



Gonadal expression profiles of sex-specific genes during early sexual differentiation in Japanese eel *Anguilla japonica*

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Abstract

The Japanese eel *Anguilla japonica* is a prominent and highly valued species in the aquaculture industry in Japan. Sex determination and sex differentiation in eels are significantly affected by environmental factors; however, the molecular mechanisms involved in sex differentiation in eels are largely unknown. In this study, to investigate the gonadal expression profiles of sex-specific genes during and after sexual differentiation in Japanese eel, we induced elvers into predominantly phenotypic males or females by rearing on a control diet or estradiol-17 β -treated diet, respectively, during the sex differentiation period. Quantitative real-time PCR showed that forkhead box L2A (*foxl2a*) and cytochrome P450 aromatase (*cyp19a1*) were more highly expressed in ovaries than in testes, whereas the expression levels of anti-Müllerian hormone (*amh*) and gonadal soma-derived factor (*gsdf*) were significantly higher in testes than in ovaries. Furthermore, *foxl2a* and *cyp19a1* displayed female-specific expression early in the sex differentiation process, while after slightly more growth *amh* and *gsdf* displayed male-specific expression during sex differentiation. Together, these results suggest that these genes have important roles in sexual differentiation and development in this species.

Keywords Sex differentiation · Estrogen · Foxl2a · Cyp19a1 · Japanese eel

Introduction

In many vertebrates, sex is determined by the genotype, and sex differentiation is governed by biochemical pathways. Many poikilothermic vertebrates, however, exhibit plasticity in sex differentiation, and the phenotypic sex can be affected by environmental conditions during sensitive periods of gonadal development (Nakamura 2010). For instance, in many reptiles, fishes, and amphibians, sex determination is affected by a combination of genetic and environmental cues (e.g. temperature, pH, relative density, social interactions)

early during development (Francis and Barlow 1993; Francis 1984; Rubin 1985; Baroiller et al. 1999).

Water temperature, as a key environmental factor during gonadal development, may override genetic sex determination mechanisms, altering the resultant phenotypic sex of many teleosts. Previously, it has been reported that genetically female progenies of Japanese flounder *Paralichthys olivaceus* (Kitano et al. 1999), medaka *Oryzias latipes* (Sato et al. 2005), and zebrafish *Danio rerio* (Ribas et al. 2017) could be sex-reversed into functional males when reared in high water temperatures during the critical periods of gonad differentiation. However, many aspects of the molecular mechanisms of environmental sex determination in teleosts remain unclear.

Sex determination in eels is highly influenced by the environment, although it is not yet known to what extent genetic factors contribute to the final sexual phenotype. Research on sex determination and differentiation in eels has been conducted since the 1980s. Environmental factors shown to affect the sex ratios of anguillid species include temperature and pH, density, and social interactions (Davey and Jellyman 2005). Several studies have examined gonadal

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morphological changes during sex differentiation in the European eel *Anguilla anguilla* (Colombo and Grandi 1996; Geffroy et al. 2013, 2016). It is generally accepted that eel gonadal development is related more to body length than to age (Colombo et al. 1984; Colombo and Grandi 1996; Oliveira and McCleave 2000; Jellyman 2001). Though previous studies of gonadal development in the European eel provide information on morphological sex differentiation in eels, the molecular mechanisms involved in sex differentiation are largely unknown.

In teleosts, sex determination and differentiation are mainly governed by the expression of sex-specific genes. The genes chosen for consideration in this study are considered to be related to the process of sex differentiation in fish. Forkhead box L2 (Foxl2) is a member of the winged-helix/forkhead family of transcription factors and is involved in ovarian development in teleosts, including Japanese medaka (Nakamoto et al. 2006) and black porgy *Acanthopagrus schlegelii* (Wu et al. 2008). Anti-Müllerian hormone (Amh), also known as Müllerian-inhibiting substance, functions in regulating germ cell proliferation and/or differentiation in male gonad development in different fish species (Yoshinaga et al. 2004; Baron et al. 2005; Haugen et al. 2012), including the Japanese eel *Anguilla japonica* (Miura et al. 2002). Gonadal soma-derived factor (Gsd) is a member of the transforming growth factor- β (TGF- β) family and was shown to play a vital role in the development of both the testis and the ovary in the medaka (Shibata et al. 2010; Zhang et al. 2016). In addition, the *gsdf* mRNA expression pattern is related to testes development in gonochoristic fish and to sex change in protogynous fish (Horiguchi et al. 2013). Consequently, *gsdf* is often used in researching sex differentiation in teleosts (Kitano et al. 2012; Kaneko et al. 2015; Robledo et al. 2015).

Aromatase (encoded by *cyp19a1*) converts androgens into estrogens and plays an important role in ovarian development in vertebrates (Nakamura 2010). *cyp19a1* is suspected to play an essential role in ovarian development in teleosts (Kitano et al. 1999). Because of teleost-specific genome duplication, most teleosts have duplicated genes for aromatase, named *cyp19a1a* and *cyp19a1b*, which are expressed predominantly in the gonads and brain, respectively (Tchoudakova and Callard 1998; Kishida and Callard 2001). *cyp19a1* encoding aromatase has been cloned in Japanese eel (Ijiri et al. 2003) and European eel (Tzchori et al. 2004). However, eels have a single form of aromatase, which is expressed both in the gonads and in the brain (Jeng et al. 2012). Differential expression of aromatase according to sex has been studied in the European eel by Tzchori et al. (2004) and Geffroy et al. (2013). Their results showed that *cyp19a1* gene expression in gonads was higher in females than in males. However, Jeng et al. (2018) found no significant difference in the transcript levels of *cyp19a1* in the

gonads of males and females of Japanese eels. Thus, little is known concerning aromatase gene expression during the early stages of gonad differentiation in the Japanese eel.

In this study, to investigate the gonadal expression profiles of sex-specific genes during early sex differentiation in Japanese eel, we first induced elvers into predominantly either phenotypic males or females, by rearing on a control diet or estradiol-17 β (E2)-treated diet, respectively, during the sex differentiation period. Next, we examined the gene expression patterns in the gonads by quantitative real-time polymerase chain reaction PCR (qPCR).

Materials and methods

Animals

Elvers (*A. japonica*) (experiment 1: body length = 5.79 ± 0.21 cm; body weight = 0.14 ± 0.02 g; experiment 2: body length = 5.78 ± 0.23 cm; body weight = 0.13 ± 0.02 g) were purchased from the Isshiki Unagi Cooperative Association of Fisheries and transferred to the aquaculture station of the Aichi Fisheries Research Institute. The experimental fish were acclimated to a freshwater pond environment (an indoor 0.8-ton fiber-reinforced plastic [FRP] tank) with natural lighting and continuous aeration. Fish were kept under warm-temperature conditions (28–30 °C) until the experiments.

Experimental treatments

In experiment 1, control ($n = 100$) and E2-treated ($n = 100$) elvers were reared in separate 0.8-ton FRP tanks. Control fish were fed a commercial eel feed powder mixed with water and ethanol. The fish were feminized by feeding a diet containing E2 at the elver stage (Chiba et al. 1993; Colombo and Grandi 1995); E2 (Sigma-Aldrich Corp., St. Louis, MO, USA) was dissolved in ethanol, then mixed with water and the commercial eel feed powder to obtain the E2-treated diet (20 mg/kg feed). Fish were fed the diet 6 days a week. Fish were collected from both groups after 120 days, when most had reached 30 cm. Gonads from individual eels were collected for gonadal histology and gene expression analysis.

In experiment 2, the controls ($n = 100$) and E2-treated elvers ($n = 100$) were reared according to the same procedure as in experiment 1. The elvers were feminized by feeding an E2-containing diet (10 mg/kg feed) because the mortality of E2-treated fish in experiment 1 was high (data not shown). To investigate expression of the sex-specific genes in the undifferentiated gonads, fish treated with or without E2 for 45 days were used. These eels were collected and divided into three body-length groups (Small, < 15.0 cm; Medium, 15–19.9 cm; Large, 20–24.9 cm) for gonadal histology and

gene expression analysis. The remaining fish from each group were collected after 120 days, when most had attained a length of at least 30 cm.

Quantitative real-time PCR (qPCR)

Total RNA from the gonadal tissues of the eels was extracted by homogenization in ISOGEN (Nippon Gene, Tokyo, Japan) as previously described (Kitano et al. 2012). Next, reverse transcription was performed at 37 °C for 30 min, using PrimeScript RT Master Mix (TaKaRa, Shiga, Japan). qPCR was performed on a LightCycler 480 system (Roche, Mannheim, Germany) using SYBR Green I Master Mix (Roche). The PCR conditions were 95 °C for 5 min, then 45 cycles of 95 °C for 5 min, 59 °C for 10 s, and 72 °C for 10 s. Finally, melting-curve analysis was performed after thermocycling to evaluate the specificity of the amplification and to verify the absence of primer dimers. Relative gene expression levels were calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). The copy number values of each gene were normalized against elongation factor 1 alpha (*ef1 α*). The primers used are listed in Table 1.

Histological analysis

The samples of eel body segments or gonadal tissues were fixed in Bouin's solution overnight at 4 °C, then dehydrated, embedded in paraffin, and serially sectioned at 5 μ m as previously described (Kitano et al. 2012). Sections were stained with hematoxylin and eosin. Finally, gonadal status was determined by examination of the tissue sections under a light microscope, using the procedure described by Jeng et al. (2018) to characterize the gonad histological structure.

Table 1 Sequences of primers used in this study

Primer name	Sequence (5'–3')	Accession no.
ef1 α -S	TGTGGGAGTCAACAAGATGGA	MH020210
ef1 α -AS	CTCAAAACGCTTCTGGCTGTA	
vasa-S	CGTGATTCAGGTGACCCAGTT	KX987991
vasa-AS	GCCCGTGGTGTTCAGGAA	
amh-S	TCTCCTAGCAACGGATTGGC	AB074569
amh-AS	CACAGGAAGTGGAAAGTCCGG	
gsdf-S	ACAGTACAGGCTTGCAAGTCTG	LC466011
gsdf-AS	AGTTCTCCCAACCAAGATCGTT	
cyp19a1-S	CAGAGAAGTTGGATGATGCTG ACT	AY616117
cyp19a1-AS	GCTCCCCGTGGTTCTGAGC	
foxl2a-S	CCACCCACTCCTATGCCCTAT	MF966994
foxl2a-AS	GCCGACAGTCCTTTGACGTT	

Statistical analysis

Experimental results were tested using Levene's test for homogeneity of variance. Data were compared using Student's *t* test or analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, using SPSS Statistics software (IBM Corp., Armonk, NY, USA).

Results

Tissue observation and gene expression patterns for gonads after sex differentiation

Eels raised to at least 120 days were sacrificed and gonadal sex was determined. Among the control fish, 91% ($n=69$) were males and 9% ($n=7$) were undifferentiated; conversely, among the E2-treated fish, 97% ($n=64$) were females and 3% ($n=2$) were undifferentiated (Fig. 1a). Spermatogonia were observed in males of the control group (Fig. 1b, c), and oocytes were observed in females of the E2-treated group (Fig. 1d, e). The expression levels of *vasa*, *foxl2a*, *cyp19a1*, *amh*, *gsdf*, and *ef1 α* were analyzed by qPCR. The germ cell marker *vasa* was expressed in both testes and ovaries (Fig. 1f). Expression of the female-specific genes *foxl2a* and *cyp19a1* was significantly higher in ovaries than in testes (Fig. 1g, h). Conversely, expression of the male-specific genes *amh* and *gsdf* was significantly higher in testes than in ovaries (Fig. 1i, j).

Tissue observation and gene expression patterns for undifferentiated gonads

Eels raised to 45 days were collected for gonadal histology; all were found to have undifferentiated gonads. Of the eels reared to at least 120 days, 88% ($n=30$) were males and 12% ($n=4$) had undifferentiated gonads in the control group, whereas 94% ($n=48$) were females and 6% ($n=3$) had undifferentiated gonads in the E2-treated group (Fig. 2a). For analysis of their gene expression profiles, the eels raised to 45 days were divided into three body-length groups (Small, Medium, and Large). Germ cells were observed in the gonads of individuals in all length groups of the controls and the E2-treated fish (Fig. 2b–g). Analysis of gene expression by qPCR revealed that the germ cell marker *vasa* was expressed in the gonads of both the controls and the E2-treated fish; however, in the Large groups, expression of *vasa* was significantly higher in the E2-treated fish than in the controls (Fig. 2h). In all length groups, expression of the female-specific gene *foxl2a* was significantly higher in gonads of the E2-treated eels than in those of control fish (Fig. 2i). In both the Medium and Large groups,

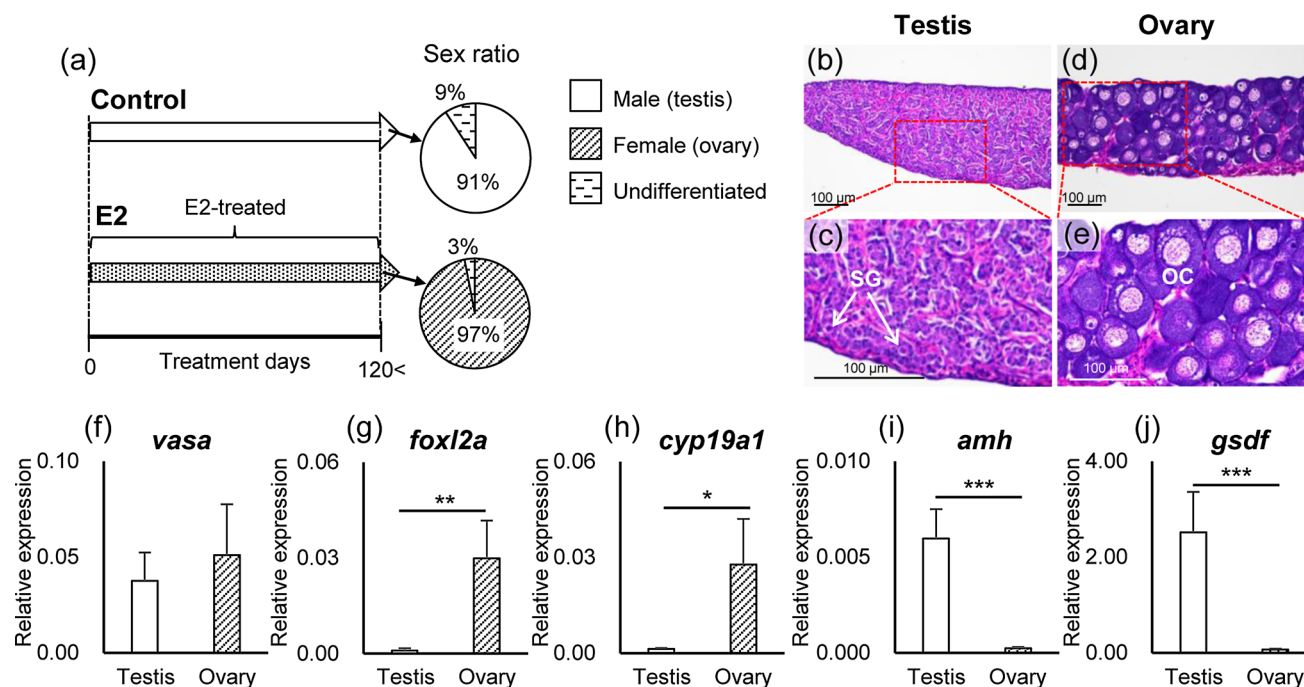


Fig. 1 Results of experiment 1. Time course of the feminization experiment through an estradiol-17 β -treated diet, and a pie chart showing the sex ratio of Japanese eels raised to at least 120 days (a). Histological sections of gonads after hematoxylin and eosin staining: testis (b, c) and ovary (d, e). Quantitative real-time PCR analy-

sis of the expression of each gene in the gonads of fish ($n=5$) (f–j). Relative expression levels of the target genes were normalized to that of *ef1a*: *vasa* (f), *foxl2a* (g), *cyp19a1* (h), *amh* (i), and *gsdf* (j). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; SG spermatogonia, OC oocyte

expression of *cyp19a1* was significantly higher in the E2-treated fish than in the control fish (Fig. 2j). In contrast, expression levels of the male-specific genes *amh* and *gsdf* were significantly higher in the gonads of the controls than in the E2-treated fish in the Large group only (Fig. 2k, l).

Discussion

In this study, we raised elvers of Japanese eel for at least 120 days to ensure a feminizing effect through E2-treated diets; gonadal histological analysis showed that almost all E2-treated fish were females, whereas no females were found among the controls. Using a similar rearing method to achieve feminization, Jeng et al. (2018) likewise found only males in the control group of Japanese eels after 6 months, and Colombo and Grandi (1995) produced a large proportion of males among laboratory-raised European eel. In the current study, based on a histological characterization of gonads following Jeng et al. (2018), the gonads of both the control and E2-treated fish raised to only 45 days (experiment 2) were all undifferentiated.

The expression of *vasa* increased with gonadal development in the controls and E2-treated fish. Robledo et al. (2015) reported that in turbot *Scophthalmus maximus*, *vasa* expression starts to increase at an early stage of gonadal

development, but with higher expression in females than in males. Our results suggest that *vasa* is a germ cell-specific marker in eels, as in some other teleosts. *Foxl2a* is a paralog of *foxl2* and has an important function in ovarian differentiation in rainbow trout *Oncorhynchus mykiss* (Baron et al. 2004). In this study, the sexually dimorphic expression of *foxl2a* in ovaries was similar to that found in other teleosts. We found a significant difference in *cyp19a1* gene expression between Japanese eel testes and ovaries, a result that agrees with a study conducted with European eel (Geffroy et al. 2013). However, Jeng et al. (2018) reported no difference between males and females in *cyp19a1* expression in the gonads of Japanese eel. When we used the primer described by Jeng et al. (2018), we likewise found no differences between *cyp19a1* expression in testes and ovaries (data not shown); therefore, we designed a new primer and used it for qPCR. Expression of *foxl2a* and *cyp19a1* in E2-treated fish increased during gonadal development, suggesting the involvement of these genes in ovarian development. In a previous study, transient transfection assay showed that the Japanese flounder *Foxl2* can directly activate the *cyp19a1* gene transcription in vitro (Yamaguchi et al. 2007), and therefore *Foxl2* may also induce *cyp19a1* expression in eels. In contrast, the male-specific genes *amh* and *gsdf* were significantly more highly expressed in testes than in ovaries. We also found higher *amh* and *gsdf*

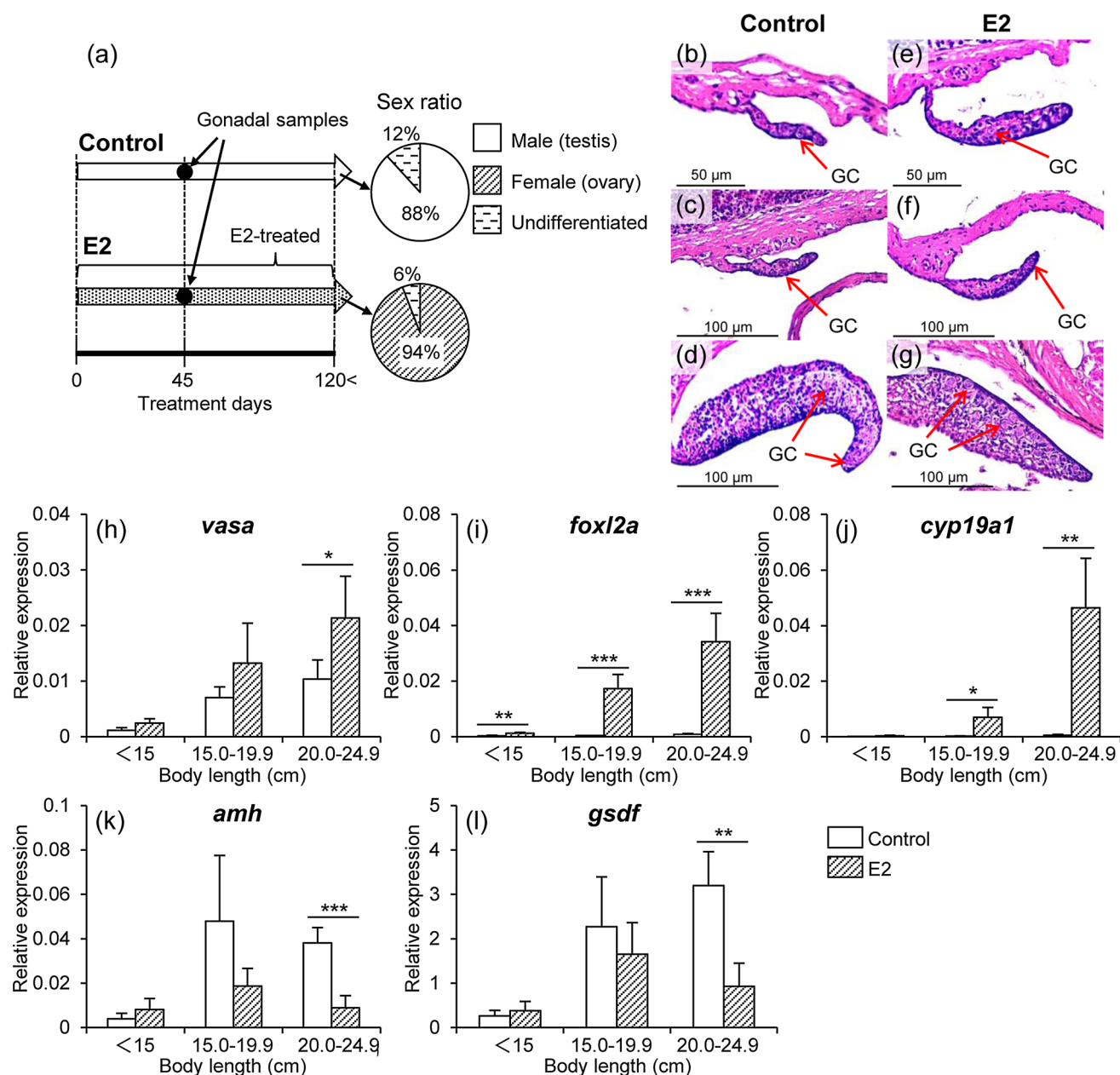


Fig. 2 Results of experiment 2. Time course of the feminization experiment through an estradiol-17β-treated diet, and a pie chart showing the sex ratio of Japanese eels raised to 45 days (a). Histological sections of undifferentiated gonads after hematoxylin and eosin staining in eels of body-length groups Small (b, e), Medium

(c, f), and Large (d, g). Quantitative real-time PCR analysis of the expression of each gene in the gonads of fish (n=5) (h–l). Relative expression levels of the target genes were normalized to that of *ef1a*: *vasa* (h), *foxl2a* (i), *cyp19a1* (j), *amh* (k), and *gsdf* (l). **p* < 0.05; ***p* < 0.01; ****p* < 0.001; GC germ cell

expression in the undifferentiated gonads of males compared with females, which is consistent with the well-described dimorphic expression in European eel (Geffroy et al. 2016). Thus, our data align with the results of previous studies, and together, the results suggest that *amh* and *gsdf* are required for testis development in eels.

Cortisol is the major glucocorticoid produced by the adrenal cortex in vertebrates, and its production can be induced

by stress. It was previously reported that cortisol suppresses the expression of *foxl2a* and *cyp19a1*, but induces the expression of *amh*, which causes masculinization of XX fish (Yamaguchi et al. 2010). Furthermore, Kitano et al. (2012) reported that exposure to cortisol inhibited the expression of *cyp19a1* and induced the expression of *gsdf* in XX gonads of medaka during gonadal sex differentiation. We hypothesize that masculinization of cultured eels may be prompted by

suppression of female-specific genes (*foxl2a* and *cyp19a1*) and induction of male-specific genes (*amh* and *gsdf*). We intend to conduct future research that focuses on the involvement of cortisol in masculinization in cultured eels.

In conclusion, in Japanese eel, *foxl2a* and *cyp19a1* display female-specific expression during early sex differentiation, while after slightly more growth, *amh* and *gsdf* significantly display male-specific expression during the early stages of sex differentiation. Together, these results suggest that these genes play important roles in sexual differentiation and development in eels.

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