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Reproductive Dysfunction in Cultured Sablefish (*Anoplopoma fimbria*)

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Abstract: Sablefish *Anoplopoma fimbria* is a ground fish native to the North Pacific Ocean that is considered a promising new species for marine aquaculture in the US. However, efforts to establish sustainable production of sablefish have been constrained by the reproductive performance of females from the first-filial (F1) generation. Although some F1 females may mature after 5+years of age, some others fail to initiate puberty in captivity. Development of methods to unblock/induce early puberty are necessary to reduce costs associated with rearing F1 female broodstock and reduce generation times for selective breeding.

Current research at the Northwest Fisheries Science Center (NOAA, Seattle, USA) integrates basic and applied biology to gain knowledge on the reproductive endocrine system of sablefish and develop approaches to unblock or reduce the age of puberty in F1 female sablefish. As part of our basic line of research, we compared the pituitary gonadotropin-ovary axis in wild-caught, maturing females and 8 year-old F1 females that had never shown signs of sexual maturation. Wild-maturing females had higher levels of pituitary gonadotropin subunit and ovarian gonadotropin receptor mRNAs and plasma sex steroids compared to F1 females, which were holding at the immature, perinucleolus ovarian stage. Anecdotal evidence from sablefish farms indicates that F1 female broodstock maintained in ~4 ℃ seawater mature in captivity. We hypothesize that culture conditions that use warmer water (10–15 ℃) suppress the pituitary gonadotropin-ovary axis in sablefish, and ultimately block the onset of puberty.

As part of our applied line of research, we conducted a series of studies to determine the ability of exogenous hormones to stimulate the reproductive axis in prepubertal F1 females. Treatments with testosterone or estradiol 17-beta (E2) increased the expression of pituitary luteinizing hormone beta subunit 40- and 185-fold, respectively, relative to control. This finding suggests that pituitaries from immature females are responsive to exogenous hormones, and that sex steroids may be an important part of a hormone therapy to stimulate the reproductive axis of F1 females. In addition, using an *in vitro* ovarian tissue culture system, we demonstrated that fragments of prepubertal sablefish ovaries incubated with human-chorionic gonadotropin increased the secretion of E2. This indicates that ovaries of prepubertal females are equipped to synthesize and release sex steroids critical for vitellogenesis under the appropriate hormone stimulation, and that the failure to initiate puberty is likely due to a lack of adequate gonadotropin signaling. These data provide the foundation for the development of hormone treatments aimed at inducing puberty in prepubertal F1 female sablefish.

Key words: sablefish, marine aquaculture, reproduction, gonadotropins

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In fishes, as in other vertebrates, reproduction is primarily controlled by the hypothalamic-pituitary-gonadal axis. The hypothalamic neuroendocrine system regulates synthesis and release of the pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). Both gonadotropins are essential in the endocrine control of reproduction by regulating steroidogenesis and germ cell development through interactions with their respective receptors in gonadal tissues (Levavi-Sivan *et al*., 2010; Zohar *et al*., 2010).

Optimal function of the reproductive endocrine system is critical for fish to complete gonadal development, maturation and spawn successfully. Under culture conditions, however, many fish species exhibit some degree of reproductive dysfunction. These can vary from inconsistent spawning to a complete failure to undergo puberty (i.e., the time during which an individual becomes capable of reproducing for the first time) (Mylonas *et al*., 2010; Taranger *et al*., 2010). Such a delay in age of puberty poses a major problem for selective breeding in finfish aquaculture and necessitates further research.

Hormone therapies are widely used to control reproduction in cultured fish species. During the past two decades, hormone therapies have been developed to induce puberty in striped bass (*Morone saxatilis*) (Holland *et al*., 2002), European sea bass (*Dicentrarchus labrax*) (Zanuy *et al*., 1999), European eel (*Anguilla anguilla*) (Vidal *et al*., 2004), and grey mullet (*Mugil cephalus*) (Aizen *et al*., 2005). Therapies for inducing puberty or to accelerate this process were based on an understanding of how the endogenous reproductive system is hormonally regulated and where the insufficiency/failure occurs in the reproductive system (e.g., brain, pituitary gland and/or gonad) that delays or blocks puberty onset. Therefore, characterization of the reproductive dysfunction in a given species helps to tailor specific strategies to optimize its reproduction in captivity.

Sablefish (*Anoplopoma fimbria*), known as *gindara* in Japan, is a groundfish native to the North Pacific Ocean ranging from Baja California to Alaska's Bering Sea and Japan. The primary market for sablefish is in Japan, where demand and prices are high, but an increasing amount of the wild catch is staying in the US market. Due to its rapid growth rate (1.5 kg after 12-months of growout) and high market value (22 USD/lb. approx. market price), sablefish has been identified as an excellent marine aquaculture species in the US. However, efforts to establish sustainable and efficient production have been constrained by the reproductive performance of females from the first filial (F1) generation (i.e., produced and maintained in captivity). Although some F1 females may mature at 5+ years old in the industry setting, others never initiate puberty in captivity (B. Campbell, Sablefish Canada Inc., personal communication). This situation compromises the development of selective breeding programs and increases costs associated with rearing female sablefish broodstock.

Our approach to this issue integrated a series of basic and applied studies aimed at gaining a basic understanding of the reproductive physiology of sablefish and developing a method that would eliminate the block or reduce the age of puberty in F1 female sablefish.

Materials and methods

Study I: Assessment of the pituitary gonadotropin-ovary axis in wild maturing and F1 non-maturing female sablefish: To understand the endocrine differences that underlie the reproductive impairment of female sablefish propagated in captivity, we first compared the pituitary gonadotropin-ovary axis of two stocks of sablefish with different reproductive status: wild-caught maturing females and 8 year-old F1 females that had never shown signs of sexual maturation.

Wild female sablefish were caught using sportfishing gear near the mouth of the Quinault River (Washington, USA) in October 2010. The fish were transported to the Manchester Research Station (Port Orchard, Washington, USA) and maintained in tanks supplied with flow-through, sand-filtered and UV-treated seawater. During February 2011, the peak spawning period along the Washington coast (Mason *et al*., 1983), the wild females showed signs of sexual maturation by ultrasound. F1 sablefish (broodyear 2003) were reared at the Manchester Research Station in net-pens until fall 2010 when

they were transported to the Northwest Fisheries Science Center (NWFSC, Seattle, Washington, USA) where they were reared on recirculated seawater. These females were maintained under similar rearing conditions as the wild-caught fish at the Manchester Research Station. In contrast to the wild females, the F1 females did not exhibit signs of ovarian maturation when assessed by ultrasound.

Wild and F1 females were sampled on 28 February 2011 and 8 March 2011, respectively. Three females from each broodstock group were deeply anesthetized with MS-222 and body weight (BW) and fork length (FL) recorded (wild, 6413.0 ± 712.9 g BW and 81.3 ± 3.1 cm FL; F1, 4160.0 ± 303.3 g BW and 69.8 ± 2.7 cm FL). Plasma was obtained by centrifugation of whole blood and stored at −20 ℃ for sex steroid analyses. The pituitary gland and a small piece of ovary (~80 mg) were also collected from each fish, frozen in liquid nitrogen and stored at −80 ℃ until RNA extraction. Genes of interest included the pituitary gonadotropin subunits (*fshb, lhb, cga*) and ovarian gonadotropin receptors (*fshr* and *lhcgr*). For histology, a middle portion of the ovary was preserved in Bouin's fixative for 48h prior to storage in 70% ethanol and processing as described elsewhere (Campbell *et al*., 2006).

Study II: Effect of sex steroids on pituitary gonadotropin gene expression *in vivo*: To determine potential effects of sex steroids on pituitary gonadotropins and whether sex steroids will be an important part of hormone therapies to reduce the age of puberty in sablefish, we evaluated the effect of testosterone (T) and estradiol-17beta (E2) on pituitary gonadotropin beta subunit (*fshb* and *lhb*) mRNA levels in prepubertal F1 females *in vivo*.

Twelve 2-year old prepubertal F1 female sablefish (1187.4 ± 49.4 g BW and 469.9 ± 5.3 cm FL) maintained at the NWFSC on 12 ℃ recirculated seawater were distributed into three groups (n=4 sablefish/group) and implanted with cholesterol-based pellets (0.2 x 0.5 mm) containing no hormone (control) or containing T at doses of 0.75 or 3.75 mg. Twenty-eight days after implantation, fish were euthanized and their pituitaries removed and snap frozen for later RNA isolation. In a parallel study, 6 females from the same cohort of fish received 6 intramuscular injections of 2 mg E2/kg dissolved in ethanol: 0.9% NaCl (1:7) or vehicle alone (control), every other day. Two days after the last injection, fish were euthanized and pituitaries removed to determine gonadotropin beta subunit gene expression. Doses of T and E2 were selected from previous studies in other fish species (Guzmán *et al*., 2008; Holland *et al*., 2002; Vidal *et al*., 2004)

Study III: Effect of gonadotropins on ovarian secretion of estradiol *in vitro*: To determine whether immature ovaries of F1 female sablefish are responsive to gonadotropin stimulation, we evaluated the effect of two gonadotropin preparations, recombinant coho salmon Fsh analog (sFsha) and human chorionic gonadotropin (hCG), on E2 production *in vitro*.

For this, a 3-year old prepubertal F1 female sablefish (2910 g BW, 67.6 cm FL) maintained at the NWFSC was euthanized and the ovaries removed. Ovarian fragments (~60 mg each) were cultured in Cortland's solution alone (control) or containing sFsha (provided by Dr. W.R. Moyle, Robert Wood Johnson Medical School, New Jersey, USA) at doses of 50 and 500 ng/ml or hCG (Sigma) at doses of 1, 10 and 100 IU/ml. After 24 h incubation at 11 ℃, the culture medium was collected and stored at -20 ℃ for E2 analysis. Doses of sFsha and hCG were selected based on previous studies in other species (Luckenbach *et al*., 2011; Sorbera *et al*., 2001).

RNA isolation, cDNA synthesis and quantitative PCR: Total RNA from sablefish pituitaries and ovarian tissue was isolated with Tri-Reagent (Molecular Research Center) using a TissueLyser II (Qiagen). An aliquot of total RNA was diluted to ~250 ng RNA/μl in nuclease-free water and DNase treated using the DNA Free kit's "rigorous" protocol (Ambion). RNA yields and quality were assessed by NanoDrop (ND-1000 Spectrophotometer) and gel electrophoresis. For reverse transcription, 1 μg of total RNA of each sample was reverse transcribed in a 20-μl reaction with the Superscript II kit (Invitrogen). Quantitative PCRs were conducted as previously described (Luckenbach *et al*., 2011). Reactions consisted of 1x Power SYBR Green PCR master mix (Applied Biosystems), 150 nM of gene-specific primers (see Guzmán *et al*., 2013 for primer sequences) and 0.5 ng of pituitary or ovarian cDNA template. Assays were run on an ABI 7700 Sequence

Detector using standard cycling conditions.

Sex steroids: Plasma levels of E2 were measured by radioimmunoassay and levels of T were quantified by enzyme-linked immunosorbent assay using protocols previously validated for sablefish (Guzmán *et al.*, 2013).

Statistical analyses: Statistical analyses were performed using Prism 5 software for Mac OSX (GraphPad Software) with the minimum level of significance set to $P<0.05$. Differences were examined using t-test or one-way ANOVA followed by a Tukey multiple comparisons test. When necessary, data were log or Ln transformed in order to comply with normality and homogeneity of variance, which were tested by Kolmogorov–Smirnov and Bartlett methods, respectively. Data are expressed as mean ± standard error of the mean (S.E.M.).

Results

Study I: Assessment of the pituitary gonadotropin-ovary axis in wild maturing and F1 non-maturing female sablefish

Endocrine parameters indicated a more active reproductive axis in wild versus F1 female sablefish (Fig. 1). At the pituitary level, *lhb* and *cga* mRNA

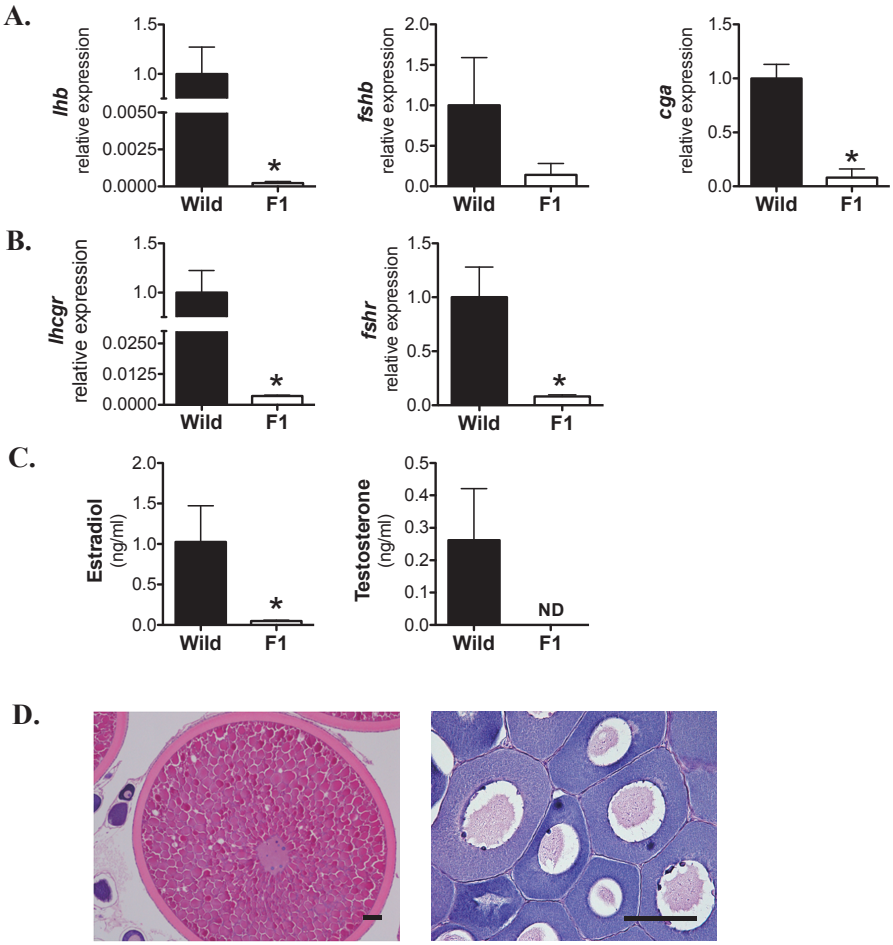

Fig. 1. Relative gene expression of pituitary gonadotropin subunits (A) and ovarian gonadotropin receptors (B), plasma sex steroid levels (C), and representative ovarian histological sections (D) of wild maturing and F1 female sablefish. Gene expression levels were determined by qPCR and normalized to *eef1a*. Levels of E2 were determined by RIA, whereas levels of T were determined by ELISA. Data are expressed as the mean ± SEM ($n=3$). Asterisks indicate significant differences (t-test, $p<0.05$). Maturing follicles with the nucleus migrating to the periphery predominated in the ovaries of wild maturing females, whereas only follicles at the perinucleolus stage were found in ovaries of F1 females (D). Scale bars: 100 μm. ND, non detectable.

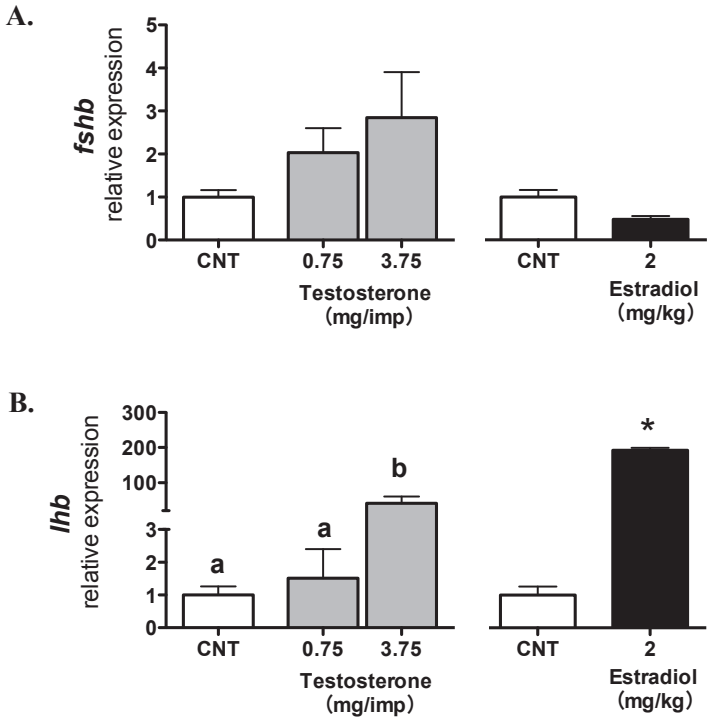

Fig. 2. Relative gene expression of pituitary follicle-stimulating hormone beta subunit (A) and luteinizing hormone beta subunit (B) in prepubertal F1 female sablefish treated with T (left graphs) or E2 (right graphs). For T treatments, females received a single implant containing hormone at doses of 0 (control), 0.75 and 3.75 mg for 28 days. For E2 treatments, females received 6 intramuscular injections of 2 mg hormone/kg dissolved in ethanol: 0.9% NaCl (1:7) or vehicle alone (control, CNT), every other day. Gene expression levels were determined by qPCR and normalized to *eef1a*. For T treatments, bars with different superscript letters are significantly different (ANOVA, $p<0.05$). For the E2 treatment, an asterisk indicates a significant difference relative to control (t-test, $p<0.05$).

levels were significantly higher in wild fish than in F1 fish. Levels of *fshb* were also elevated in wild fish relative to F1 fish (7.1-fold), although differences were not statistically significant due to the high variance across individuals. In the ovary, transcripts for both gonadotropin receptors, *fshr* and *lhcgr*, were significantly higher in wild than in F1 females. Plasma levels of E2 were also significantly higher in wild than in F1 females, whereas only wild females had detectable levels of T in plasma.

The ovary of wild females consisted of post-vitellogenic preovulatory follicles characterized by the nucleus migrating to the periphery and yolk coalescence, as well as another clutch of follicles at the perinucleolus stage. In contrast, ovaries from F1 females were characterized by the sole presence of follicles at the perinucleolus stage.

Study II: Effect of sex steroids on pituitary gonadotropin gene expression *in vivo*

The effect of treatment with T or E2 on *in vivo* pituitary gonadotropin beta subunit (*fshb* and *lhb*) mRNA levels is shown in Fig. 2. Transcripts for *fshb* were not significantly affected by treatment with T or E2. In contrast, transcripts for *lhb* increased significantly in fish treated with the highest dose of T or multiple injections of E2 (40- and 185-fold, respectively, relative to control).

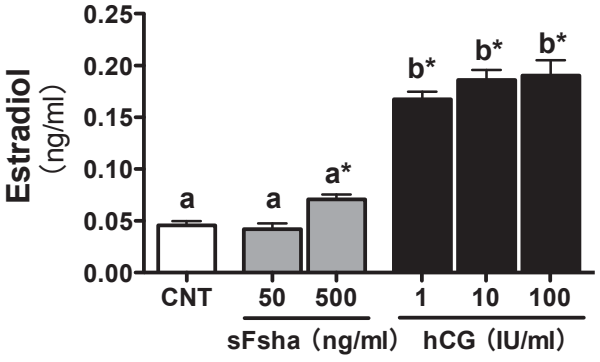

Fig. 3. Effect of sFsha or hCG on E2 production by ovarian fragments obtained from prepubertal F1 female sablefish. Ovarian fragments were cultured in Cortland's solution alone (control, CNT) or containing sFsha at doses of 50 and 500 ng/ml or hCG at doses of 1, 10 and 100 IU/ml, at 11 ℃ for 24h. Levels of E2 were determined by RIA. Bars with different superscript letters are significantly different (ANOVA, $p<0.05$), whereas asterisks indicate significant differences relative to control (t-test, $p<0.05$).

Study III: Effect of gonadotropins on ovarian secretion of estradiol *in vitro*

The effect of sFsha or hCG on ovarian secretion of E2 *in vitro* is shown in Fig. 3. Incubation with sFsha had a subtle effect on the secretion of E2 with only the highest dose of sFsha having a significant effect compared to control. In contrast, treatment with hCG regardless of dose significantly stimulated E2 secretion (>4-fold).

Discussion

Currently, the emerging aquaculture industry for sablefish is mostly dependent on spawning wild-caught individuals. The expansion and optimization of this industry requires a closer understanding of the reproductive physiology of this species and the development of protocols to control the age of puberty in cultured broodstock.

In Study I, we demonstrated that transcript levels of pituitary *lhb* and ovarian *lhcgr* were higher in wild-caught maturing females than in non-maturing F1 females. This is in accordance with the predominant role of Lh in the regulation of gonadal maturation, ovulation and spawning in fishes (Gothilf *et al*., 1997; Swanson *et al*., 2003; Yaron *et al*., 2003). Interestingly, ovarian *fshr* mRNA levels were also higher in maturing females and a similar tendency was observed for the pituitary *fshb* mRNA levels. Little is known about specific roles of Fsh during gametogenesis in fishes, although it is accepted that Fsh is important for early gonadal development and vitellogenesis (Lubzens *et al*., 2010; Luckenbach *et al*., 2011). The elevated levels of pituitary *fshb* and ovarian *fshr* that we observed in wild females may indicate that a population of perinucleolar oocytes within the ovary were preparing for the transition to vitellogenic growth, or that Fsh also plays a role in final oocyte maturation. Since the ovary of the wild mature females had a mixed population of oocytes it is not possible to distinguish these two possibilities. Plasma levels of E2 and T were also higher in wild females than F1 females as expected from the observed differences in pituitary gonadotropin subunit and ovarian receptor mRNA levels. This demonstrates classical gonadotropin-mediated sex steroid secretion (Lubzens *et al*., 2010) by the ovaries of wild females and indicates a lack of gonadotropin signaling in F1 females.

Although F1 females from Study I were 8 years old, they remained arrested in a prepubertal stage. Interestingly, it has been recently demonstrated that some F1 females can initiate vitellogenesis (i.e., onset of puberty) if they are maintained in cold water (~4 ℃) after 5 years of age (B. Campbell, personal communication). It is generally accepted that every fish species has an optimal range of water temperatures for vitellogenin synthesis, and both higher and lower temperatures affect the normal progression of vitellogenesis (Guzmán *et al*., 2008; Kim and Takemura, 2003). Considering that sablefish is a deep-water species and the onset of vitellogenesis may naturally occur at very low temperatures, it is possible that the temperature at which fish were maintained (fluctuated between 8 and 15 ℃ throughout the year) had a deleterious effect on reproductive axis function, and ultimately on the onset of vitellogenesis in F1 females.

Although some F1 females maintained in cold water can achieve puberty after 5+ years, the development of methods to reduce and synchronize the age of puberty is essential for reliable and cost effective sablefish breeding programs. Hormone

treatments are widely used in aquaculture to improve the reproductive performance of captive fishes, including activation of the reproductive endocrine system and promotion of gonadal development in immature stages (Holland *et al*., 2002; Zanuy *et al*., 1999). We conducted Studies II and III to determine the ability of exogenous hormones to stimulate the reproductive axis in prepubertal F1 sablefish.

In Study II we demonstrated that treatment with T or E2 significantly elevated transcripts for pituitary *lhb*, but did not affect levels of *fshb* mRNA. Effects of sex steroids on levels of the pituitary gonadotropin subunit mRNAs have been demonstrated in a number of fishes, although results vary among species. For example, treatment with T elevated pituitary transcripts of *lhb* in European sea bass during the period of "sexual resting," while T and E2 reduced pituitary *fshb* mRNA levels (Mateos *et al*., 2002). In immature European eel (*Anguilla anguilla*), treatments with E2 but not T elevated transcripts for *lhb*, whereas no effect was observed on *fshb* mRNA levels (Aroua *et al*., 2007). Lastly, treatments with T and E2 increased pituitary transcript levels for *fshb* and *lhb* in zebrafish (*Danio rerio*) (Lin and Ge, 2009). Our results suggest that pituitaries from prepubertal female sablefish are responsive to exogenous hormones, and that sex steroids may be an important part of a hormone therapy to stimulate the reproductive axis of F1 females. In line with this, treatments with sex steroids were necessary to induce puberty in European sea bass (Zanuy *et al*., 1999), striped bass (Holland *et al*., 2002) and European eel (Vidal *et al*., 2004).

In Study III, we demonstrated that treatment with hCG, an Lh-like gonadotropin, stimulates ovarian secretion of E2 *in vitro*. Treatments with gonadotropin preparations, which act directly on the gonad, have proven effective in the stimulation of gonadal maturation, ovulation and spawning in a number of fish species (Zohar and Mylonas, 2001). Most importantly, in species from the genus *Anguilla*, captive fish often remain arrested in a prepubertal stage in captivity. However, multiple injections with pituitary extracts can trigger ovarian vitellogenesis, maturation and ovulation in females (Ohta and Tanaka, 1997), while multiple injections of hCG can induce testicular development and spermiation in males (Dou *et al*., 2007). Our data suggest that, as in anguillid eels, immature ovaries of F1 female sablefish are equipped with the ability to synthesize and release sex steroids (critical for vitellogenesis) under appropriate hormone stimulation, and that treatment with gonadotropin preparations may effectively stimulate ovarian function in this species.

In conclusion, wild-maturing females had elevated gonadotropin signaling (pituitary and ovarian gene expression and plasma steroid levels) compared to 8 year-old F1 females, which were holding at the immature, perinucleolus ovarian stage. Anecdotal evidence indicates that F1 females maintained in ~4 ℃ seawater may mature in captivity after 5 years of age. It might be possible that culture conditions that use warmer water (10–15 ℃) suppress the pituitary gonadotropin-ovary axis in sablefish, and ultimately block the onset of puberty. Our results indicate that hormone treatments, such as sex steroids and gonadotropin preparations, can stimulate the reproductive endocrine axis in prepubertal females and therefore should be considered as candidates to reduce the age of puberty in this species.

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