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Initiation of sex change and gonadal gene expression in black sea bass (*Centropristis striata*) exposed to exemestane, an aromatase inhibitor

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Abstract

Many teleost fishes exhibit sequential hermaphroditism, where male or female gonads develop first and later undergo sex change. Model sex change species are characterized by social hierarchies and coloration changes, which enable experimental manipulations to better understand these processes. However, other species such as the protogynous black sea bass (*Centropristis striata*) do not exhibit these characteristics and instead receive research attention due to their importance in fisheries or aquaculture. Black sea bass social structure is unknown, which makes sex change sampling difficult, and few molecular resources are available. The purpose of the present study was to induce sex change using exemestane, an aromatase inhibitor, and assess gonadal gene expression using sex markers (*amh*, *zpc2*) and genes involved in steroidogenesis (*cyp19a1a*, *cyp11b*), estrogen signaling (*esr1*, *esr2b*), and apoptosis or atresia (*aen*, *casp9*, *fabp11*, *parg*, *pdcd4*, *rif1*). Overall, dietary exemestane treatment was effective, and most exposed females exhibited early histological signs of sex change and significantly higher rates of ovarian atresia relative to control females. Genes associated with atresia did not reflect this, however, as expression patterns in sex changing gonads were overall similar to those of ovaries, likely due to a

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whole ovary dilution effect of the RNA. Still, small but insignificant expression decreases during early sex change were detected for ovary-related genes (*aen*, *casp9*, *fabp11*, *zpc2*) and anti-apoptotic factors (*parg*, *rif1*). Exemestane treatment did not impact spermatogenesis or testicular gene expression, but testes were generally characterized by elevated steroidogenic enzyme and estrogen receptor mRNAs. Further research will be needed to understand these processes in black sea bass, using isolated ovarian follicles and multiple stages of sex change.

Keywords

Black sea bass; sex change; exemestane; hermaphroditism; protogynous; aromatase; estrogen; atresia

1. Introduction

Hermaphroditism refers to the development of both male and female reproductive structures, and may be simultaneous or sequential, in which male (protandry) or female (protogyny) organs develop first and later undergo sex change (Policansky, 1982; Jalabert, 2005). Protandrous and protogynous sex change occurs in many teleost fishes that exhibit dramatic plasticity in reproductive strategies (Devlin and Nagahama, 2002; Nakamura et al., 2005). The pivotal events related to gonadal reorganization, however, are largely conserved across teleosts and mediated by major sex steroids, 11-ketotestosterone (11KT) and 17β-estradiol (E2) (Frisch, 2004; Guiguen et al., 2010). For example, in many protandrous species, E2 administration can induce ovarian development and sex change is often associated with increases in gonadal aromatase (Cyp19a1a), the enzyme required for estrogen production (Godwin and Thomas, 1993; Frisch 2004; Wu et al., 2010b; Wu and Chang, 2018). In contrast, protogynous sex change is characterized by elevated 11KT and aromatase inhibition as testicular tissue develops (Cardwell and Liley, 1991; Nakamura et al., 2003; Bhandari et al., 2005). Gonadal fate is also influenced by other mechanisms associated with sex steroid pathways, including epigenetic modification of the *cyp19a1a* promoter and glucocorticoid interactions (Fernandino et al., 2013; Zhang et al., 2013).

Endocrine control of sex change has largely been studied in haremic species with easily manipulated social structures and dramatic coloration changes (Cardwell and Liley, 1991; Godwin and Thomas, 1993; Frisch 2004). One such species is the protogynous bluehead wrasse (*Thalassoma bifasciatum*), in which removal of the dominant male will induce behavioral and functional sex change in the largest female within 1–2 weeks (Warner and Swearer, 1991). However, not all species exhibit such well understood systems and instead receive considerable research attention related to sex change due to their importance in aquaculture or fisheries management (Yeh et al., 2003; Frisch et al., 2004).

Black sea bass (*Centropristis striata*) is a commercially important protogynous species along the U.S. Atlantic coast (Mercer, 1989). These fish are in high demand but only seasonally available, which has contributed to broad research toward aquaculture development (Berlinsky et al., 2000; Atwood et al., 2003; Watanabe et al., 2003; Berlinsky et al., 2004; King et al., 2005; Alam et al., 2008b). Black sea bass change sex between 2–5 years of age, though this process is often accelerated in culture, and juveniles can undergo precocious sex

change (Shepherd and Idoine 1993; Howell et al., 2003; Colburn et al., 2009). While social cues are known to influence sex ratios, black sea bass social dynamics are poorly understood and sex change progression is not associated with predictable coloration changes (Benton and Berlinsky, 2006; Provost et al., 2017). In addition, endocrine mechanisms in this species are not well-studied and there are few available molecular resources, such as gonadal sequences or transcriptomic databases (Breton et al., 2015; Morin et al., 2015). A better understanding of endocrine mechanisms and sex change processes could enhance aquaculture practices, to more effectively maintain viable broodstock populations, or to possibly improve growth rates by restricting energy use for gonadal reorganization.

The objective of the present study was to induce the initiation of female-to-male sex change in black sea bass using short-term treatment with exemestane, a third generation aromatase inhibitor previously used for sex reversal in only a few fish species (Ruskana et al., 2010; Horiguchi et al., 2013). Gonads of exemestane-exposed and control fish were histologically characterized, and gene expression was assessed in male, female, and early sex changing fish using a suite of candidate genes involved in steroidogenesis, estrogen signaling, and follicular atresia.

2. Materials and Methods

2.1. Exemestane treatment and animal sampling

Black sea bass (~1–2 years old, n = 48, 296.0 ± 16.4 g, 262 ± 50 mm total length) were wild-caught from Rhode Island (USA) waters in October 2014 and transported to a 2,200 L recirculating seawater system at the University of New Hampshire (UNH) Aquaculture Research Center. All fish were maintained under UNH Institutional Animal Care and Use Committee guidelines, held at 17–20°C, and fed a commercially prepared marine finfish diet (Skretting, Stavanger, Norway). After acclimation to culture conditions for two months, fish were randomly separated into two independent 1,200 L systems that each consisted of three replicate tanks (n = 8 fish/tank). Black sea bass in one system were fed pellets top-coated with the fat-soluble, steroidal inhibitor exemestane (Horiguchi et al., 2013), while fish in the other system received a control diet. Control and exemestane diets were prepared using established protocols (Mankiewicz et al, 2013; DiMaggio et al, 2014). Briefly, gelatin (Knox, Kraft Foods Global, Inc., Northfield, IL, USA; 0.008g/g diet) and exemestane (Selleck Chemicals, Houston, TX, USA, 1 mg/g diet) were dissolved in hot water (17 ml/g gelatin) and ethanol (0.067 ml/mg exemestane), respectively, combined, and mixed vigorously with Skretting Europa 6 mm feed. The control diet was made in a similar manner using gelatin and ethanol, but without exemestane. Both diets were air-dried overnight, stored at −20°C, and fed to each system twice daily to satiation for 16 days. The dosage and treatment duration were based on previous trials in three-spot wrasse to maximize fish sampling at the onset of sex change (Horiguchi et al., 2013). Following treatment, all fish were euthanized with 200 mg/L buffered tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA) and gonads were immediately removed. One gonad from each fish was preserved in 10% neutral buffered formalin (Sigma Aldrich, St. Louis, MO, USA) for 24–48 h, and longitudinal sections were processed for routine hematoxylin and eosin staining. The other gonad was immediately placed into a sterile 1.5

ml cryovial (Wheaton Science Products, Millville, NJ, USA), snap frozen in liquid nitrogen, placed on dry ice, and stored at −70°C until RNA extractions were performed. Histological sections were examined using a compound microscope to identify male, female, and sex changing fish. Gonads with predominantly ovarian tissue (~80–90%) and visible spermatocyte and spermatid development only in the posterior gonad were classified as early sex change, while late sex change was identified as predominantly testicular tissue (>90%) with few remaining oocytes (Cochran and Grier, 1991; Breton et al., 2015). Atresia stages were characterized using established criteria in other teleosts (Miranda et al., 1999; Blazer, 2002; Kurita et al., 2003). To quantify follicular atresia in females, pictures were taken of each ovary section and gonadal area was determined using ImageJ (Abramoff et al., 2004). Atretic follicles were counted manually throughout the gonad using a compound microscope and expressed relative to the total area.

2.2. RNA extractions and cDNA synthesis

RNA extractions were performed using gonads from 29 fish, including males (n = 6) and females (n = 6) fed the control diet, and males (n = 6), females (n = 5), and early sex changing fish from the exemestane treatment (n = 6). Due to large sizes, gonads were homogenized using a 15 ml glass mortar and pestle (Kontes Glass Co., Vineland, NJ, USA) with 1–3 ml of cold Tri Reagent (depending on size). Homogenized tissues were stored at −70°C prior to RNA extraction. Briefly, homogenized tissue (500 μl) was added to Tri Reagent (500 μl; Sigma Aldrich) and extractions were performed using standard phenol/chloroform procedures (Molecular Research Center, Cincinnati, OH, USA). Total RNA quantity and quality were assessed using an ND 1000 NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 1.0% agarose gel electrophoresis. All samples exhibited no contamination (260/280 nm and 260/230 nm ratios ~2.0) and intact rRNA banding patterns. Total RNA (2.5 μg) was DNase-treated using the Promega RQ1 RNase-free DNase kit (Promega Corp., Madison, WI, USA) and cDNA synthesis was performed using 1.8 μg DNase-treated RNA, 2.5 μM oligo dT primer (20mer), and 200 units of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Complementary DNA samples were stored at −20°C.

2.3. Candidate gene identification and primer design

To identify candidate genes for expression analyses, we used a black sea bass gonadal transcriptome sequence read archive (SRA) previously generated by our group and deposited in NCBI (SRA acc. no.: **SRP135689**). The SRA database consisted of male, female, and sex changing juvenile black sea bass cDNAs sequenced using Illumina HiSeq 3000 technology (Illumina Inc., San Diego, CA, USA) by the Oregon State University Center for Genome Research and Computing (Corvallis, OR, USA). Gonadal sequences were quality-trimmed using Trimmomatic (v.0.36) and trimmed reads were used to generate a partial and preliminary *de novo* transcriptome assembly using Trinity (v.2.0.6) (Grabherr et al., 2011; Harding et al., 2013; Bolger et al., 2014). The transcriptome assembly consisted of 181,263 unique contigs (mean length = 496 bp) that were annotated using blastn and blastx against NCBI's nt (partially non-redundant nucleotide) and nr (non-redundant protein) databases, respectively. Contigs were parsed manually to identify coding domain sequences (cds) for 12 genes of interest. Several sequences were identified that corresponded to well-studied genes

involved in steroidogenesis and estrogen signaling, including estrogen receptor alpha (*esr1*), an estrogen receptor beta (*esr2b*), gonadal aromatase (*cyp19a1a*), and 11β-hydroxylase (*cyp11b*). Four genes previously associated with differential expression during follicular atresia in fish were also used, including caspase 9 (*casp9*), programmed cell death protein 4 (*pdcd4*), fatty acid binding protein 11 (*fabp11*), and replication timing regulatory factor 1, also known as RAP1 interacting protein 1 (*rif1*) (Agulleiro et al., 2007; Tinguad-Sequeira et al., 2009; Yamamoto et al., 2011, 2016). Partial sequences for apoptotic genes poly(ADP-ribose) glycohydrolase (*parg*) and an apoptosis-enhancing nuclease (*aen*) were also included (Affar et al., 2000; Kawase et al., 2008; Shirai et al., 2013). Lastly, fragments corresponding to zona pellucida C (*zpc2*) and anti-Mullerian hormone (*amh*) were used as a potential ovarian or testicular marker, respectively (Onichtchouk et al., 2003; Wu et al., 2010a; Smith et al., 2013).

To confirm putative cds fragments obtained from Illumina sequencing and assembly, primers were designed and used in polymerase chain reaction (PCR) (Table 1). Each reaction consisted of 30 μl total volume, with 4 μl cDNA template from a control fish, 1X Promega GoTaq Flexi PCR buffer (Promega, Madison, Wisconsin USA), 2.0 mM $MgCl_2$, 0.2 μg/μl bovine serum albumin, 0.28 mM deoxynucleoside triphosphates, 0.7 μM of each primer, and 0.2 U of GoTaq Flexi DNA polymerase. PCR was conducted in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and consisted of an initial denaturation step (95°C for 5 min), followed by 40 cycles of 95°C for 30 sec, 58 or 60°C for 30 sec, and 72°C for 40 sec, and a final extension step (72°C for 5 min). PCR reactions using *cyp19a1a* primers required a 58°C annealing temperature for amplification, while all other primer sets were effective at 60°C. PCR products were treated with ExoSAP-IT PCR Product Cleanup Reagent (Affymetrix, Inc., Santa Clara, CA, USA) and transported to the MDI Biological Laboratory (Bar Harbor, ME, USA) for sequencing using the dideoxy chain termination method on an Applied Biosystems 3130xl Genetic Analyzer (Foster City, CA, USA). All PCR products were sequenced in both directions using forward and reverse primers, and sequence chromatograms were trimmed for quality prior to manual assembly and analysis using blastn and blastx against NCBI databases. Confirmed partial cds fragments for all targeted genes were deposited in GenBank (Table 1) and used to design qPCR primers in NCBI Primer-BLAST (Table 2). Previously developed black sea bass-specific primers for *eef1a* were also included as a reference gene for qPCR analyses (Breton et al., 2015).

2.4. Real time quantitative PCR (qPCR)

Relative quantification SYBR Green qPCR assays were performed using a StepOne Plus Real Time PC System and the FAST SYBR™ Green Master Mix (Applied Biosystems). Each reaction consisted of 10 μl total volume, with 1.33 μl diluted template and 0.1–2.5 μM primer concentration, depending on the assay. Each assay was run under standard cycling conditions (95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min), except for *cyp19a1a*, which required a 58°C annealing temperature. All assays were followed by dissociation curve analysis and exhibited only single peak amplification. All samples were assayed in duplicate (diluted 1/40, 1/50, or 1/100, depending on assay) and triplicate relative standard curves (diluted 1/5–1/1280) were made from pooled cDNA samples represented

across treatments. Optimized linear standard curves consisted of four to eight points, and all assays exhibited approximately 90–110% PCR efficiency. PCR products from each assay were also electrophoresed in 2% agarose gels to confirm amplification of the intended target, and no contamination was evident in all standard qPCR negative controls (no template and no reverse transcriptase).

2.5. Statistical analyses

Quantitative PCR results were analyzed using the Pfaffl method for relative quantification (Pfaffl, 2001). Individual expression levels were: 1) calibrated to the sex change (SC) group mean, 2) normalized to *eef1a*, to compensate for differences in cDNA synthesis efficiency among samples, and 3) expressed relative to the SC group (set to 1.0), to enhance data presentation (Luckenbach et al., 2008). Relative expression values of some assays were log-transformed prior to analyses to satisfy assumptions of normality or equal variance among groups. To group similar qPCR expression profiles and individual gonad samples, the online program Heatmapper was used with the average linkage and Pearson methods for clustering and distance measurements, respectively (Babicki et al., 2016). To include all gene assays in the cluster analysis, individuals with mRNA levels below quantifiable ranges were arbitrarily set to 0. Percentage data associated with tank sex ratios or ovarian stages were arcsine square root-transformed, and atretic follicle data were log-transformed. All data were expressed as mean ± standard error and analyzed using one-way ANOVAs in JMP 13.0 (SAS Institute, Cary, NC, USA), followed by Tukey's post hoc tests to identify significant differences ($p < 0.05$) among reproductive stages and treatments.

3. Results

3.1. Exemestane-induced initiation of sex change

Following treatment, the proportion of males per tank did not differ between control and exemestane-treated groups (Fig. 1A). Female proportions were more variable but also not significant, while significantly more exemestane-treated fish were changing sex ($p = 0.0177$). Females also exhibited significant histological differences in ovarian composition by treatment. Control female ovaries were largely in the cortical alveolus (CA) stage (early secondary growth) ($p = 0.0120$, Fig. 1B), while exemestane-treated females exhibited significantly more atresia ($p < 0.0001$, Fig. 1C).

Overall, black sea bass fed the control diet exhibited normal ovarian or testicular development (Fig. 2A-B). The majority of female ovarian tissue was comprised of primary growth oocytes and CA stage follicles, while males were characterized by multiple stages of spermatogenesis, including spermatocyte and spermatid development, as well as the presence of mature spermatozoa. Exemestane fed males were similar to control males, while females exhibited largely primary growth oocytes (Fig. 2C-D). Most sex changing fish in the exemestane treatment group were in early sex change (Fig. 2E). Only one fish per treatment was in late sex change (data not shown) and had both likely initiated sex change before the experiment started. For this reason, these fish were not included in later molecular analyses.

To more fully characterize ovarian development and atresia in black sea bass, individual follicles were examined in both control and exemestane-treated females (Fig. 3). In control females, CA stage follicles exhibited a clearly defined nucleus and zona pellucida, and the presence of small, peripheral follicle cells (Fig. 3A). Only exemestane-treated females exhibited high proportions of atretic follicles, which were largely at initial or mid atresia stages (Fig. 3B), with clear nuclear and zona pellucida degeneration, and a vacuolated or fragmented cytoplasm. No follicles were identified in later stages of atresia, typically characterized by complete oocyte cytoplasm phagocytosis or accumulation of yellow-brown bodies (Rizzo and Bazzoli, 1995; Miranda et al., 1999; Blazer, 2002; Kurita et al., 2003).

3.2. Gene expression patterns

All 12 gene fragments matched their respective putative transcriptome assembly sequences with high similarity (96–100% identity) and exhibited significant matches to respective nucleotide and protein sequences of related teleost species, including orange-spotted grouper (*Epinephelus coioides*), European sea bass (*Dicentrarchus labrax*), Japanese sea bass (*Lateolabrax japonicus*), and common clownfish (*Amphiprion ocellaris*). Relative transcript levels for each gene varied, and some individuals in some treatments had non-detectable levels of *amh*, *esr2b*, or *rif1*. Expression of *amh* could not be quantified in any female or early sex changing fish, as well as one control male, while *esr2b* and *rif1* expression could not be quantified in three exemestane-treated females and three early sex changing fish, respectively. In contrast, reference gene expression (*eef1a*) was largely stable and did not significantly differ among gonadal stages.

Clustering analysis grouped the 12 gene expression patterns into three clusters, broadly divided into genes that exhibited: 1) largely similar patterns between testes and ovaries, 2) a testis-dominated expression profile, or 3) an ovary-dominated profile (Fig. 4). In addition, individual sample clustering identified a division between fish with either primarily testes or ovaries. There was little variance in control and exemestane-treated males, while females and early sex changing fish clustered separately as a more variable group.

Cluster 1 genes exhibited largely stable expression patterns with a few small decreases evident in exemestane-treated females and sex changing fish (Fig. 5). For example, *esr1* exhibited approximately equal expression in males and variable expression in control females. Exemestane-treated females and sex changing fish, however, exhibited somewhat lower expression (2–3 fold) with evidence of weak differences overall ($p = 0.0402$). Pairwise significant differences among groups were not detected, though, due to the conservative nature of the post-hoc analysis. Similarly, *parg* expression was overall equal among males and females, with a small decrease in early sex changing fish that was significantly different from control females ($p = 0.0272$). In contrast, the *pdcd4* transcript showed a slightly more testis predominant profile, while exemestane-treated females and early sex changing fish were characterized by a 2–3 fold decrease in expression relative to males only ($p = 0.0001$).

Cluster 2 genes exhibited a testis-dominated expression profile, and three of the five genes were characterized by non-quantifiable or extremely weak levels in some female and sex changing fish (Fig. 6). Expression of *amh* was weakly detected in most males but could not be detected in any gonad with predominantly ovarian tissue (i.e., F and SC fish). Transcript

levels for *cyp19a1a*, *esr2b*, and *cyp11b* were highly upregulated in males, including 4-, 5-, and 60-fold changes, respectively, compared to females and early sex changing fish ($p < 0.0001$). Expression of *rif1* was also testis predominant ($p = 0.0151$), with non-quantifiable levels in most early sex changing fish, but fold changes were variable and not significantly different in post-hoc analysis.

Cluster 3 genes exhibited an ovary-dominated profile, with early sex changing fish typically characterized by intermediate expression levels (Fig. 7). Transcript levels for *zpc2* and *fabp11* were high in all ovarian samples, with approximately 50- and 10-fold higher levels in females compared to males, respectively ($p < 0.0001$). Expression levels in early sex changing fish were overall intermediate but more similar to ovaries than testes. In contrast, *aen* and *casp9* differences were comparatively less than those observed for other genes in this cluster, but still downregulated 2–3 fold in males ($p < 0.0001$ and $p = 0.0038$, respectively) and intermediate in early sex changing fish.

4. Discussion

The initiation of sex change was induced in black sea bass using exemestane, a third generation, irreversible aromatase inhibitor commonly used during breast cancer treatment in postmenopausal women (Geisler et al., 1998; Miller et al., 2008; Goss et al., 2011). The older, reversible inhibitor fadrozole has previously been used in fish to induce masculinization (Kitano et al., 2000; Kwon et al., 2000), including black sea bass (Benton and Berlinsky, 2006), but is no longer commercially available and is generally less potent than third generation inhibitors (Lønning, 2011). Recently, the third generation reversible inhibitor letrozole has been used for sex reversal/inhibition in a variety of fishes, but little research has focused on steroidal inhibitors with similar potency, such as exemestane (Li et al., 2005; Lønning, 2011; Singh, 2013). In fishes, exemestane use has been limited only to masculinization of juvenile, gonochoristic Nile tilapia (*Oreochromis niloticus*) and sex change induction in adult protogynous three-spot wrasse (*Halichoeres trimaculatus*) (Ruskana et al., 2010; Horiguchi et al., 2013, 2018). Female black sea bass (present study) and wrasse (Horiguchi et al., 2013) were exposed to an identical dose and short duration (1 mg/g diet, 15 or 16 days) and both species were characterized by similar sex change stages. In other studies, male wrasse were only exposed to low exemestane doses (0.002 and 0.2 mg/g diet) that negatively impacted spermatogenesis (Kobayashi et al., 2011, 2014). Male black sea bass in this study, however, were exposed to a higher dose (1 mg/g diet) for a shorter duration (16 days vs. 10 weeks) and exhibited no testicular degeneration. As a result, effects on spermatogenesis likely require longer-term exposure to manifest histologically. Overall though, responses to exemestane in adult black sea bass were largely similar to the three-spot wrasse and reflect its potency in masculinizing fishes.

4.1 Atresia and apoptotic processes

Exemestane-treated females were characterized by increased ovarian atresia, with follicles at initial or mid stages of degeneration. These apoptotic processes are often induced by factors that influence the hypothalamic-pituitary-gonadal (HPG) axis, such as environmental stressors and starvation, or through decreased plasma E2 (Afonso et al., 1999; Janz et al.,

2001; Ankley et al., 2002; Sato et al., 2005; Yamamoto et al., 2011; Nakamoto et al., 2018). The atresia we observed in black sea bass is consistent with estrogen signaling dysfunction, and longer exemestane exposure would likely lead to greater proportions of atretic follicles and sex change in most, if not all, females.

Expression patterns associated with atresia in gonochoristic species were not detected in black sea bass. For instance, atresia in coho salmon (*Oncorhynchus kisutch*) was characterized by elevated expression of both *casp9* and *pdcd4*, which are well-studied genes in intrinsic, mitochondria-mediated apoptosis pathways and translational repression, respectively (Lankat-Buttgereit and Göke, 2009; Tait and Green, 2010; Yamamoto et al., 2011, 2016). However, black sea bass did not exhibit elevated levels of either mRNA in atretic gonads. Instead, *pdcd4* and *casp9* only exhibited either a testicular or ovarian-dominated profile, respectively. *Pdcd4* is implicated in gonadal germline stem cell differentiation in invertebrates, but the functional significance of testicular *pdcd4* in vertebrates is unknown (Onishi et al., 1998; Lankat-Buttgerreit and Göke, 2003; Cash and Andrews, 2012). *Casp9* in ovaries, in contrast, may indicate high constitutive expression in growing vertebrate oocytes to regulate meiotic progression (Ene et al., 2013). Transcripts for *aen* and *fabp11* also exhibited elevated ovarian expression and likely reflect normal oocyte functions associated with RNA processing and lipid transport, respectively (Liu et al., 2003; Kawase et al., 2008; Wu et al., 2010a). These patterns were also similar to *zpc2*, which is not associated with atresia but is an ovary gene essential to proper zona pellucida formation (Onichtchouk et al., 2003). Overall, genes previously associated with atresia in gonochoristic species may not be effective markers in black sea bass due to their basal functions in normal ovarian development or functional differences associated with alternative reproductive strategies like hermaphroditism.

All black sea bass ovaries exhibited similar transcript levels, irrespective of treatment and despite significant histological differences. In these samples, atretic follicles only represented a small proportion of each ovary, which were largely dominated by primary growth oocytes that undergo massive transcription (Song and Wessel, 2005; Breton and Berlinsky, 2014). This produces a gonadal dilution effect in species like iteroparous black sea bass, which exhibit multiple populations of different oocyte stages (Goetz et al., 2006; Breton et al., 2012). In contrast, semelparous species such as coho salmon exhibit a homogenous oocyte population that more easily enables stage-specific transcript analyses (Luckenbach et al., 2008; Breton and Berlinsky, 2014). Therefore, the ability to detect relatively small, atresia-specific RNA differences within intact black sea bass gonads was likely limited, especially across such a short-term treatment. A longer exemestane exposure, in contrast, would likely produce detectable expression differences among tissues due to greater proportions of atretic follicles and sex changing tissue in the homogenized ovary.

Testicular signals in early sex changing gonads were also likely minimized due to the same gonadal dilution effect. This is similar to patterns in protogynous honeycomb grouper (*Epinephelus merra*) in which early sex change was characterized by oocyte degeneration and spermatocyte development, yet gene expression mirrored that of ovaries (Alam et al., 2008a). In the present study, however, many ovary-predominant genes exhibited weak decreases in expression during early sex change that may indicate onset of a progressive loss

of ovarian phenotype. In addition, when compared to females, sex changing fish were also characterized by a significant decreases in *parg* and largely non-detectable levels of *rif1*, which are both anti-apoptotic factors. Parg functions in cell survival and hydrolyzes poly(ADP-ribose), a well-characterized death signal that accumulates in damaged or stressed cells (Andrabi et al., 2008). Rif1 is a DNA repair protein that acts in a protective manner to limit progression of caspase-dependent apoptosis (Wang et al., 2009) and is downregulated during atresia in Senegalese sole (*Solea senegalensis*) (Tinguad-Sequeira et al., 2009). These small changes suggest that decreases in expression of anti-apoptotic genes may occur early in sex change, but this requires further research using later sex change stages and isolated ovarian follicles.

4.2 Steroidogenesis and estrogen receptors

Transcript levels associated with steroid hormone signaling and gonadal differentiation were largely elevated in testes relative to ovaries and sex changing gonads. For instance, *amh* and *cyp11b* were elevated in males, which is consistent with sexually dimorphic patterns in other species (Rodríguez-Marí et al., 2005; Wang and Orban, 2007; Smith et al., 2013). Both genes also commonly exhibit elevated profiles during early testicular development, while gonadal aromatase (*cyp19a1a*) is higher during ovarian differentiation (Luckenbach et al., 2005; Wang and Orban, 2007; Smith et al., 2013). In adults, however, expression profiles are more complex, as *amh* is also expressed in granulosa cells in ovarian follicles, and estrogen signaling is important in spermatogenesis (Rodríguez-Marí et al., 2005; Carreau et al., 2011).

Gonadal estrogens are likely important in black sea bass testes, as both *cyp19a1a* and estrogen receptor beta (*esr2b*) were upregulated. Estrogen receptor alpha (*esr1*) was only slightly elevated, but may exhibit greater upregulation later during spermiation, as in rainbow trout (*Oncorhynchus mykiss*) (Bouma and Nagler, 2001; Delalande et al., 2015). These data are largely consistent with elevated levels of estrogen receptor transcripts in testes of other teleosts, including zebrafish (*Danio rerio*) and red porgy (*Pagrus pagrus*) (Menuet et al., 2002; Tsakogiannis et al., 2018). Estrogens are also critical in ovarian development and maintenance, but *cyp19a1a* expression is relatively low during primary oocyte growth, and decreased gene expression in female black sea bass likely reflects these early stages (Kobayashi et al., 2004; Breton and Berlinsky, 2014). Lastly, both males and females exposed to exemestane exhibited no significant changes in estrogen-related transcripts. This is consistent with gonadal *cyp19a1a* expression in other species, which is not directly responsive to E2 levels, but is instead regulated by the HPG axis and other local processes (Kishida et al., 2001; Guiguen et al., 2010) that were likely not impacted in the present study.

5. Conclusions

The initiation of female-to-male sex change was induced in the protogynous black sea bass using dietary exemestane treatment. Early sex change was marked by significant increases in the number of atretic ovarian follicles and development of testicular tissue in the posterior gonad. Gonadal effects were overall similar to those previously documented in three-spot

wrasse, and longer duration of exemestane treatment (30 days; Horiguchi et al., 2013) may also induce functional testis development and complete sex change in black sea bass, but this requires further study. Gonadal expression of sex markers and candidate genes involved in steroidogenesis, estrogen signaling, atresia, and apoptosis were largely divided into testis- or ovary-dominated patterns. Small changes associated with early sex change were mostly not detected, possibly due to a whole gonad dilution effect. Some small, insignificant expression shifts, however, were observed that suggest both decreases in ovarian phenotype and some apoptosis-related factors at the onset of sex change. These will require further investigations using manually isolated follicles, or laser-capture microdissections, coupled with apoptotic (TUNEL) assays and *in situ* hybridization to better characterize atresia and identify transcript changes likely occurring in oocytes and follicle cells.

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Highlights:

- Exemestane induced the initiation of female-to-male sex change in black sea bass.
- Exemestane-exposed females exhibited significantly greater ovarian atresia than control fish.
- Males did not exhibit gonadal effects from exemestane treatment.
- All testes exhibited elevated steroiogenic enzyme and estrogen receptor mRNAs.
- Small decreases in apoptosis-related mRNAs were evident in sex changing gonads.

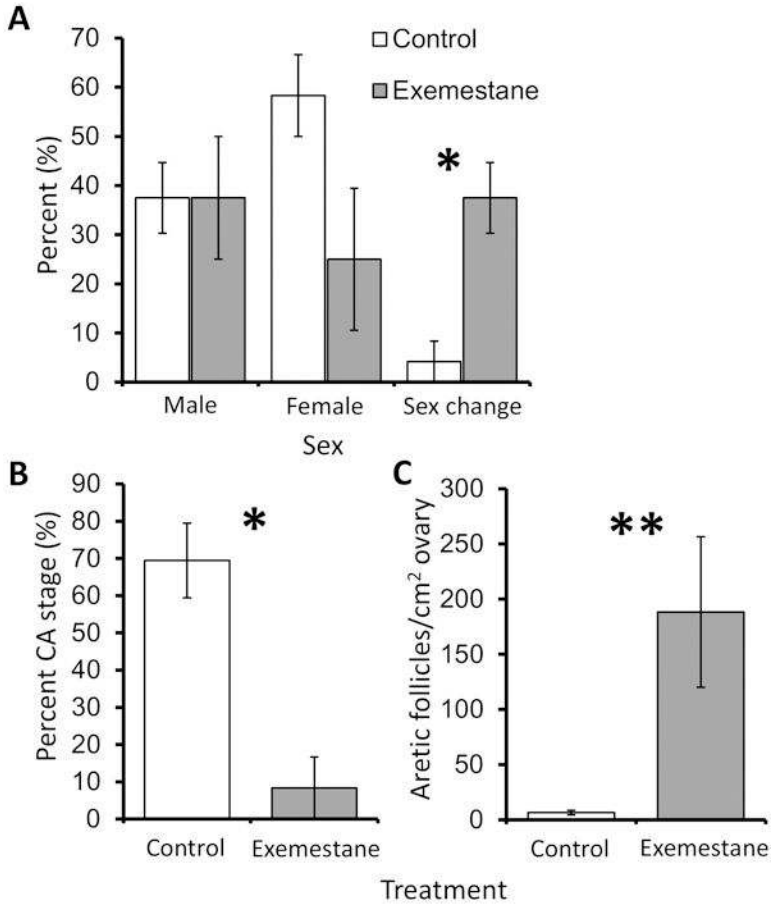

Fig. 1.

Mean percentage of fish (A) in each tank (± standard error) that were either male, female, or sex changing, as determined by gonadal histology. In females, (B) the mean percentage of fish per tank (± standard error) with some proportion of cortical alveolus (CA) stage oocytes was determined, as well as (C) mean number of atretic follicles/cm^2 ovary (± standard error). White and gray bars refer to control and exemestane treatments, respectively. Asterisks indicate either (*) $p < 0.05$ or (**) $p < 0.0001$.

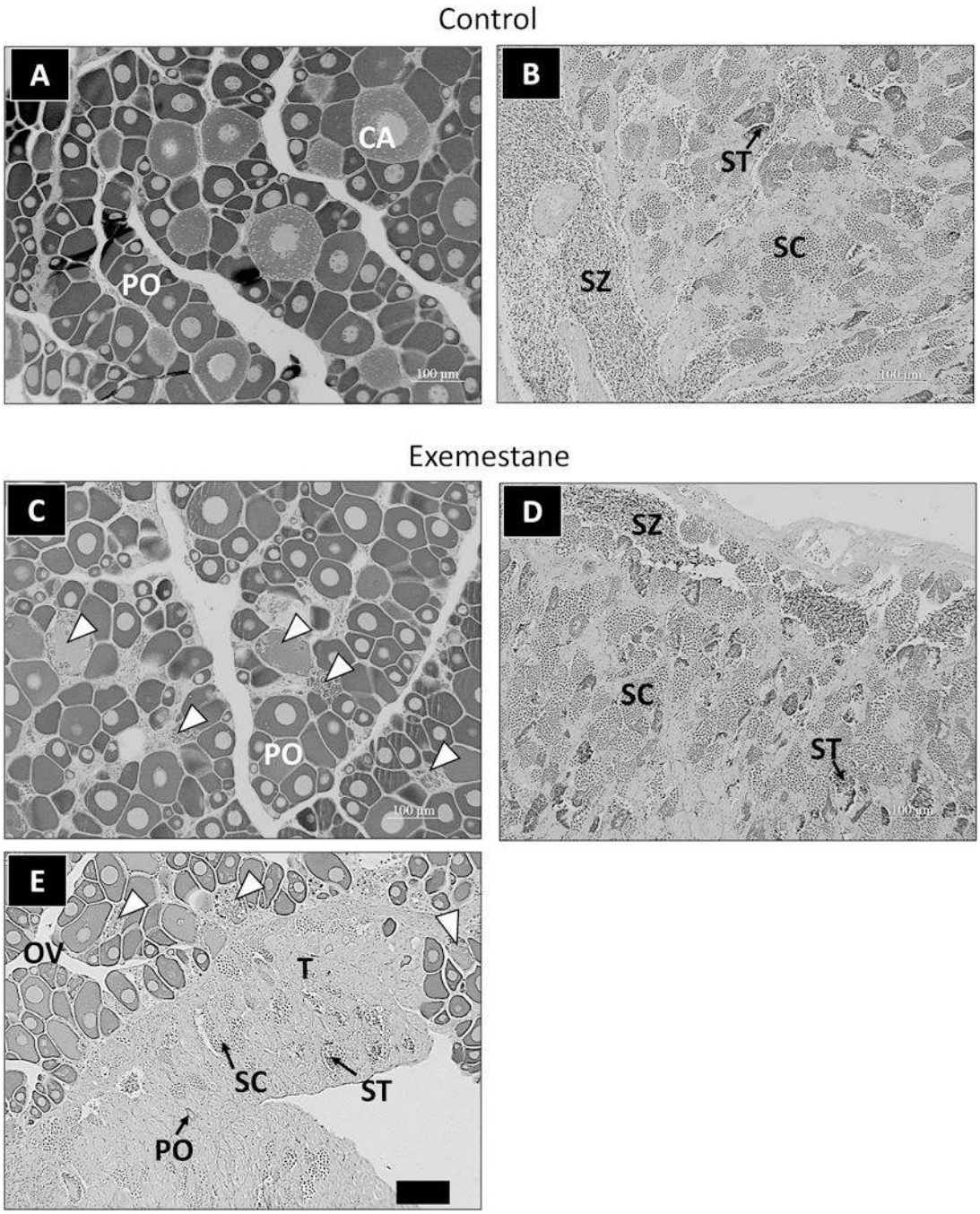

Fig. 2.

Representative gonadal histology of control (A) female and (B) male fish, and exemestane-treated (C) female, (D) male, and (E) early sex changing black sea bass. Scale bar represents 100 µm. Arrowheads indicate atretic follicles. PO, primary growth oocyte; CA, cortical alveolus, or early secondary growth stage oocyte; SZ, spermatozoa; ST, spermatids; SC, spermatocytes; OV, ovarian tissue; T, testicular tissue.

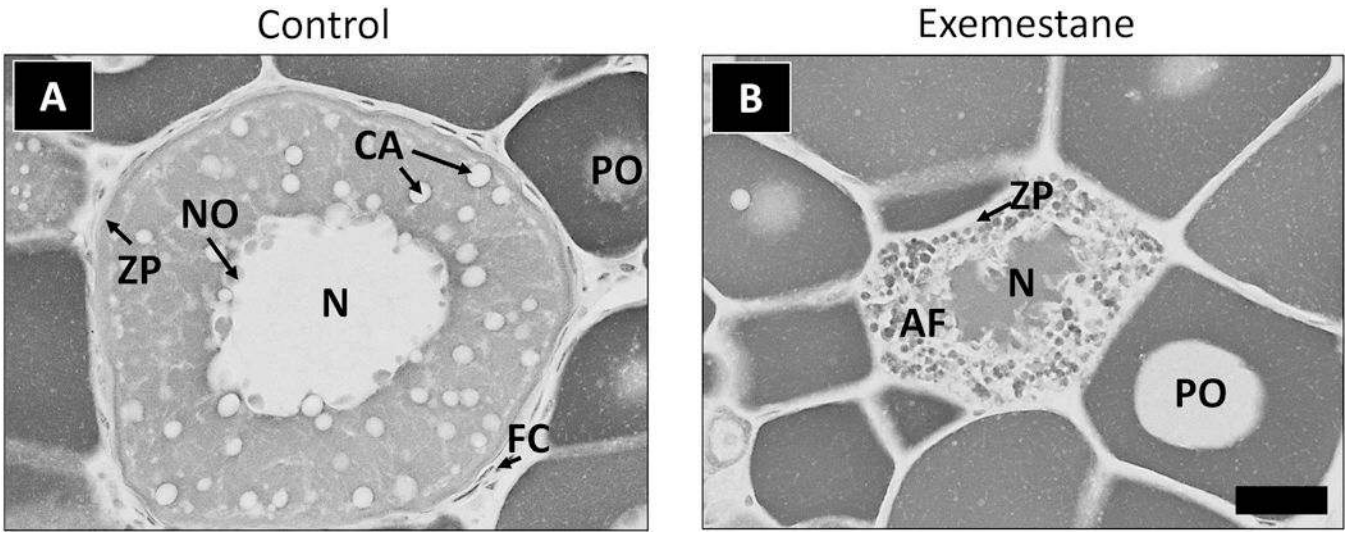

Fig. 3.

Representative gonadal histology of (A) cortical alveolus stage follicle in control females and (B) atresia in exemestane-fed females. Scale bar represents 20 μm. ZP, zona pellucida; NO, nucleolus; N, nucleus; CA, cortical alveolus; PO, primary growth oocyte; FC, follicle cells; AF, atretic follicle.

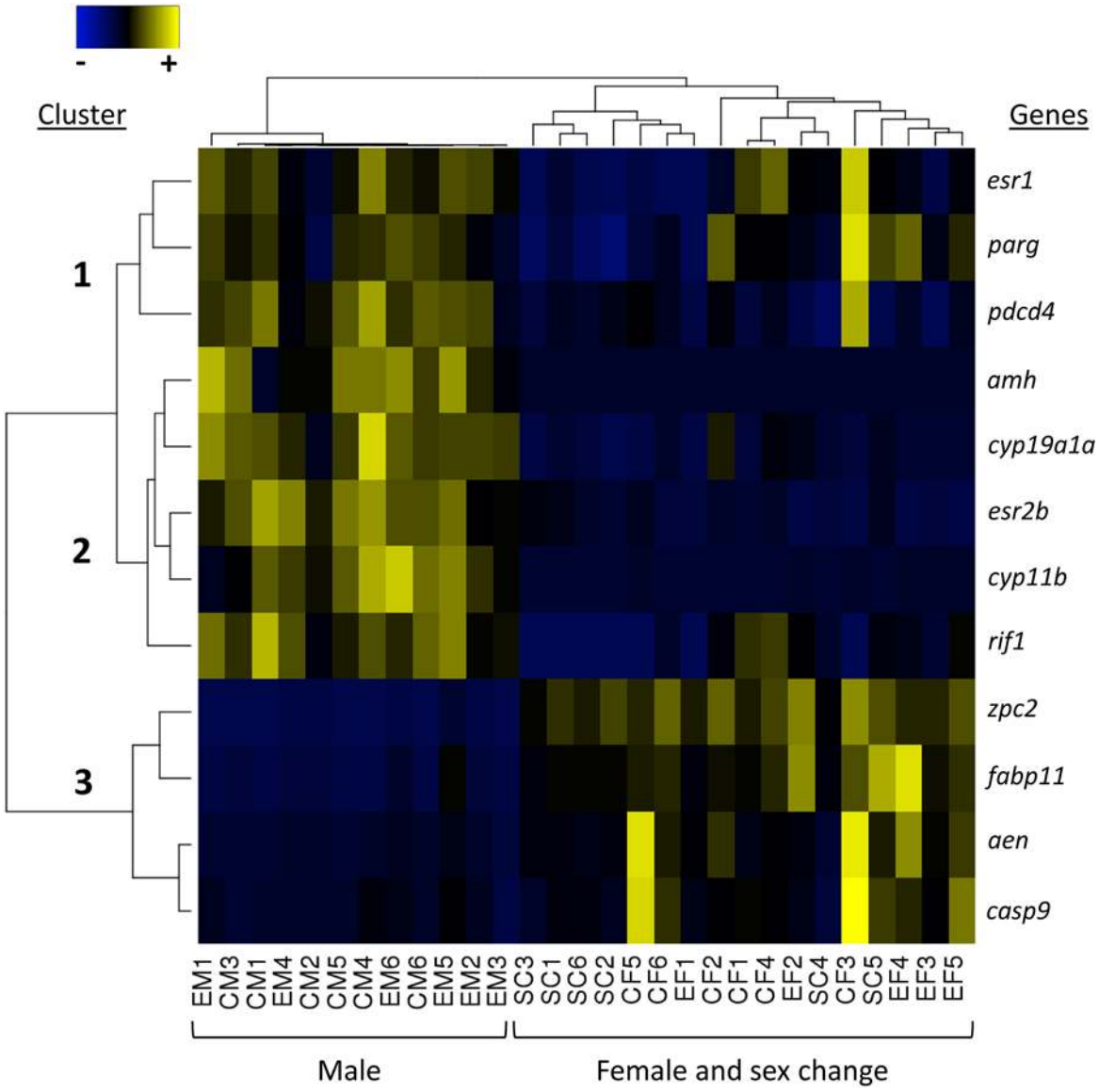

Fig. 4.

Cluster analysis of 12 gonadal gene expression patterns in control male (CM), control female (CF), exemestane-treated male (EM), exemestane-treated female (EF), and exemestane-induced early sex changing fish (SC). Each row represents a single gene (labeled at right) and each column represents an individual (labeled at bottom). Colored blocks represent relative gene expression on a scale of high (yellow), medium (black), or low (blue), based on Pearson distance measurements. Brackets below surround either entirely testicular (male) or ovarian-dominated samples (F and SC), and bold numbers to the left indicate major gene clusters.

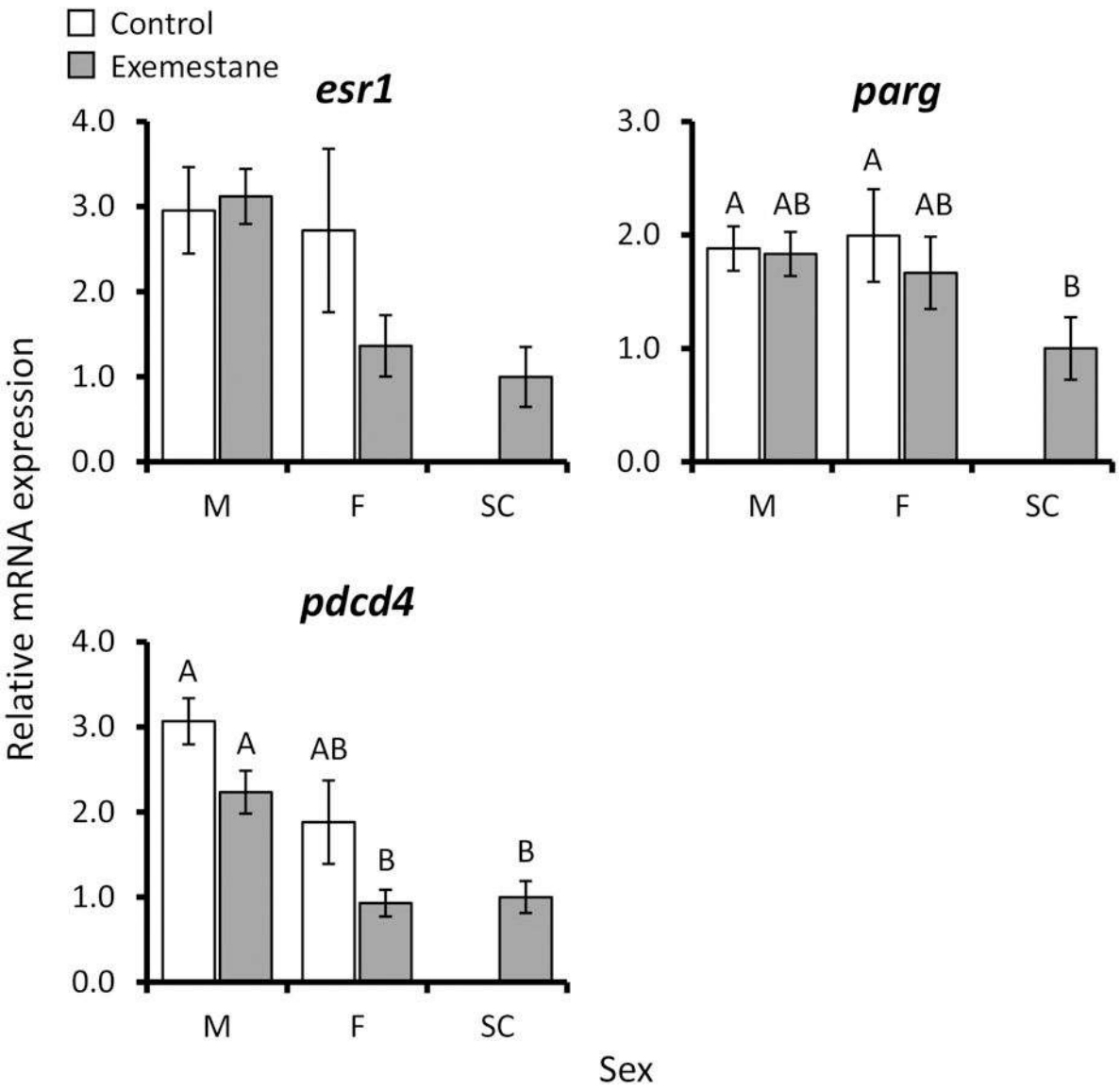

Fig. 5.

Relative mRNA expression (mean ± standard error, normalized to *eef1a*) of cluster 1 genes (*esr1*, *parg*, and *pdcd4*) in male (M), female, (F), and early sex changing (SC) fish. White and gray bars refer to control and exemestane treatments, respectively. Different letters denote significant differences.

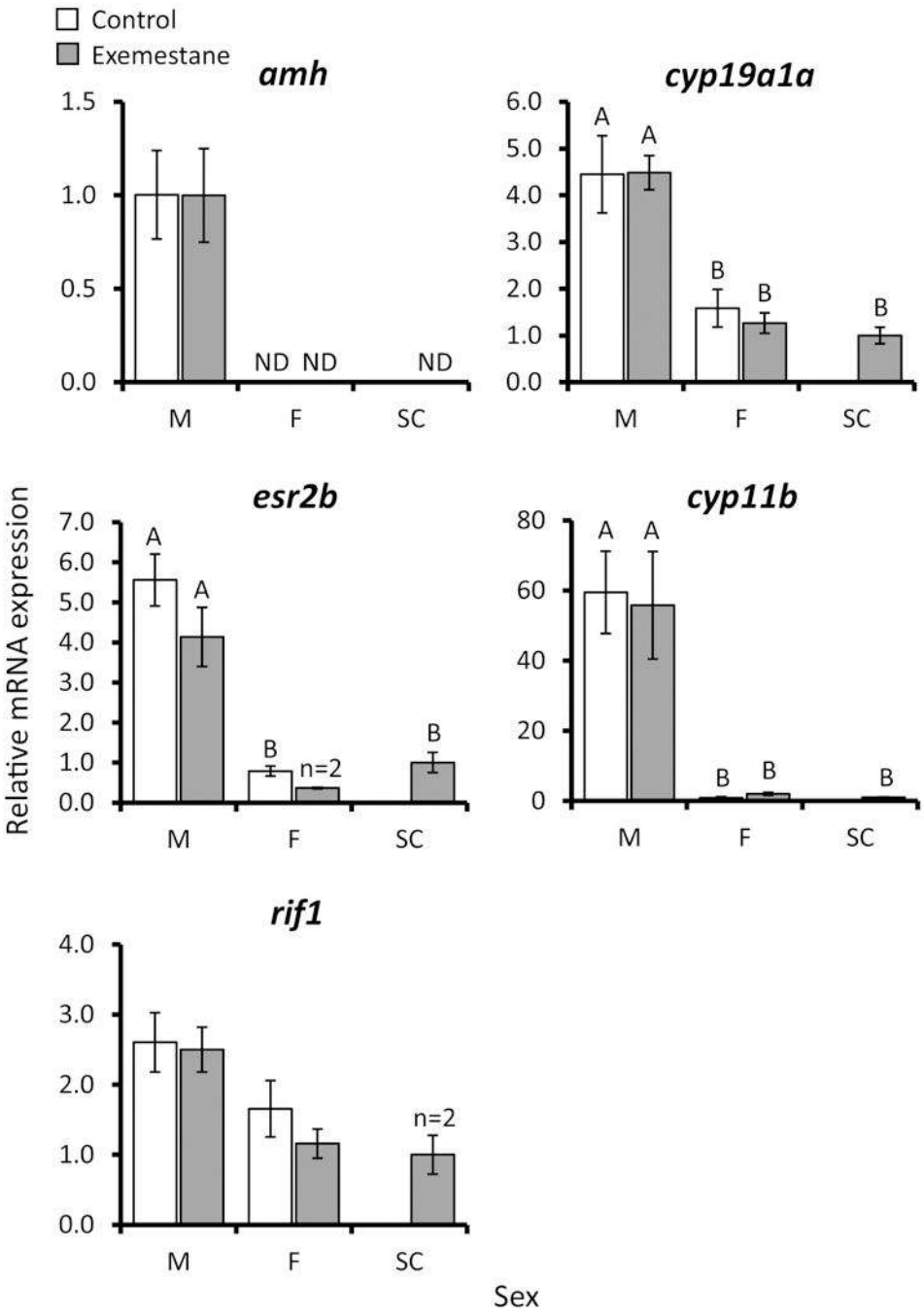

Fig. 6.

Relative mRNA expression (mean ± standard error, normalized to *eef1a*) of cluster 2 genes (*amh*, *cyp19a1a*, *esr2b*, *cyp11b*, and *rif1*) in male (M), female, (F), and early sex changing (SC) fish. White and gray bars refer to control and exemestane treatments, respectively. Different letters denote significant differences.

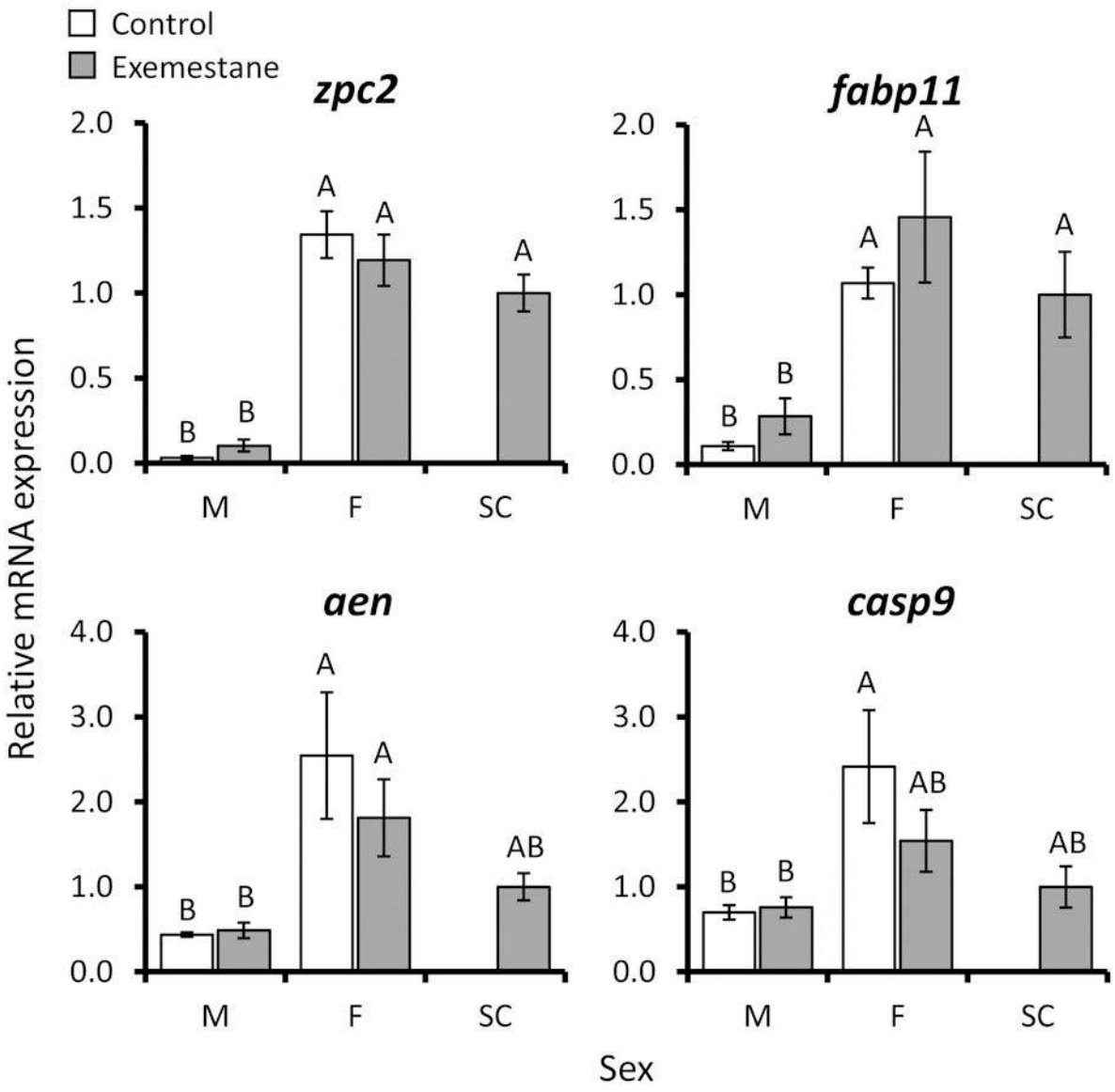

Fig. 7.

Relative mRNA expression (mean ± standard error, normalized to *eef1a*) of cluster 3 genes (*zpc2*, *fabp11*, *aen*, and *casp9*) in male (M), female, (F), and early sex changing (SC) fish. White and gray bars refer to control and exemestane treatments, respectively. Different letters denote significant differences.

Table 1.

Gene symbols, identities, putative functions, primer sequences, product sizes (bp), and GenBank accession numbers for partial coding domain sequence (cds) fragments of 12 candidate genes in black sea bass. Zebrafish Information Network (ZFIN) nomenclature and selected gene ontology (GO) Biological Function terms were used when possible.

Gene	Gene identity	Putative function	GO ID or reference	Primer sequence (5′-3′)	bp	GenBank
aen	apoptosis-enhancing nuclease	apoptosis	Kawase et al., 2008	F - AACAGGAAGATCCAGGCGG	431	**MH308015**
				R - CACAGCAGAAAATGTGCGGA		
amh	anti-Mullerian hormone	gonad development	GO:0008406	F - GGTCACTGCATCCTGACACA	681	**MH308019**
				R - CTCGGTACTGGCTCTCTCCT		
casp9	caspase 9	apoptotic process	GO:0006915	F - AATCGCAAGGGGTCCAACAT	623	**MH308017**
				R - ATTGTGGCCAAGTCATCGGT		
cyp11b	11-beta hydroxylase	11-KT synthesis	Baroiller et al., 1999	F - CTTTCGCTCCCTGGCGTTT	232	**MH308014**
				R - TGGTGATGGCATGACTCTGT		
cyp19a1a	gonadal aromatase	female gonad development	GO:0008585	F - GGACCTCGTACGGGTTGTTT	592	**MG979051**
				R - GTCCAGGTGAGTCTGTGTGG		
esr1	estrogen receptor alpha	cellular response to estrogen stimulus	GO:0071391	F - CTGTCCCGGCAAACTCATCT	580	**MH476361**
				R - GTTCTCGTGGCTGACTCTGG		
esr2b	estrogen receptor beta 2	response to estrogen	GO:0043627	F - GATGTCCCTCACCAACCTGG	672	**MH476362**
				R - GCGTCACAGTAAGTGTCGGA		
fabp11	fatty acid binding protein 11	ovarian follicular atresia	Agulleiro et al., 2007	F - CTTCACGGGTGATCCTGTCA	501	**MH308016**
				R - AAGCTCAGTGCATTTGGGGT		
parg	poly (ADP-ribose) glycohydrolase	poly (ADP-ribose) degradation	Shirai et al., 2013	F - GCAAGCACCTCTCTGCACTA	662	**MG979052**
				R - GGCCTAACAGCAACCAGGAT		
pdcd4	programmed cell death protein 4	apoptosis	Shibahara et al., 1995	F - CGTAGACGCCGAGAAGAGTG	504	**MH308018**
				R - GCTTGGCTTTGGCTTTGAGT		
rif1	replication timing regulatory factor 1	regulate DNA replication	Foti et al., 2016	F - TGAAGCACTCCAAGGTACACA	250	**MG979053**
				R - AGCGCTGATCACAGCTGATT		
zpc2	zona pellucida glycoprotein C2	egg envelope component	Spargo and Hope, 2003	F - AATGCAGACATGGTGCCTCA	611	**MH476363**

Table 2.

qPCR primer sequences, product sizes (bp), PCR efficiencies (%), and mean cycle threshold (Ct) values for *eef1a* and 12 candidate gene assays in black sea bass. Mean Ct refers to the mean diluted 1/20 standard curve point in each assay.

Gene symbol	qPCR primer sequence (5'–3')	Product size (bp)	PCR efficiency (%)	Mean Ct
aen	F - GAGAGCTGCAGGACAAACT R - GTGACATCATCGGGCCAGTA	104	94.0	21.8
amh	F - CAAGACCACCCTGAATCCCC R - CTGGAGGAGAGGCCTAGTGT	80	97.0	30.0
casp9	F - GCACGTCCCAGTTCAGTACA R - GGACACCTCAAAGCCTGTGT	130	97.0	26.9
cyp11b	F - TGTGTCGGGAGGAGAATTGC R - TGCAGGATGAGCGTGACTTT	122	89.8	26.7
cyp19a1a	F - GGTCCGTCTTTCTGTCTGGG R - GTAGTTGCTGGCTGTGCCTA	84	97.5	26.9
eef1a	F - GTGACAACGTCGGCTTCAAC R - ATTGGTGGGTCGTTCTTGCT	91	102.4	22.8
esr1	F - CTGTCCCGGCAAACTCATCT R - GGTTTGAGTTTGAGCAGGCG	135	87.6	26.6
esr2b	F - GCCTGCTGGACTCTGTGATT R - AGCATGGTGAGGTGTCCAAG	100	104.1	29.2
fabp11	F - GCGAAGCCTAACCTGGTGAT R - TGACGGTCTTGGTCTTTCGG	148	88.3	15.5
parg	F - GCAAGCACCTCTCTGCACTA R - TGTGAAGTACGCCACGTCTC	128	98.2	23.0
pdcd4	F - CATGGCGACCGAAGTGGATA R - GATGGTTAGCCTCTGCCAGG	89	89.2	23.7
rif1	F - CTCCAAACGGACTCTCAACCA R - AAGCACTTCAGCAGATGGCA	84	89.4	26.8
zpc2	F - GGTTCACCCCATCATCAGCA R - CCGTGACCGAACTTGAAGGA	126	90.3	16.5