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Expression profiles of *dmrts* and *foxls* during gonadal development and sex reversal induced by 17α-methyltestosterone in the orange-spotted grouper

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ABSTRACT

The orange-spotted grouper, *Epinephelus coioides*, is a marine protogynous hermaphrodite fish of commercial importance. There are many examples of sex change species among marine fish, but the molecular basis for the sex change is still unknown. Gonadal expression patterns of the *dmrts* and *foxls* genes in *E. coioides* have pointed to sexual dimorphism in this species and it has been shown that mRNA levels of *dmrts* and *foxls* to vary significantly during reproduction cycles. The steroid 17α-methyltestosterone was used to induce sex reversal in these fish, during which *dmrts* and *foxls* levels changed significantly and subsequently reverted to normal when 17α-methyltestosterone was withdrawn. Interestingly, the expression of *dmrt2b* and *dmrt3* was not affected by this steroid. We speculate that the role of *foxl2* in reproduction may be conserved via regulation of early differentiation of the ovary by the hypothalamus–pituitary–gonad axis, and *dmrt2* may have a significant role in premature ovarian differentiation and maintenance in *E. coioides*. *dmrt1* and *foxl3* played a role in the development of the testes and are believed to be potential male regulatory genes.

1. Introduction

Sex development is a multistep process involving sex determination, initiation, differentiation, and maintenance, and culminating in the production of sperm or eggs for germline transmission (Zhang et al., 2016). Sex determination in vertebrates, encompassing both genetic and environmental sex determination, is a relatively uncharacterized biological phenomenon in nature (Graves, 2008). There are several sex determination mechanisms in animals, but in comparison to other vertebrates, sex determination mechanisms in fish are more diverse (Guiguen et al., 2010). For example, gonochoristic species possess purely ovarian or testicular tissues, while hermaphroditic species can mature either, as a male (protandrous) or a female (protogynous). Genetic, environmental, behavioral, and physiological factors affect the fate of both somatic and germ cells within the primordial gonad (Brykov, 2014; Devlin and Nagahama, 2002). Some hermaphroditic animals, such as annelids, echinoderms, mollusks, and fish, can also undergo sex reversal (Allsop and West, 2010; Vega-Frutis et al., 2014). Many genes involved in gonadal differentiation have been identified in vertebrates (Picard et al., 2015; Takatsu et al., 2013; Xia et al., 2007). Nonetheless, the molecular mechanism involved in gonadal differentiation in fish is still unclear. Exogenous sex steroids induce sex reversal, a process that has been widely studied (Piferrer, 2001; Ryosuke et al., 2014). Gonadal differentiation is sensitive to steroids, and phenotypic sex can be changed by hormonal treatment (Takatsu et al., 2013). Treatment of zebrafish during gonadal differentiation with either the nonsteroidal aromatase inhibitor fadrozole or 17α-methyltestosterone (MT) altered gonad morphological differentiation and *CYP19* expression patterns (Fenske and Segner, 2004). Mature females were induced to change to functional males by an aromatase inhibitor, accompanied by decreases in estrogen levels (Guiguen et al., 1999; Kitano et al., 2000; Uchida et al., 2004; Wu et al., 2015). Similarly, androgens can induce females to change to functional males (Wu et al., 2015).

The doublesex and mab-3 related transcription (*dmrt*) gene family has been extensively studied and comprised major transcription factors involved in vertebrate sex determination, neurogenesis, and

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differentiation pathways (Ma et al., 2016; Picard et al., 2015; Wexler et al., 2014). The family members are widely expressed in a variety of organisms, and play key roles in growth, development, sex differentiation, organ development and maintenance, and somite differentiation (Wexler et al., 2014). One member of the family, *dmrt1*, is evolutionarily conserved and is involved in sex determination and differentiation, playing important roles in spermatogenesis and testicular development (Agbor et al., 2013; Parlier et al., 2013; Sheng et al., 2014; Úbeda-Manzanaro et al., 2014). Other *dmrt* family members are expressed in both gonadal and extragonadal tissues. Another member, *dmrt2*, is expressed in the dermomyotome of developing vertebrate somites (Seo et al., 2006). *In situ* hybridization studies have shown that *dmrt2* and *dmrt3* are both expressed in germ cells of the developing swamp eel (Sheng et al., 2014).

Similarly, *foxl2* encodes a forkhead transcription factor implicated in ovarian differentiation and development (Caburet et al., 2012; Crisponi et al., 2001; Pisarska et al., 2011). Two *foxl2* paralogs, *foxl2a* (*foxl2*) and *foxl2b* (*foxl3*), have been discovered in several teleost species and were considered as fish-specific duplicates (Baron et al., 2004; Crespo et al., 2013; Geraldo et al., 2013). Owing to evolutionary inputs, *foxl3* has been lost in tetrapods (Christoffels et al., 2004; Geraldo et al., 2013). A recent study has focused on Foxl3 in medaka (Nishimura et al., 2015), however more studies are required to enhance our understanding of *foxl3* roles, especially in vertebrate sexual differentiation (Gao et al., 2016; Geraldo et al., 2013).

Gonad development in the grouper undergoes transition from ovaries to intersexual gonads and into testes, therefore *Epinephelus coioides* is an ideal model for studying vertebrate sex determination or differentiation (Zhou and Gui, 2010). The MT steroid has been widely used to induce masculinization; however, the process underlying sex reversal induced by MT exposure and subsequent withdrawal has been under-researched. In this study, we investigated gonadal histology and expression profiles of the *dmrt* and *foxl* genes during gonadal development and sex reversal induced by MT exposure and subsequent withdrawal. These investigations revealed that both *dmrt* and *foxl* genes showed differential expression patterns in *E. coioides* tissues.

2. Materials and methods

2.1. Animals and samples

Orange-spotted groupers aged 1 year (body mass, 0.09–0.24 kg; body length, 14.9–22.8 cm) and 6–36 months (body mass, 0.9–4.6 kg; body length, 33.7–58.5 cm) were obtained from the Guangdong Marine Fishery Experiment Center (Huizhou City, Guangdong, People's Republic of China) from May to September 2013 and during the grouper reproductive season (April–October 2016). Fish were fed twice daily at a rate of 4–5% body weight, which was regulated according to feed intake. The groupers were lightly anesthetized with 8 mg/L eugenol before dissection. Fifteen specimens were killed by cervical dislocation to collect tissues for gene cloning and tissues investigations. Various tissues (brain, pituitary, heart, liver, intestine, stomach, kidney, muscle, gill, spleen, ovaries, and testes) were collected and placed into sterile tubes containing appropriate volumes of RNAlater® (Thermo Scientific, Waltham, MA, USA). All samples were stored at −20 °C for downstream RNA isolation experiments. A small piece of gonadal tissues was also placed into a tube containing Bouin's fluid for histology to confirm the gonadal development stage. All animal experiments conformed to directives from the Institutional Animal Care and Use Committee, South China Agricultural University.

2.2. RNA isolation and reverse transcription

Total RNA was extracted from the ovaries and testes of groupers using a Hi-pure Universal RNA Kit (Magen, Guangzhou, China) according to the manufacturer's instructions. Nucleic acid concentrations were quantified by measuring the absorbance at 260 nm on a Nanodrop ND-1000 spectrophotometer (Quawell Technology, San Jose, CA, USA). Approximately 1 μg of total RNA from each sample was used to synthesize first-strand cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Table 1
Primers used in this study.

Primer	Primer sequence (5′ → 3′)
dmrt2-F	GAAAGGAATATCTGGGAAACA
dmrt2-R	AGATGAGATGCCCTCTGACT
dmrt2b-F	AAGTCTGGTCAGCGTTAG
dmrt2b-R	CGTGTTCTGGAAATGGTT
dmrt3-F	CTCCATCAACCTGCCCTTCA
dmrt3-R	CGGATGTCCGTCCACAATA
foxl2-F	CTGGGCTTACAGAGTCACAA
foxl2-R	ATGACTTCTTTACCTCCAGAT
foxl3-F	GACAATGGAGGGGACCGTAAG
foxl3-F	AGGTAGAGGGGCTCGGGATA
dmrt1-QF	ACGCCACCAAATGAATAA
dmrt1-QR	TCAACTAAGGCACAGAAAA
dmrt2-QF	CGAACTGCCTGGATCTGTC
dmrt2-QR	CGAACTGCCTGGATCTGTC
dmrt2b-QF	CAACCATCTCACGCTCTATT
dmrt2b-QR	TCTGAGCACCAGCCCTTA
dmrt3-QF	AACGACGTGACTCTGTGGAATA
dmrt3-QR	AGTACAGGCCGGGTTTGG
foxl2-QF	CGGAAGGTTGGCAGAACAGTA
foxl2-QR	GGTAAAAGTGCGTCGGTGGA
foxl3-QF	GACAATGGAGGGGACCGTAAG
foxl3-QF	AGGTAGAGGGGCTCGGGATA
β-*actin*-QF	ACCATCGGCAATGAGAGGTT
β-*actin*-QF	ACATCTGCTGGAAGGTGGAC

2.3. Cloning and sequencing of dmrt1, dmrt2, dmrt2b, dmrt3, foxl2, and foxl3 cDNA

The *E. coioides dmrt1* sequence was obtained from the National Center for Biotechnology Information (NCBI reference sequence EF017802.1). We obtained the open reading frame (ORF) sequences for *E. coioides dmrt2, dmrt2b, dmrt3, foxl2*, and *foxl3* (data not shown) and the conserved regions from known cDNA sequences (*Oreochromis niloticus*, NCBI reference sequence NM_001279778; *Cynoglossus semilaevis*, NCBI reference sequence NM_001294199; *Danio rerio*, NCBI reference sequence NM_001317761). Primers for *dmrt1* were designed according to Xia et al. (2007). The remaining primers were designed using Primer 5.0 software (Biosoft International, Palo Alto, CA, USA). All primers used in this study are listed in Table 1.

PCR amplifications were performed in a volume of 20 μL containing 5 μL of 10× buffer, 2 μM dNTPs, 0.5 μL of cDNA template, 1 μM of each primer, and 0.2 U of Blend Taq polymerase (Toyobo, Tokyo, Japan). The PCR parameters were as follows: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min; and then 72 °C for 10 min. Negative controls without the template were treated in the same way. All PCR amplification products were electrophoresed on 1.5% agarose gels. Bands reflecting appropriate product sizes were excised and purified using an E.Z.N.A®Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA), then subcloned into the pTZ57R/T vector (Fermentas, Hanover, MD, USA). Positive clones were sequenced by the Invitrogen Biotechnology Company (Shanghai, China). The *dmrts*, *foxl2*, and *foxl3* genes of the *E. coioides* transcriptome were identified by BLAST analysis using the NCBI web servers (www.ncbi.nlm.nih.gov/). Multiple alignment of amino acid sequences was performed using CLUSTAL W (www.ebi.ac.uk/clustalw/). MEGA 5.1 (www.megasoftware.net/) was applied to construct phylogenetic trees using the neighbor-joining method with 1000 bootstrap replicates.

2.4. Tissues distributions of dmrt1, dmrt2, dmrt2b, dmrt3, foxl2, and foxl3

Total RNA was extracted (as described earlier) from mature male and female *E. coioides* tissue, including the brain, pituitary, heart, liver, intestine, stomach, kidney, muscle, gill, spleen, ovaries, and testes. The RNA quality and concentrations for each sample were checked, as previously described. Approximately 1 μg of total RNA from each sample was used to synthesize first-strand cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche). Semi-quantitative RT-PCR was used to investigate the tissues distribution of *dmrt1*, *dmrt2*, *dmrt2b,* and *dmrt3* using Blend Taq® Plus (Toyobo). PCR was performed in a 20 μL reaction mix using the following cycling parameters: 95 °C for 3 min; 32 cycles of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 1.5 min; and then 72 °C for 10 min. Negative controls without the template were treated in the same way.

To investigate the tissues distribution of *foxl2* and *foxl3*, quantitative real-time PCR (qPCR) analyses were performed on an ABI7900 480 real-time PCR system (ABI, Waltham, MA, USA) using SYBR®Green real-time PCR Master Mix (Toyobo) according to the manufacturer's instructions. Real-time PCR conditions were as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 20 s. Negative controls without the template were treated in the same way. After amplification, fluorescence data were converted to cycle threshold (CT) values. We chose β-actin as a reference gene for sample normalization. The qPCR primers were designed using Primer 5.0 software (Table 1). All samples were analyzed in triplicate. The formula $2^{-\Delta\Delta Ct}$ was used to calculate relative transcript quantities.

2.5. Gonadal histology

Gonads were fixed in Bouin's solution for 24 h at room temperature and stored in 70% ethanol, until histological processing. After dehydration in ascending ethanol grades and embedding in paraffin, tissues blocks were sectioned at 7 μm and stained with hematoxylin and eosin for analysis (Liu and de Mitcheson, 2009). Different stages of gonadal development were identified using light microscopy (Nikon IQ50, Tokyo, Japan) according to the classification of gonadal morphology by Liu and de Mitcheson (2009) and Chen et al. (2011).

2.6. Expression patterns of dmrt1, dmrt2, dmrt2b, dmrt3, foxl2, and foxl3 during gonadal development

Total RNA from gonads from different development periods were used to synthesize first-strand cDNA, as described earlier. qPCR assays were performed on an ABI7900 480 real-time PCR system, as previously described. Negative controls without the template were treated in the same way. Real-time PCR conditions were as follows: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 20 s. The formula $2^{-\Delta\Delta Ct}$ was used to calculate relative transcript quantities.

2.7. Sex reversal during MT feeding and MT withdrawal

We randomly divided groupers into two groups: aMT feeding group and a control group. The two groups were cultivated separately. The MT feeding group (n = 60) was fed with MT formula feed (10 mg/kg; Sigma Aldrich, San Mateo, CA, USA), while the control group (n = 30) was simultaneously fed a standard diet. To explore the effects of MT withdrawal, we separated half of the groupers after 3 months of treatment and withdrew the MT diet, replacing it with a standard diet. The gonads of five groupers were collected each month for qPCR and gonadal histology.

2.8. Analysis of gonadal differentiation induced by MT

We used MT to induce grouper gonadal differentiation for a period of 90 days after hatching; MT induces male development. Groupers were separated into two groups: an MT feeding group and a control group. The MT feeding group (n = 25) was fed with MT feed (10 mg/kg; Sigma Aldrich) and the control group (n = 25) was fed a standard diet. Gonad samples from six groupers from each group were taken at 20, 60, and 90 days after treatment (dat) for qPCR analysis.

Table 2
Pairwise comparison of amino acid sequences for Dmrt and Fox proteins in *E. coioides* and other species.

Protein	Species	Amino acid identity	Accession no.
Dmrt2	*Monopterus albus*	90%	AGJ00067.1
Dmrt2	*Oreochromis niloticus*	90%	NP_001266696.1
Dmrt2	*Takifugu rubripes*	83%	CAC42780.1
Dmrt2	*Danio rerio*	69%	NP_571027.1
Dmrt2	*Xenopus tropicalis*	59%	NP_001093726.1
Dmrt2	*Chelonia mydas*	58%	EMP30458.1
Dmrt2	*Homo sapiens*	58%	AAI36494.1
Dmrt2	*Columba livia*	57%	EMC85639.1
Dmrt2	*Glandirana rugosa*	57%	BAF44084.1
Dmrt2	*Bos taurus*	56%	NP_001179302.1
Dmrt2	*Mus musculus*	56%	NP_665830.1
Dmrt2	*Bos mutus*	56%	ELR53306.1
Dmrt2	*Rattus norvegicus*	54%	NP_001101067.1
Dmrt2b	*Oreochromis niloticus*	73%	NP_001266402.1
Dmrt2b	*Monopterus albus*	73%	AGJ00068.1
Dmrt2b	*Salmo salar*	48%	NP_001133069.1
Dmrt2b	*Carassius carassius*	43%	ABK27325.1
Dmrt2b	*Danio rerio*	40%	NP_001073445.1
Dmrt3	*Monopterus albus*	94%	ADI24451.1
Dmrt3	*Takifugu rubripes*	87%	NP_001033034.1
Dmrt3	*Megalobrama amblycephala*	70%	AJD87235.1
Dmrt3	*Danio rerio*	68%	AAU89440.1
Dmrt3	*Glandirana rugosa*	61%	BAF44085.1
Dmrt3	*Xenopus tropicalis*	61%	NP_001243149.1
Dmrt3	*Rattus norvegicus*	61%	NP_001099828.1
Dmrt3	*Mus musculus*	60%	NP_796334.2
Dmrt3	*Homo sapiens*	60%	NP_067063.1
Foxl2	*Epinephelus merra*	100%	ACD62374.1
Foxl2	*Monopterus albus*	98%	AGJ01120.1
Foxl2	*Paralichthys olivaceus*	97%	BAF69017.1
Foxl2	*Oreochromis niloticus*	97%	NP_001266707.1
Foxl2	*Oryzias latipes*	96%	NP_001098358.1
Foxl2	*Oncorhynchus mykiss*	93%	NP_001117957.1
Foxl2	*Cynoglossus semilaevis*	91%	NP_001281128.1
Foxl2	*Rugosa rugosa*	83%	BAG69483.1
Foxl2	*Gallus gallus*	81%	NP_001012630.1
Foxl2	*Xenopus laevis*	79%	NP_001128256.1
Foxl2	*Leptosomus discolor*	74%	KFQ03440.1
Foxl2	*Mus musculus*	67%	NP_036150.1
Foxl2	*Homo sapiens*	65%	AAY21823.1
Foxl2	*Bos taurus*	62%	NP_001026920.1
Foxl3	*Danio rerio*	69%	NP_001304690.1
Foxl3	*Oncorhynchus mykiss*	38%	NP_001117956.1
Foxl3	*Dicentrarchus labrax*	78%	AFV13295.1
Foxl3	*Kryptolebias marmoratus*	63%	APD78564.1
Foxl3	*Oryzias latipes*	52%	XP_004070713.1

2.9. Statistical analysis

All data are expressed as the mean ± standard error (SE). Significant differences were estimated using one-way analysis of variance (ANOVA; Graphpad Prism 7; GraphPad Software, La Jolla, CA, USA). A probability level of $p < 0.05$ using Duncan's multiple range test indicated a significant difference.

3. Results

3.1. E. coioides dmrt1, dmrt2, dmrt2b, dmrt3, foxl2, and foxl3 cDNA sequence and phylogeny

The complete ORFs for *dmrt2*, *dmrt2b*, and *dmrt3* were 1401, 1344,

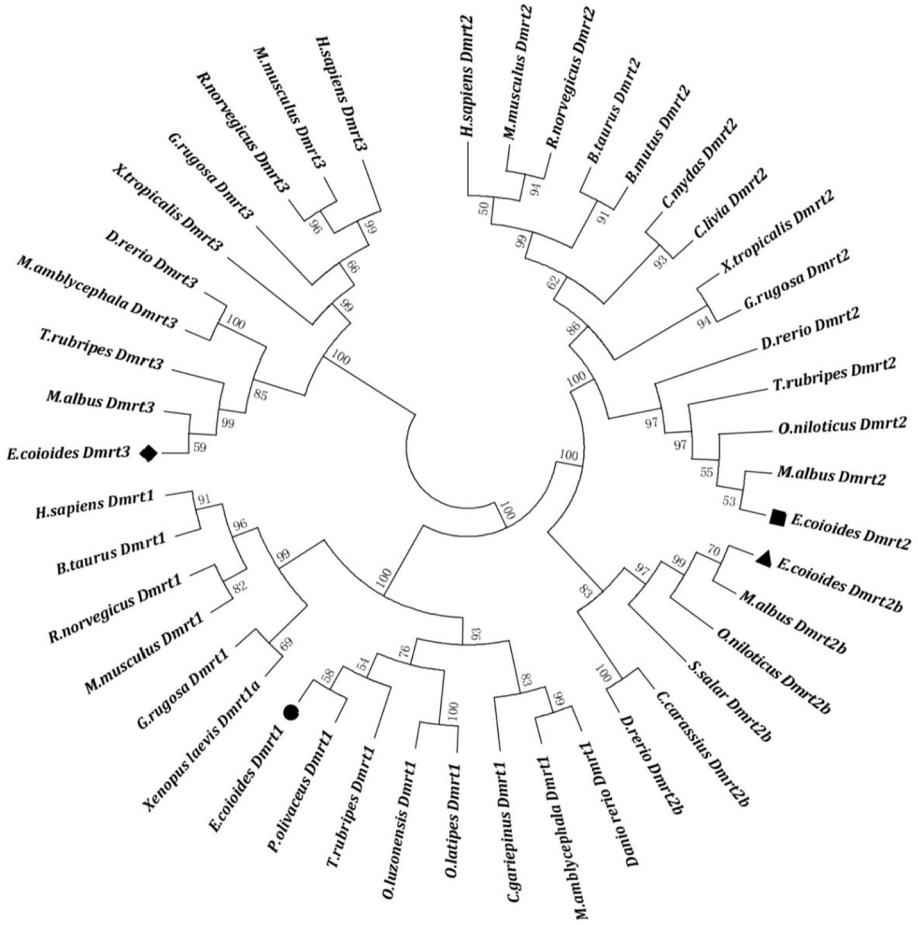

Fig. 1a. Phylogenetic analysis of *E. coioides* Dmrt proteins. Accession numbers for these sequences are given in Table 2.

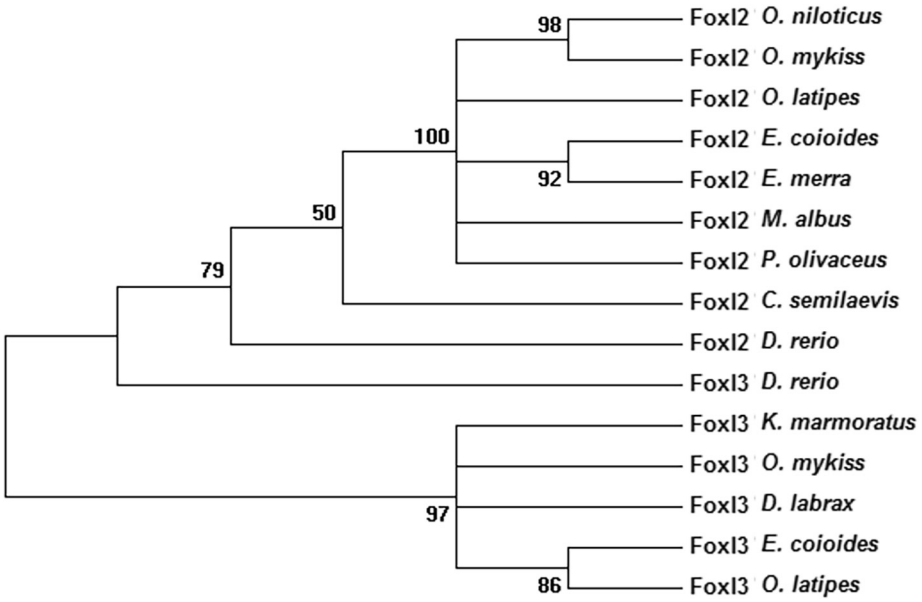

Fig. 1b. Phylogenetic analysis of *E. coioides* Foxl2 and Foxl3 proteins. Accession numbers for these sequences are given in Table 2.

and 1422 bp, encoding proteins of 466, 448, and 473 amino acids, respectively (Fig. S1A–C). The complete cDNA of *E. coioides foxl2* was 1413 bp and contained an ORF of 921 bp encoding a protein of 306 amino acids. The ORF was preceded by a 5′ untranslated region (UTR) of 280 bp; the 3′ UTR was 212 bp (Fig. S1D). *foxl3* cDNA was 1656 bp and contained a 795-bp ORF encoding a protein of 264 amino acids, with a 259-bp 5′ UTR and a 602-bp 3′ UTR containing a typical polyadenylation signal (AATAAA) (Fig. S1E).

Multiple sequence alignment revealed 54–90% identity between the deduced Dmrt2 protein in *E. coioides* and other vertebrates, with 40–73% identity for Dmrt2b and 60–94% for Dmrt3 (Table 2). Foxl2 showed 62–100% and Foxl3 showed 52–60% identity with other

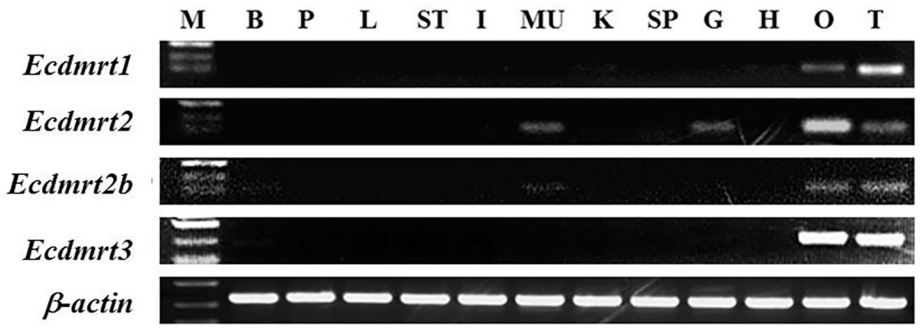

Fig. 2a. RT-PCR analysis of *dmrt* gene expression in *E. coioides* tissue. M: 2-kb DNA ladder; B: brain; P: pituitary; L: liver; ST: stomach; I: intestines; MU: muscle; K: kidney; SP: spleen; G: gill; H: heart; O: ovaries; T: testis.

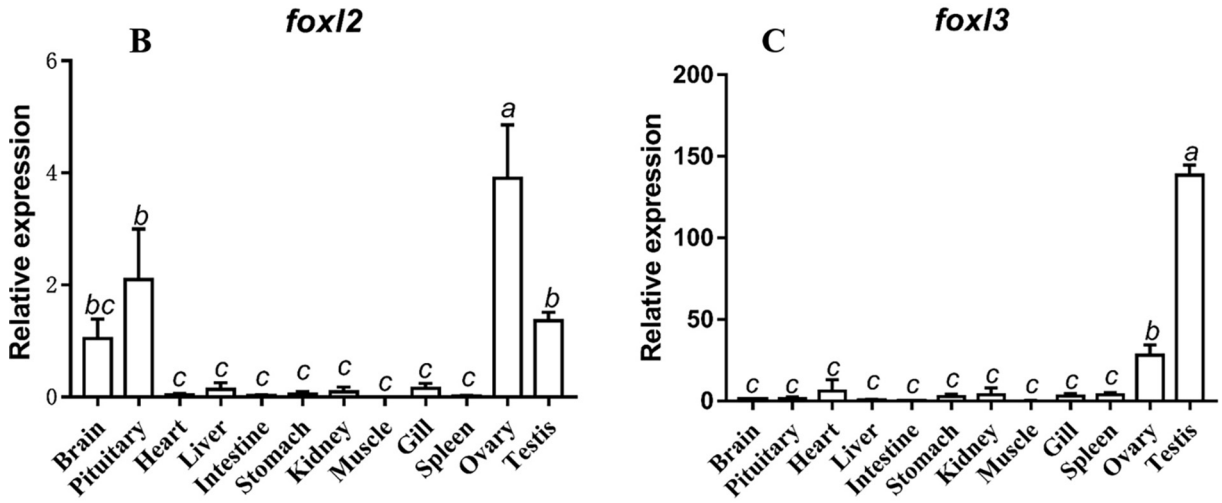

Fig. 2b–c. . shows qPCR analysis of the tissues distribution of *foxl2* and *foxl3* in *E. coioides*. Significant differences among tissues are indicated using lowercase letters (a, b and c). *foxl2* expression was detected in the ovaries and was significantly higher in this tissue when compared with other tissues, while *foxl3* was predominantly expressed in the testes and ovaries, with higher levels in the testes and low expression levels in the ovaries and other tissues.

species (Table 2).

Phylogenetic analyses were performed to elucidate the evolutionary relationship of *E. coioides* with respect to other vertebrates (Fig. 1a). In vertebrates, Dmrt shows strong evolutionary conservation. Phylogenetic analysis revealed that *E. coioides* Dmrt1, Dmrt2, Dmrt2b, and Dmrt3 proteins segregate into four different branches. *E. coioides* Foxl2 clusters with *Epinephelus merra* (bootstrap 92), *Oryzias latipes*, *Monopterus albus*, and *Paralichthys olivaceus* (bootstrap 100) proteins (Fig. 1b). *E. coioides* Foxl3 clusters with *O. latipes* (bootstrap 86), *Danio rerio*, *Kryptolebias marmoratus*, and *Oncorhynchus mykiss* (bootstrap 97) proteins (Fig. 1b).

3.2. Tissues distributions of dmrt1, dmrt2, dmrt2b, dmrt3, foxl2, and foxl3

dmrt1 expression levels were higher in testes when compared to ovaries (Fig. 2a). *dmrt2* was expressed in muscle, gill, testes and ovaries. *dmrt2b* was expressed in muscle, testes and ovaries, while *dmrt3* was mainly expressed in testes and ovaries (Fig. 2a). *foxl2* expression was detected in the ovaries and was found to be significantly higher in this tissue when compared to other tissues ($p < 0.05$; Fig. 2b), while *foxl3* was predominantly expressed in the testes and ovaries, with higher levels in the testes and low expression levels in the ovaries and other tissues ($p < 0.05$; Fig. 2c). From these observations, we deduced that the role of *foxl2* in reproduction may be conserved, mainly via the regulation of early differentiation of the ovaries by the hypothalamus–pituitary–gonad axis. While *foxl3* may participate in testes development, its role in reproduction may be regulated by the testes, suggesting that *foxl3* is a potentially male-specific regulatory gene. To understand the possible roles of *E. coioides dmrt1*, *dmrt2*, *dmrt2b*, and *dmrt3* genes during gonadal development, mRNA expression levels were determined in gonads at different growth stages in both natural and artificially sex-reversed *E. coioides*.

3.3. The six stages of gonad development

According to gonadal morphology characteristics (Chen et al., 2011; Liu and de Mitcheson, 2009), ovary development involves six stages. In juveniles, the gonadal primordium with gonium (G) develops and the ovarian lumen (OL) forms (ov-I; Fig. 3a). Upon completion of the OL and G proliferation, G occupies almost most of the gonad, and the primary growth stage of oocytes occurs (O1), therefore O1 becomes dominant (ov-II; Fig. 3b). Before the breeding season, the gonads develop and cortical-alveolus stage oocytes (O2) become dominant (ov-III; Fig. 3c). During the breeding season, vitellogenic stage oocytes (O3) become dominant (ov-IV; Fig. 3d). After the breeding season, O3s degenerate (Fig. 3e) and the gonads transition to a bisexual stage, with the appearance of spermatogonia (Fig. 3F). At this stage the gonad develops into a testis (Fig. 3G).

3.4. Gene expression profiles during gonadal development

To investigate the relationship between *dmrts* and *foxls* expression and sex reversal in *E. coioides*, we analyzed gene expression at different gonadal developmental stages. The highest levels of *dmrt1* were detected in the ovotestis (Fig. 4A), while *dmrt2* was highly expressed at stage II ovary development ($p < 0.05$; Fig. 4B). The highest levels of

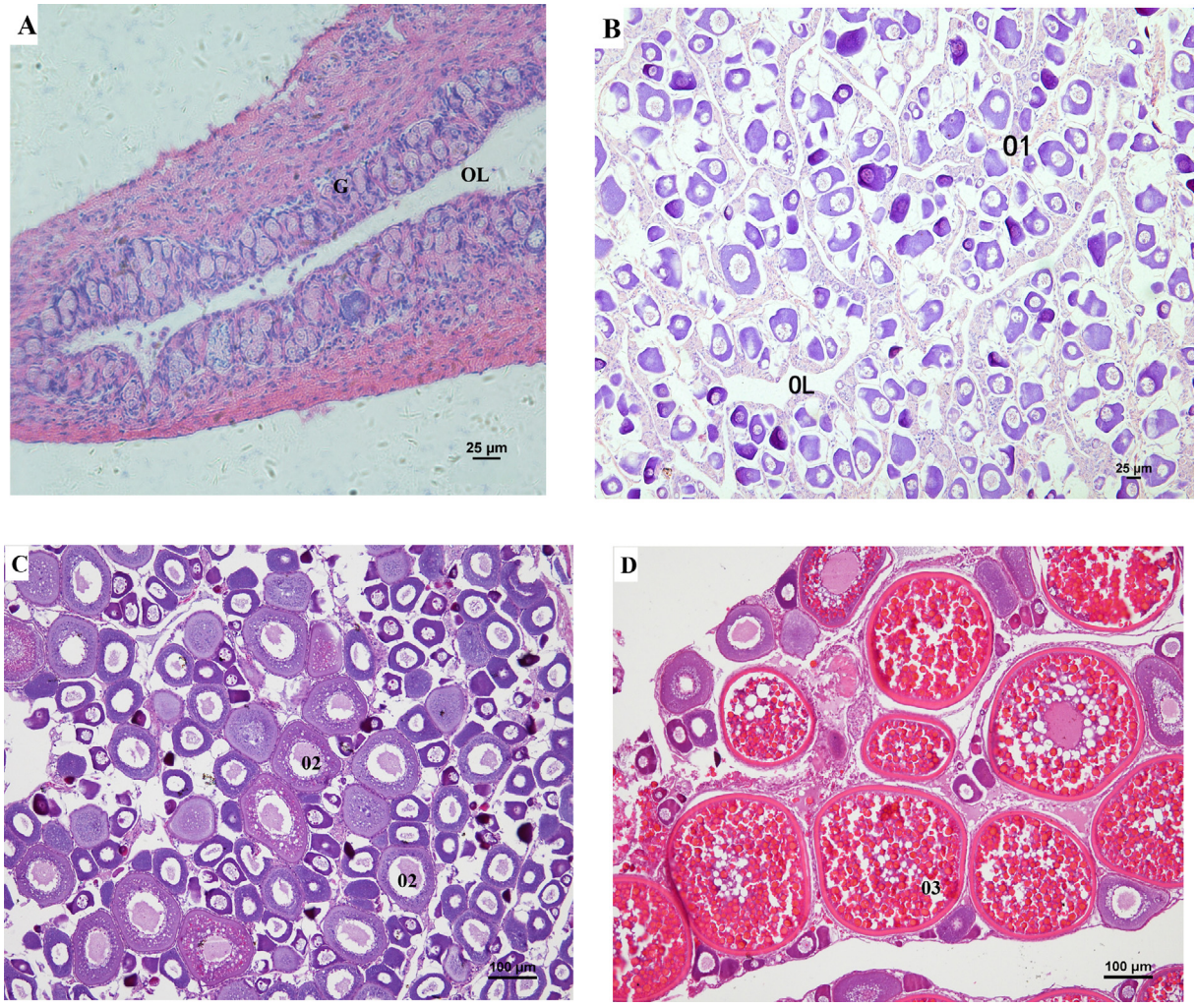

Fig. 3. Histology sections from *E. coioides* during gonadal development. OL, ovarian lumen; G, gonium; O1, primary-growth stage oocyte; O2, cortical-alveolus stage oocyte; O3, vitellogenic stage oocyte; aO3, atretic vitellogenic stage oocyte; SC, spermatogenic cyst; SG, spermatogonia; ST, spermatid.

dmrt2b and *dmrt3* were detected in the ovotestis (Fig. 4C and D). *foxl2* expression was significantly upregulated during ovarian maturation ($p < 0.05$), suggesting that *foxl2* may play an important role at the ovaries (Fig. 4E). *foxl3* expression in the ovary was very low. However, with gonadal development, *foxl3* expression levels increased in the bisexual gonads and peaked in the testes ($p < 0.05$; Fig. 4F).

3.5. Gonadal histology during MT feeding and withdrawal

Gonadal histology was assessed in the MT-fed and control groups. During months 1–3, gonads from the control group contained oocytes that had a cortical-alveolus stage appearance (O2; Fig. 5A1–A3) and stage O1 oocytes (Fig. 5A4–A5), while spermatogonia were dominant in the MT-fed group (Fig. 5B1–B3). In the fourth month, gonads in the continuously MT-fed group developed into a male phenotype. During the fourth and fifth months, there were large numbers of sperm cells in the gonads of both MT-fed groups (Fig. 5B4–B5). However, spermatogonia and spermatids gradually disappeared in the gonads of the MT withdrawal group (Fig. 5C1–C2).

3.6. Gene expression profiles during MT feeding and withdrawal

dmrt1 expression increased significantly when compared to the control group ($p < 0.05$) (Fig. 6A). During the fourth and fifth month, *dmrt1* expression levels in the continuously fed MT group were higher than the MT withdrawal group and control group (Fig. 6B). *dmrt2* expression in the control group and the MT withdrawal group were significantly higher than the continuously fed MT group. Nevertheless, there were no significant differences in *dmrt3* expression in these three groups (Fig. 6C). *foxl2* expression was significantly decreased ($p < 0.05$) by the MT induced diet, however upon halting MT feeding, *foxl2* expression levels were restored to levels seen in the control group (Fig. 6D). These results suggested that gonads reverted to the female developmental track, after MT withdrawal.

3.7. foxl2 and foxl3 expression during MT-induced sex reversal in the grouper gonadal differentiation period

To investigate *foxl2* and *foxl3* expression profiles during gonadal differentiation and the potential roles of these genes in *E. coioides*, fish were fed MT in the 90-day gonadal differentiation period after hatching. *foxl2* expression was significantly lower in the 60- and 90-dat groups when compared to the control group ($p < 0.05$; Fig. 7A), while *foxl3* expression was significantly higher in the experimental groups than in the control group ($p < 0.05$; Fig. 7B). From these data, we speculate that groupers develop into males directly after MT induction in the 90-day gonadal differentiation period after hatching,

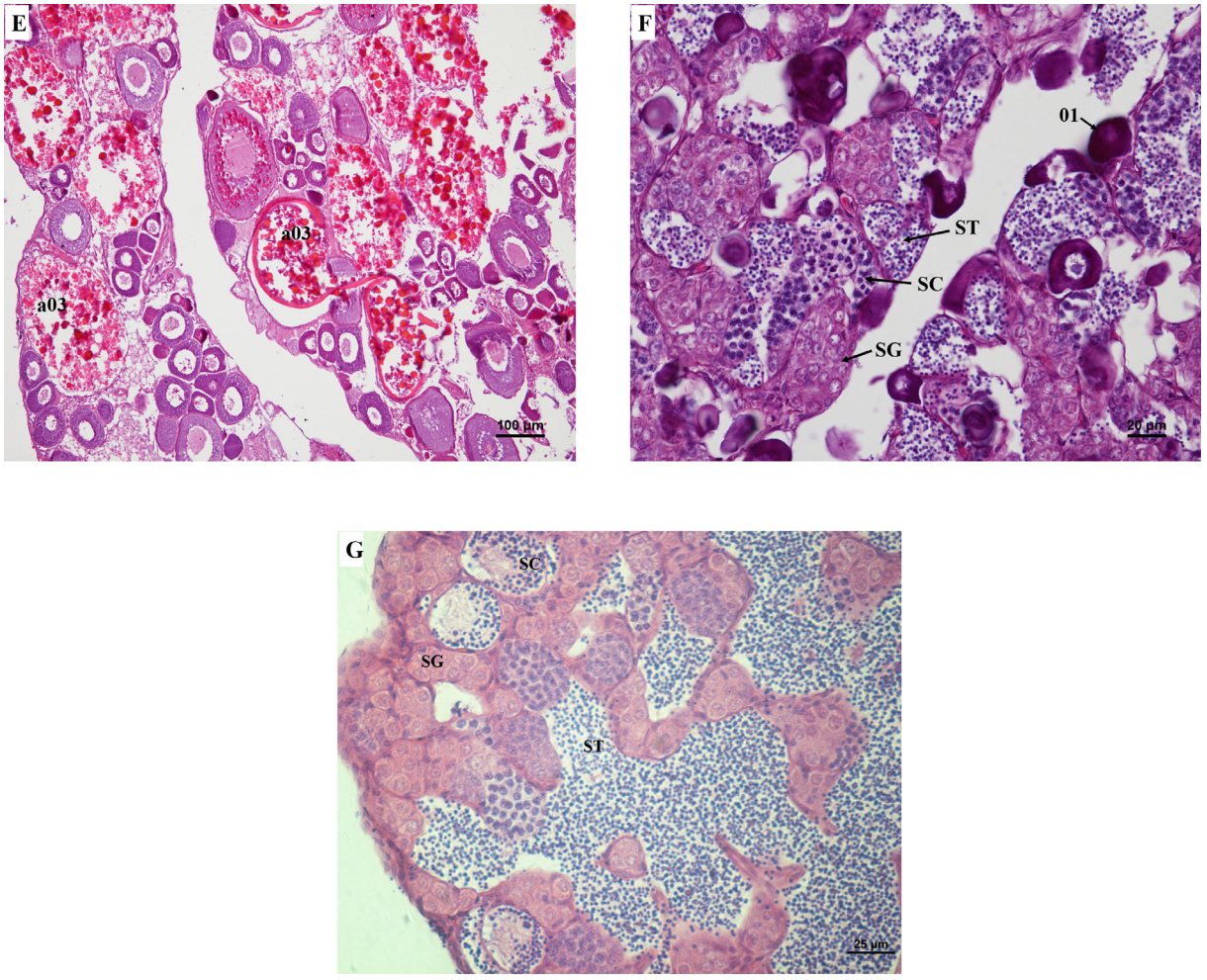

Fig. 3. (*continued*)

4. Discussion

The *dmrts*, *foxl2*, and *foxl3* genes were identified by BLAST analysis of the *E. coioides* transcriptome. The majority of mammalian genomes contain multiple *DMRT* genes with different functions and expression patterns (Mason and Rabinowitz, 2008; Miller et al., 2003; Raymond et al., 1999; Tresser et al., 2010; Yu et al., 2011). In both invertebrates and vertebrates, *dmrt1* is a relatively conserved gene related to sex differentiation (Fujitani et al., 2016). Previous studies have shown that *E. coioides dmrt1* has no introns and no duplicate gene (Xia et al., 2007). The *dmrt2* gene has two subtypes: *dmrt2a* and *dmrt2b*. Both forms originated from the second round of whole-genome duplication of the ancestral *dmrt2* gene, and interestingly both have acquired different functions (Johnsen and Andersen, 2012). The *dmrt2a* gene is present in all vertebrates, while *dmrt2b* is unique to fish (Johnsen and Andersen, 2012). Bioinformatics analysis of *dmrt2* has revealed that it is highly conserved among several fish species (Seo et al., 2006). In vertebrates, *dmrt2* plays a role in somitogenesis, and in zebrafish it regulates left–right axial patterning (Liu et al., 2009). No sexual dimorphism in *dmrt2* expression levels has been observed (Wexler et al., 2014). The amino acid sequence of *E. coioides* Dmrt2b showed lower conservation when compared to *S. salar*, *C. carassius*, and *D. rerio*. Multiple sequence alignment revealed that the grouper Dmrt3 DNA binding domain (DM domain) is highly conserved among fish (*M. albus*, *O. niloticus*, *D. rerio*, and *T. rubripes*).

The prevalence of *foxl2* and *foxl3* genes agrees with evidence of a duplication event in fish (Christoffels et al., 2004). Nevertheless, it was believed that *foxl3* was lost in eutherian species as a result of evolution (Geraldo et al., 2013). The duplicate forms of *dmrt2a* and *foxl2* may be due to the evolution of ray-finned (actinopterygian) fish, whereby a whole-genome duplication event occurred. Evidence for this is a fish-specific genome duplication before the origin of teleosts, which resulted in an extra set of paralogs for all fish genes (Christoffels et al., 2004).

Sexually dimorphic phenotypes are present in numerous mammals. A number of hermaphrodite fishes have sex reversal in their life history. However, the molecular mechanisms underlying sex reversal induced by exogenous steroid treatment is still unclear, even though it has been widely studied in hermaphroditic fish (Marino et al., 2001; Wu et al., 2015). Aromatase inhibitor treatment could cause retraction of the ovaries after the appearance of testes-like organs, and cyst structures have been observed in these tissues, demonstrating that mature ovaries still retain sex plasticity (Takatsu et al., 2013).

During sex reversal in *E. coioides*, *dmrt1* and *dmrt2* expression changed notably in the gonads, suggesting that *dmrt1* and *dmrt2* may be involved in gonadal development and differentiation in this species. It has been shown that the *dmrt1* gene plays a key role in sex determination and development in metazoans (Ma et al., 2016). Gene expression increased significantly in the bisexual period, indicating that *dmrt1* may have significant roles in the protogynous hermaphroditic gonad in stimulating spermatogenesis (Wexler et al., 2014). *E. coioides dmrt1* was highly expressed in the testes. The same expression patterns have been reported for other species such as *E. merra* (Alam et al., 2008), *D. rerio* (Guo et al., 2005), and *Sebastes schlegeli* (Ma et al., 2014). In addition, *dmrt1* expression was significantly higher than in the control group ($p < 0.05$). After MT withdrawal, *dmrt1* expression levels decreased sharply. Fujitani et al. (2016) found that *dmrt1* increased in male

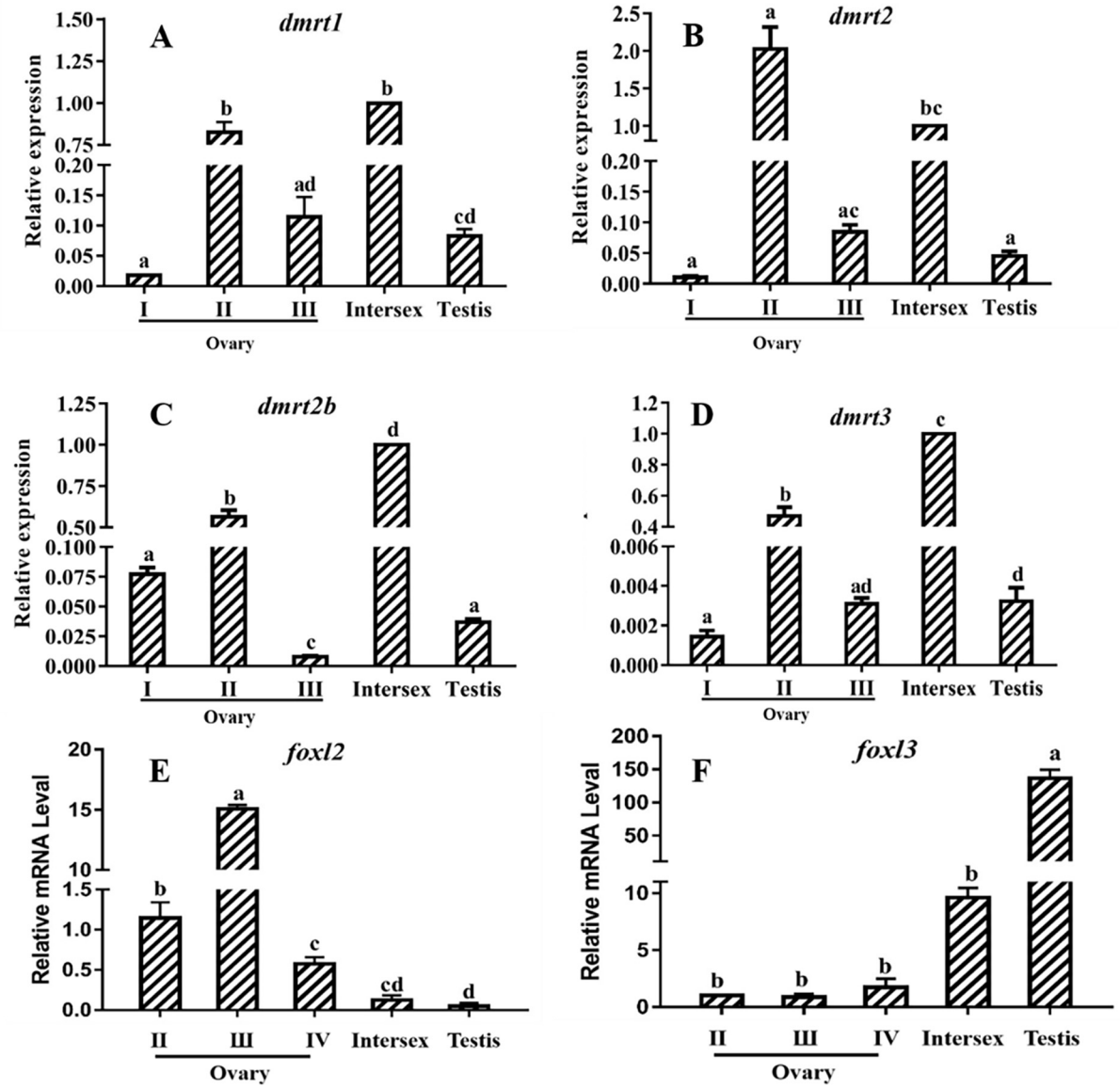

Fig. 4. Relative expression of *dmrts* and *foxls* in *E. coioides* gonads. Each bar represents the mean ± S.E. β-*actin* was used as a reference gene. Differences were determined using ANOVA. Different letters over the bars indicate significant differences ($p < 0.05$). I, stage I ovaries; II, stage II ovaries; III, stage III ovaries; IV, stage IV ovaries; Intersex, Intersex gonad.

gonads of *Xenopus laevis* after metamorphosis, but decreased in female gonads. In conclusion, we conjecture that *dmrt1* is affected by sex steroids.

The patterns of *dmrt2* expression in different species are dissimilar. Highest *dmrt2* expression in *E. coioides* was observed in the ovaries, indicating a potentially important role in ovary development. In the third month, O1-stage oocytes began to proliferate, with significant simultaneous increases in *dmrt2* levels, suggesting that elevated *dmrt2* expression may be related to the proliferation of O1-stage oocytes. *dmrt2* was also expressed in muscle, gill, and testis, and may be involved in the formation and development of muscle and gills. Notwithstanding these data, it is believed that *dmrt2* may be functionally involved in male gonadal development; for example, in *Chlamys nobilis* males injected with different MT concentrations, *dmrt2* was upregulated in the spawning stage (Shi et al., 2014). *dmrt2* is also mainly expressed in male gonads in *Pinctada martensii* (Yu et al., 2011) and the adult medaka *Oryzias latipes* (Mariko Kondoa et al., 2002). According to our data, *E. coioides dmrt2b* was faintly expressed in muscle and gonads. *dmrt2b* contributes to divergent functions in somitogenesis through the Hedgehog pathway and maintains common functions in left–right asymmetry establishment (Liu et al., 2009). Our data show that *E. coioides dmrt3* was mainly expressed in gonads and at lower levels in the brain, similar to *dmrt3* expression patterns observed in adult zebrafish (Li et al., 2008). Expression of *E. coioides dmrt3* in gonads was not affected by MT, suggesting that *dmrt3* may have coordinated with other factors during the development of grouper gonads. Abundant *dmrt3* expression was detected in adult *T. rubripes* testes, but at lower levels in the ovaries (Yamaguchi et al., 2006). *dmrt3* expression was detected in developing sperm cells in the testes and in developing oocytes in the ovaries in *M. albus* (Sheng et al., 2014). *dmrt3* was highly expressed in the head of Atlantic cod larvae and was also expressed in the testes (Johnsen and Andersen, 2012). This evidence and our study results demonstrate that *dmrt3* may be associated with gonadal development and may be involved in brain and embryo development in fish.

The *foxl2* gene was significantly upregulated during ovarian maturation ($p < 0.05$), suggesting that *foxl2* may play an important role in the ovary. Notably, *foxl3* expression levels were very low in the ovary. With gonadal development, *foxl3* expression levels increased in

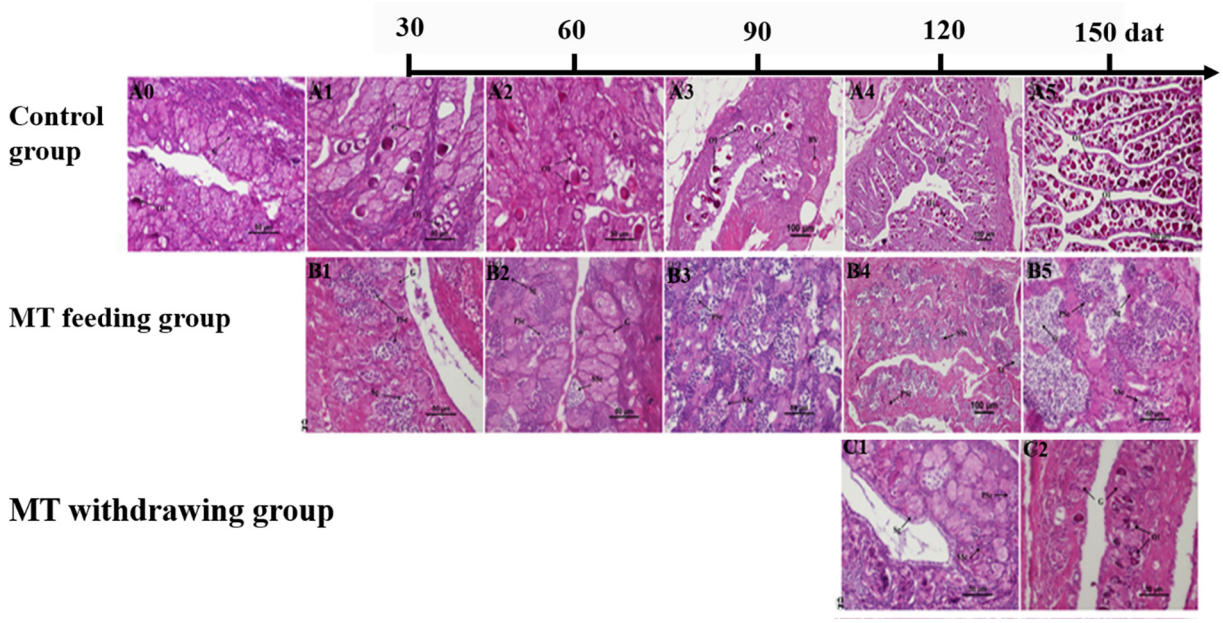

Fig. 5. Sections of *E. coioides* gonad development by MT induced. G, Gonocyte; BV: Blood vessel; PSc: Primary sperm cell; SSc: Secondary spermatocyte; Sg: Spermatogonia; St: Spermatid; O1: Primary oocyte.

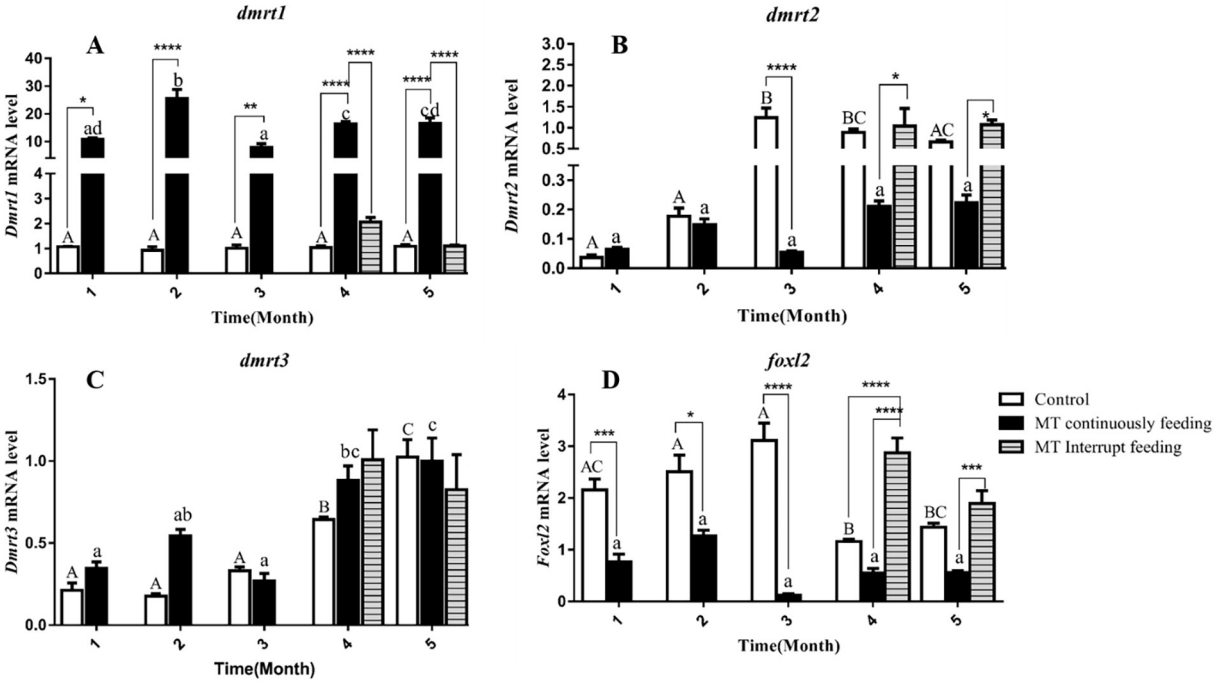

Fig. 6. Relative expression of *dmrts* and *foxl2* in *E. coioides* treated with MT. Note: Significant differences among MT-fed groups (ANOVA, $p < 0.05$) are indicated by different lowercase letters (a, b, c and d); significant differences among control groups (ANOVA, $p < 0.05$) are indicated by different uppercase letters (A, B and C); significant differences between MT-fed and control groups (ANOVA, $p < 0.05$) are indicated by *, with the number of asterisks denoting the significance level. All values are presented as the mean ± S.E. (n = 3).

the bisexual gonad and peaked in the testes ($p < 0.05$). When MT was used to induce sex reversal in the gonadal differentiation period of 90 days after hatching, groupers developed directly into males. *foxl2* expression in the experimental group was significantly lower than in the control group ($p < 0.05$), while *foxl3* expression in the experimental group was significantly higher than in the control group ($p < 0.05$). In the rainbow trout (*O. mykiss*), *foxl2* expression correlated with aromatase levels, a key enzyme in estrogen production (Baron et al., 2004). We deduce that *foxl2* plays an important role in the development of the ovary. The role of *foxl2* in reproduction may be conserved by regulating early differentiation of the ovary through the hypothalamus–pituitary–gonad axis. *foxl2* is necessary for preventing transdifferentiation of mature ovaries to testes via repression of the Sertoli cell–promoting gene *sox9* (Uhlenhaut et al., 2009). A study of *foxl3* in female (XX) and male (XY) Japanese medaka gonads, suggested that *foxl3* was dispensable for testes development. However, *foxl3* suppressed spermatogenesis in females given that ovaries produced functional sperm in XX Foxl3-knockout gonads. The expression patterns

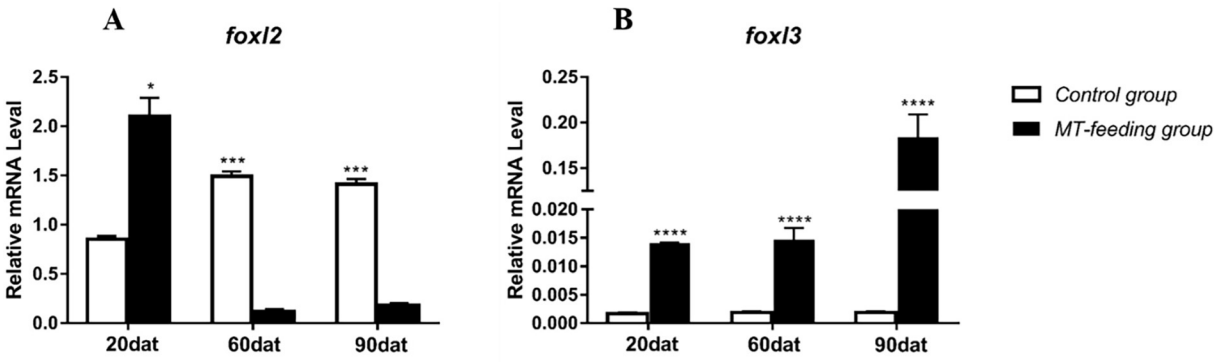

Fig. 7. Expression patterns of *foxl2* and *foxl3* in gonads during MT-induced sex reversal in *E. coioides*. *foxl2* expression was significantly lower in the 60- and 90-dat groups when compared to the control group, while *foxl3* expression was significantly higher in the experimental groups when compared to the control group. Significant differences between MT-fed and control groups (ANOVA, $p < 0.05$) are indicated by *, with number asterisks denoting significance levels. All values are presented as the mean ± S.E. (n = 3).

of *foxl3,* during the natural sex change process in groupers, were different from *foxl2 I* patterns, which may have been due to a species gap during evolution (Nishimura et al., 2015). While *foxl3* may participate in testis development, its role in reproduction may be regulated by the testes, suggesting that *foxl3* may be a potential male-specific regulatory gene. All the data indicate that *foxl2* and *foxl3* may have complementary roles in gonadal differentiation (Bertho et al., 2016).

MT influenced the expression levels of *dmrt1*, *dmrt2*, *foxl2*, and *foxl3* in gonads, indicating a direct link between these genes and estrogens/androgens (Kim et al., 2009). In the current study, groupers fed with MT showed clear masculinization characteristics. After MT was removed from the diet, oogonium was detected in the gonads, along with feminization characteristics. Similar results have been reported for *E. marginatus* (Glamuzina et al., 1998), *E. malabaricus* (Ryosuke et al., 2014), and *E. coioides* (Wu et al., 2015). The expression of male-related genes increased and that of female-related genes decreased after MT treatment. This demonstrates that sexual fate determination (secondary sex determination) was regulated by endogenous sex steroid levels (Wang et al., 2017). Under MT induction in the 90-day gonadal differentiation period after hatching, groupers could develop directly into males. This could involve MT inhibition of aromatase expression, thereby inhibiting estrogen synthesis. Considering the expression profiles of *foxl2* and *foxl3* during gonadal development and the MT-induced sex-reversal period, we speculate that estrogen is the main regulator of ovarian differentiation in teleosts and that androgens could interfere with ovarian development by inhibiting estrogen synthesis. Notwithstanding these observations, the function of *dmrts* and *foxls* in grouper gonadal development and sex reversal requires further and more in-depth study.

5. Conclusions

We speculate that *dmrt1* is affected by sex steroids, *dmrt2* may be involved in the formation and development of muscle and gills, and *dmrt3* may be associated with gonadal development and involved in brain and embryo development in fish. We deduced that *foxl2* plays an important role in the development of the ovary. The role of *foxl2* in reproduction may be conserved by regulating early differentiation of the ovary through the hypothalamus–pituitary–gonad axis. While *foxl3* may participate in testes development, its role in reproduction may be regulated by the testes, suggesting that *foxl3* may be a potential male-specific regulatory gene.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.12.014.

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