Epinephelus akaara*). Fish Physiol. Biochem. 32, 25–33.

Lokman, P.M., Harris, B., Kusakabe, M., Kime, D.E., Schulz, R.W., Adachi, S., Young, G., 2002. 11-Oxygenated androgens in female teleosts: Prevalence, abundance, and life history implications. Gen. Comp. Endocrinol. 129, 1–12.

Lokman, P.M., George, K.A., Divers, S.L., Algie, M., Young, G., 2007. 11-Ketotestosterone and IGF-I increase the size of previtellogenic oocytes from shortfinned eel, *Anguilla australis*, in vitro. Reproduction 133, 955–967.

Marino, G., Azzurro, E., Finoia, M.G., Messina, M.T., Massari, A., Mandich, A., 2001. Reproduction in the dusky grouper from the southern Mediterranean. J. Fish Biol. 58, 909–927.

Mitcheson, Y.S., Liu, M., 2008. Functional hermaphroditism in teleosts Fish. Fish 9, 1–43.

Miura, T., Miura, C.I., 2003. Molecular control mechanisms of fish spermatogenesis. Fish Physiol. Biochem. 28, 181–186.

Murata, R., Kobayashi, Y., Karimata, H., Kishimoto, K., Kimura, M., Shimizu, A., Nakamura, M., 2012. The role of pituitary gonadotropins in gonadal sex differentiation in the protogynous Malabar grouper, *Epinephelus malabaricus*. Gen. Comp. Endocrinol. 178, 587–592.

Mylonas, C.C., Gissis, A., Magnus, Y., 1997. Hormonal changes in male white bass (*Morone chysops*) and evaluation of milt quality after treatment with a sustained-release GnRHa delivery system. Aquaculture 153, 301–311.

Nakamura, I., Evans, J.C., Kusakabe, M., Nagahama, Y., Young, G., 2005. Changes in steroidogenic enzyme and steroidogenic acute regulatory protein messenger RNAs in ovarian follicles during ovarian development of rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 144, 224–231.

Nakamura, M., Kobayashi, T., Chang, X., Nagahama, Y., 1998. Gonadal sex differentiation in teleost fish. J. Exp. Zool. 281, 362–372.

Navarro-Martín, L., Blázquez, M., Piferrer, F., 2009. Masculinization of the European sea bass (*Dicentrarchus labrax*) by treatment with an androgen or aromatase inhibitor involves different gene expression and has distinct lasting effects on maturation. Gen. Comp. Endocrinol. 160, 3–11.

Norris, D.O., 2007. Vertebrate Endocrinology, 4th ed. Elsevier.

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

Olsvik, P.A., Lie, K.K., Jordal, A.E.O., Nilsen, T.O., Hordvik, I., 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol. Biol. 6, 21–29.

Parhar, I.S., Soga, T., Ogawa, S., Sakuma, Y., 2003. FSH and LH-β subunits in the preoptic nucleus: ontogenic expression in teleost. Gen. Comp. Endocrinol. 132, 369–378.

Piferrer, F., Blázquez, M., Navarro, L., González, A., 2005. Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). Gen. Comp. Endocrinol. 142, 102–110.

Rodrigues-Filho, J.A., Sanches, E.G., Garcia, C.E.O., Pannuti, C.V., Sebastiani, E.F., Moreira, R.G., 2009. Threatened fishes of the world: *Epinephelus marginatus* (Lowe, 1834) (Serranidae: Epinephelinae). Environ. Biol. Fish. 85, 301–302.

Sanches, E.G., Oliveira, I.R., Serralheiro, P.C.S., 2009. Inversão sexual da garoupa-verdadeira *Epinephelus marginatus*. Rev. Bras. Saúde Prod. Anim. 10, 198–209.

São Paulo, 2008. Decreto Estadual no 53494-2008 de 02 de outubro de 2008. In: Diário Oficial do Estado de São Paulo 118(187). Secretaria do Meio Ambiente.

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

Schulz, R.W., França, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.

Setiawan, A.N., Ozaki, Y., Shoae, A., Kazeto, Y., Lokman, P.M., 2012. Androgen-specific regulation of FSH signaling in the previtellogenic ovary and pituitary of the New Zealand shortfinned eel, *Anguilla australis*. Gen. Comp. Endocrinol. 176, 132–143.

Singh, A.K., 2013. Introduction of modern endocrine techniques for the production of monosex population of fishes. Gen. Comp. Endocrinol. 181, 146–155.

Spedicato, M.T., Boglione, C., 1999. Main constraints in the artificial propagation of the dusky grouper *Epinephelus marginatus* (Lowe, 1984): Three years experimental trials on induced spawning and larval rearing Cah. Options Méditerr. 47, 227–234.

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

Sun, L., Shao, X., Chi, J., Hu, X., Jin, Y., Fu, Z., 2011. Transcriptional responses in the brain, liver and gonad of Japanese ricefish (*Oryzias latipes*) exposed to two anti-estrogens. Comp. Biochem. Physiol. C 153, 392–401.

Tam, J.K.V., Lee, L.T.O., Cheng, C.H.K., Chow, B.K.C., 2011. Discovery of a new reproductive hormone in teleost: pituitary adenylate cyclase-activating polypeptide-related peptide (PRP). Gen. Comp. Endocrinol. 173, 405–410.

Thomas, P., Tubbs, C., Detweiler, C., Das, S., Ford, L., Breckenridge-Miller, D., 2005. Binding characteristics, hormonal regulation and identity of the sperm membrane progestin receptor in Atlantic croaker. Steroids 70, 427–433.

Tosaka, R., Todo, T., Kazeto, Y., Lokman, P.M., Ijiri, S., Adachi, S., Yamauchi, K., 2010. Expression of androgen receptor mRNA in the ovary of Japanese eel, *Anguilla japonica*, during artificially induced ovarian development. Gen. Comp. Endocrinol. 168, 424–430.

Varsamos, S., Xuereb, B., Commes, T., Flik, G., Spanings-Pierrot, C., 2006. Pituitary hormone mRNA expression in European sea bass *Dicentrarchus labrax* in seawater and following acclimation to fresh water. J. Endocrinol. 191, 473–480.

Yeh, S.L., Kuo, C.M., Ting, Y.Y., Chang, C.F., 2003. Androgens stimulate sex change in protogynous grouper, *Epinephelus coioides*: spawning performance in sex-changed males. Comp. Biochem. Physiol. C 135, 375–382.

Young, G., Kusakabe, M., Nakamura, I., 2005. Gonadal steroidogenesis in teleost Fish. In: Melamed, P., Sherwood, N. (Eds.), Hormones and their Receptors in Fish Reproduction, 4. World Scientific, p. 155.

Zhang, W., Zhang, Y., Zhang, L., Zhao, H., Li, X., Huang, H., Lin, H., 2007. The mRNA expression of P450 aromatase, gonadotropin β-Subunits and FTZ-F1 in the Orange-Spotted Grouper (*Epinephelus coioides*) during 17α-Methyltestosterone-Induced precocious sex change. Mol. Reprod. Dev. 74, 665–673.