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Inhibition of ovarian development and instances of sex reversal in genotypic female sablefish (*Anoplopoma fimbria*) exposed to elevated water temperature

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ABSTRACT

This study determined high temperature effects on ovarian development in a marine groundfish species, sablefish (*Anoplopoma fimbria*), with potential application in sex reversal or sterilization for aquaculture. Monosex female (XX-genotype) sablefish larvae (~30 mm) were randomly divided into three groups and exposed to control (15.6 °C ± 0.8 °C), moderate (20.4 °C ± 0.5 °C), or high (21.7 °C ± 0.5 °C) temperatures for 19 weeks. Treated fish were then tagged and transferred to ambient seawater (11.2 °C ± 2.3 °C) for one year to determine whether temperature effects on reproductive development were maintained post-treatment. Fish were periodically sampled for gonadal histology, gene expression and plasma 17β-estradiol (E2) analyses to assess gonadal development. Short-term (4-week) exposure to elevated temperatures had only minor effects, whereas longer exposure (12–19 weeks) markedly inhibited ovarian development. Fish from the moderate and high treatment groups had significantly less developed ovaries relative to controls, and mRNA levels for germ cell (*vasa*, *zpc*) and apoptosis-associated genes (*p53*, *casp8*) generally indicated gonadal degeneration. The high treatment group also had significantly reduced plasma E2 levels and elevated gonadal *amh* gene expression. After one year at ambient temperatures, however, ovaries of moderate and high treatment fish exhibited compensatory recovery and were indistinguishable from controls. Two genotypic females possessing immature testes (neomales) were observed in the high treatment group, indicating sex reversal had occurred (6% rate). These results demonstrate that extreme elevated temperatures may inhibit ovarian development or trigger sex reversal. High temperature treatment is likely not an effective sterilization method but may be preferable for sablefish neomale broodstock production.

1. Introduction

Temperature is an environmental factor that can influence, redirect, or block early reproductive development in some organisms. For example, sex in many reptiles is directly determined by temperature (Temperature-dependent Sex Determination, TSD; Pieau et al., 1999). In contrast, mammals and birds display a pattern of strict genetic sex determination (GSD), where the sex of an individual is fixed by the genes inherited at fertilization. Still, even among species with GSD, sexual plasticity is often observed under application of high temperature (GSD with thermal effects), particularly in teleost fishes, in which the process of gonadal sex differentiation may be easily overridden by exogenous factors (Baroiller and D'Cotta, 2016; Devlin and Nagahama, 2002; Luckenbach and Yamamoto, 2018; Ospina-Álvarez and Piferrer, 2008).

At the molecular level, effects of temperature can be observed in a suite of genes commonly associated with sexual differentiation. For instance, aromatase (Cyp19a1a) is principally responsible for 17β-estradiol (E2) biosynthesis by the gonads and considered a key driver of ovarian differentiation (Luckenbach et al., 2009; Piferrer and Guiguen,

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2008; Shen and Wang, 2014). Elevated temperatures have been shown to suppress *cyp19a1a* expression in gonadal somatic cells and in turn, E2 production, causing masculinization of genotypic female fish (Karube et al., 2007; Kitano et al., 1999; van Nes and Andersen, 2006).

Elevated temperatures have also been shown to upregulate programmed cell death (apoptosis)-associated genes such as caspases (i.e., cysteine proteases) and to promote germ cell degeneration and follicular atresia, which may have masculinizing or sterilizing effects (Byerly et al., 2005; Lee et al., 2009; Linares-Casenave et al., 2002; Pandit et al., 2015; Strüssmann et al., 1998; Takle and Andersen, 2007; Uchida et al., 2004). Although the exact mechanisms linking temperature to gonadal apoptosis are not clearly understood, suppression of *cyp19a1a* gene expression and E2 production and activation of gonadal apoptotic pathways are signatures of this process.

Temperature-induced sterility or sex reversal may be particularly beneficial in aquaculture species. The utilization of reproductively sterile fish populations addresses many concerns of escapement and genetic introgression associated with finfish aquaculture (Crozier, 1993; Skaala et al., 2006). Sex reversal is also widely used for commercial aquaculture purposes, typically to capitalize on the superior performance (e.g., growth rate or morphology) of one sex relative to the other (Baroiller and D'Cotta, 2016; Devlin and Nagahama, 2002). Sex reversal of gonochoristic species which display sexually dimorphic growth may allow for the production of monosex populations through specific broodstock crosses (e.g., sex reversed XX-genotype fish [neomales] crossed with normal XX-genotype fish), ultimately maximizing efficiency and profit for commercial aquaculture. The synthetic androgen 17α-methyltestosterone (MT) is widely used to induce sex reversal in fishes for the generation of neomale broodstock (Devlin and Nagahama, 2002; Pandian and Sheela, 1995). However, exposure to elevated temperatures, which has been shown to induce sex reversal in some fishes, may be a more eco-friendly and chemical-free method to generate monosex or sterile fish (D'Cotta et al., 2001; Ospina-Álvarez and Piferrer, 2008; Shen and Wang, 2014). Despite the abundance of temperature-related research, studies documenting temperature effects on reproductive development of marine teleosts have not been widely conducted.

Sablefish (*Anoplopoma fimbria*) is an economically important groundfish in commercial fisheries of the Pacific Northwest, USA (National Marine Fisheries Service, 2016) and an emerging aquaculture species in the United States and Canada. Sablefish naturally range from northern Mexico to the Bering Sea and Japan and can live > 100 years (Beamish and McFarlane, 2000). This species exhibits sexually dimorphic growth with females growing significantly faster and larger than males (Echave et al., 2012; Luckenbach et al., 2017). In terms of early reproductive development, sablefish is a gonochoristic species that utilizes an XX/XY GSD system with gonadal sex differentiation typically occurring when juveniles are ~75–150 mm fork length (FL) (Luckenbach and Fairgrieve, 2016; Luckenbach et al., 2017). To obtain monosex female sablefish stocks, juvenile XX-genotype fish were treated with MT during the sensitive period of sex differentiation to induce female-to-male sex reversal (Luckenbach et al., 2017). The resulting neomales ultimately produced monosex, all-female progeny when crossed with normal female (XX) broodstock. Being able to produce neomale broodstock via high temperature instead of MT treatment would represent a significant improvement upon current methodology. Moreover, methodology for monosex female production paired with reproductive sterilization (i.e., sterile all-female lines) would be particularly beneficial from a social and economic perspective.

The objective of this study was to determine effects of elevated water temperatures on early gonadal development of genotypic female sablefish. We sought to gain greater insight into mechanisms that regulate sex differentiation and development including temperature effects on E2 production and a suite of gonadal sex differentiation- and apoptosis-associated genes. We also sought to assess for the first time the utility of high temperature treatment for induction of female-to-male sex reversal and/or reproductive sterilization (i.e., germ cell loss). Successful application would aid commercial sablefish aquaculture and could potentially be applied to other marine finfish species.

2. Materials and methods

2.1. Experimental animals and general rearing conditions

Monosex (all-XX genotype) female sablefish were obtained from colleagues at the Northwest Fisheries Science Center's (NWFSC) Manchester Research Station (Port Orchard, WA, USA) and originated from a mix of ten unique crosses between seven wild-caught females and four neomale broodstock. Wild female broodstock were captured by hook-and-line off the coast of Washington while neomale broodstock were produced prior to the present study according to Luckenbach et al. (2017) and maintained at the NWFSC. Wild females and neomales were strip spawned and *in vitro* fertilization was conducted over a 6-day period. Eggs and larvae were maintained using methods detailed by Cook et al. (2015). Briefly, fertilized eggs and developing larvae were held in incubators and silos, respectively, in a recirculating seawater (5 °C) system until yolk sac adsorption was complete and they were ready for exogenous feeding. First-feeding larvae were then transferred to a 1.02 m diameter fiberglass tank (0.618 m^3) continuously supplied with heated (14.6 °C ± 0.1 °C) hatchery water in a flow-through system until they were 0.2–0.5 g and fully weaned onto dry hatchery feed (BioVita, BioOregon Inc., Longview, WA).

2.2. Experimental conditions

Fish near 30 mm FL (30.5 ± 3.7 mm FL; 0.3 ± 0.08 g body weight [BW]; mean ± SD) and ~90 days post-fertilization were targeted for experimentation since their gonads are sexually undifferentiated at this stage (Luckenbach and Fairgrieve, 2016). At time zero, 975 fish were randomly pooled and divided equally (n = 325 fish per tank) into three identical 1.02 m diameter (0.618 m^3) fiberglass tanks. The tanks were located indoors with 24-h lighting via overhead LED tubes (5000°K; Espen Technology Inc., Santa Fe Springs, CA, USA). The temperature in each tank was adjusted to one of three targeted treatment temperatures: control at 15 °C (15.6 °C ± 0.8 °C), moderate at 20.5 °C (20.4 °C ± 0.5 °C), and high at 22 °C (21.7 °C ± 0.5 °C). Hatchery water was heated using digitally-controlled immersion heaters (Finnex, Countryside, IL, USA). To acclimate the fish, the water temperature was increased by approximately 2 °C each day until target temperatures were reached. Targeted temperatures were based on previous studies with larval and juvenile sablefish, which identified 15 °C as an optimal temperature for survival and growth and 24 °C as lethal (Cook et al., 2018; Lee et al., 2017; Sogard and Olla, 2001). Furthermore, sablefish larvae reared at temperatures up to 18 °C did not show signs of sex reversal or sterilization (Lee et al., 2017).

Fish were exposed to treatment temperatures for approximately 19 weeks (136 days, or nearly 5 months). During the treatment phase they were fed a commercial salmon diet (BioVita, Bio-Oregon Inc., Longview, WA, USA) to apparent satiation by hand 3–4 times daily, except on sampling days, when feed was withheld. Water in the tanks was continuously filtered (Cascade 1000, Penn-Plax Inc., Hauppauge, NY, USA) to remove suspended solids. Uneaten feed, feces and other settleable solids were removed once daily by siphon. At 6-h intervals, 30–50% of the water was drained from each treatment tank and replaced with preheated, filtered, and UV-treated water from dedicated reservoirs by means of timer-controlled (Coleman Cable Inc., Waukegan, IL, USA) submersible pumps (TAAM Inc., Amazon, Seattle, WA, USA) equipped with float switches (FloTec, Delavan, WI, USA). Water temperature was checked twice daily with a hand-held digital thermometer (Bel-Art – SP Scienceware, Wayne, NJ, USA) and continually recorded at 15 min intervals using Hobo Pendant temperature loggers (Onset Computer Corporation, Bourne, MA, USA). During the

course of the 19-week treatment period, 38.5% of the number stocked (325 fish per treatment) were lethally sampled. Mortality totaled 30.2%, 26.5% and 43.7% of the number stocked from the control, moderate and high temperature groups, respectively.

At the end of the treatment period, approximately 40 fish were randomly selected from each treatment group, implanted with passive integrated transponder (PIT) tags (Biomark, Inc, Boise, ID, USA), and pooled into a common 2.46 m diameter (3.37 m^3) fiberglass tank with flow-through (60 L/min) ambient seawater (11.2 °C ± 2.3 °C) located outdoors and covered with shade cloth. The fish were held for one year under these conditions and provided a commercial salmon grower diet (EWOS Dynamic, EWOS Canada Ltd., Surrey, BC, Canada) 7 days per week to slight excess using a clockwork (belt) feeder (Pentair Aquatic Eco-Systems, Inc., Apopka, FL, USA) that operated 16–20 h per day.

2.3. Sample collection

All fish were handled by NOAA NWFSC staff during experimentation in accordance with the National Research Council guidelines for aquatic animals (National Research Council, 2011) and American Veterinary Medical Association (AVMA, 2007). When sampling, fish were first euthanized using a lethal dose of Tricaine-S (200 ml/L; Western Chemical, Ferndale, WA, USA), then decapitated.

Fish were randomly sampled from each treatment group at week 4. However, because of the known influence of body size on reproductive development and gonadal stage in this species (Luckenbach and Fairgrieve, 2016), attempts were made to size-match fish for sample collection and analysis at subsequent time points. At each time point (week 4, 12, and 19), 10 fish from each group were sacrificed for paraffin histology and 10–15 fish for quantitative reverse transcription-PCR (qRT-PCR). Overall, these sampling time points targeted body sizes between 60 and 180 mm FL, capturing critical developmental periods of molecular and morphological sex differentiation that may reflect sex reversal or sterility (Hayman et al., in preparation; Luckenbach and Fairgrieve, 2016; Smith et al., 2013). One year post-treatment, 35 fish from each treatment group were sampled for gonadal histology and RT-PCR in order to assess whether treatment effects on reproductive development were permanent. Morphological deformities were recorded at one year post-treatment according to Lee et al. (2017).

Gonads were isolated from the fish via one of two dissection methods depending on the type of analysis and fish size. During the treatment period, trunk sections of the body (i.e. the gonads and surrounding kidney and muscle tissue) were dissected for histology to maintain the position and integrity of the gonads. For gene expression analysis, the gonads were directly extracted from the coelomic cavity using fine forceps. To locate the gonads for either dissection method, the head was severed at the insertion of the dorsal fin and the caudal peduncle excised just anterior to the anus. An incision was made parallel to the coelomic cavity and viscera removed, exposing the gonads. One year post-treatment, the second dissection method was used exclusively. Gonadal tissues were either fixed in Bouin's solution for paraffin histology or preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) for molecular analysis.

2.4. Gonadal histology

Methods for sablefish gonadal histology and staging followed Luckenbach and Fairgrieve (2016). Briefly, gonads were fixed in Bouin's solution for at least 48 h, dehydrated in a series of ethanol dilutions, cleared with xylene, embedded in paraffin, cross-sectioned at 5 µm thickness, prepared onto slides, and stained with hematoxylin and eosin. Histological sections were first examined for any signs of female-to-male sex reversal or sterility and notes recorded on gonadal stages observed. Fish exhibiting fusion of the distal ends of the ovarian lobes to form ovarian cavities were also noted. Sections were photographed with a Nikon digital camera (Melville, NY, USA) and analyzed as follows using NIS Element image software version 4.0 (Nikon).

To compare ovary size across treatment groups, cross-sectional areas of the ovaries were measured in triplicate photographed sections (anterior to posterior) for each fish and the average calculated for each individual (n = 10 fish/group; 300 total observations). To assess the degree of ovarian differentiation/development, the proportion of fish with fused or unfused distal ends of the ovarian lobes (to form ovarian cavities) was recorded. Cross-sectional areas of perinucleolus stage oocytes (PN, the most advanced stage of oocytes observed) were measured across triplicate sections for each fish (n = 10 fish/group; 300 total observations) and individual means calculated. Only centrally-sectioned oocytes with the nucleus and surrounding nucleoli visible were measured. Finally, PN oocyte density (i.e., percentage of the tissue composed of PN oocytes) was determined for each fish by measuring the relative area of all the PN oocytes in triplicate ovarian sections.

For fish sampled one year post-treatment, due to the much larger size of the ovaries, the PN oocyte density was determined in triplicate photographs of the same magnification that did not include the entire cross-sectional area of the ovaries. Fish that had testes (not ovaries) were not included in this histological assessment and were subjected to further testing to verify genetic sex as described below.

2.5. Gene expression analysis

2.5.1. Targeted genes

Ovarian and testicular-associated gene markers were selected to assess phenotypic sex and gonadal status. Complementary DNA sequences for targeted genes were obtained from a 454-pyrosequencing project focusing on differentiating ovaries and testes of sablefish (Hayman et al., in preparation) and some previously reported assays (Smith et al., 2013). Gonadal somatic cell genes associated with ovarian development included *foxl2a (forkhead box L2a) and cyp19a1a (cytochrome P450 family 19 subfamily A polypeptide 1a)*, somatic cell genes associated with testicular development included *dmrt1 (doublesex and mab-3 related transcription factor 1)* and *amh (anti-Mullerian hormone)*, and germ cell-specific genes included *vasa* (*DEAD (Asp-Glu-Ala-Asp) box polypeptide 4*) and *zpc (zona pellucida protein c)*. Apoptosis-associated genes were also targeted because signs of ovarian degeneration were observed in the moderate and high treatment groups. These included *casp8 (caspase 8, apoptosis-related cysteine peptidase)*, *casp3 (caspase 3, apoptosis-related cysteine protease)*, *casp9 (caspase 9, apoptosis-related cysteine peptidase)*, and *p53*, a tumor suppressor gene (Table 1).

2.5.2. RNA isolation and reverse transcription

Methods for RNA isolation and RT followed Luckenbach et al. (2011). Briefly, gonads were homogenized using a TissueLyser II (Qiagen, Germantown, MD, USA), and total RNA isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. RNA samples were diluted to ~250 ng/µl and then treated with DNase to eliminate genomic DNA (Turbo DNA Free kit; Life Technologies, Carlsbad, CA, USA). RNA samples were assessed for quality and quantity using a ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). DNase-treated RNA was reverse transcribed using SuperScript II (Life Technologies) with random primers (Promega, Madison, WI, USA) and 250 ng of RNA in 10 µl reactions. Approximately 10% of the DNase-treated RNA samples were randomly selected as no amplification controls (NACs), in which water was added in place of RT enzyme, to confirm that genomic DNA had been eliminated.

2.5.3. Quantitative PCR

Quantitative PCR was used to determine steady-state mRNA levels for targeted genes in gonad samples collected at weeks 12 and 19 using methods described in Luckenbach et al. (2011). PCR primers were designed using Primer3 in MacVector software (Accelrys, San Diego, CA, USA) and redesigned if an assay displayed multiple products in melt

Table 1
Sablefish primer sequences for RT-PCR and quantitative PCR.

Transcript	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Product size (bp)
cyp19a1a	CATCTGGACTGGTATAGGCACA	TCCATTCTTCAGTACATGGTGC	136
cyp11b	GGGCAGCTATCTTTAGACCTCA	GATCTCCTTGATTGTGCCTTTC	228
foxl2a	CCTACTCCTACGTCGCTCTCAT	GTCCAGTAGTTCCCCTTCCTCT	220
dmrt1	CTGAGGTGATGGTGAAGAATGA	AATAGGAAGTTTCCAGCAGCAG	183
amh	TACATATTGCTGACGGGACAAG	TATCGTATCCTCTTTCCCCAGA	181
zpc	ATCTGGTACTGTTTGGTGCTGT	GATGTCATGGGTAGCCAGGTAG	154
vasa	TCCATTTTTGCCCACTACGAGA	GTTTCTTTTCAGGGACTCGCAC	138
casp8	GAAACGACTTGCACTCTGACAC	CTGTTCTTCATCCAAGCATGTC	196
casp3	ACAGGCATGAATCAACGAAACG	TCTCCGTGACTCAACAGAACAC	194
casp9	TACCGATGACTTGGTCAGAATG	CTTGGGTTTGAAAGTGGAAAG	131
p53	TGCCATTCGTTTAGATTCACAC	CGATTGACAGCCTAAAGGAGAG	115
btf3	GAGATTAGCAGAGACCCTTCCC	TGTTTGTTAGTTTGCCTCGTCC	142
eef1a	ACCGGTCACCTGATCTACAAGT	TAATACCTGCCGGTCTCAAACT	188
actb	TGCGTGACATCAAGGAGAAG	AGGAAGGAAGGCTGGAAGAG	175

curve analyses. Quantitative PCRs were conducted in 384-well plates using 2X Power SYBR Green Master Mix (Life Technologies; final conc.), 150 nM of each primer, and 0.5 ng of cDNA, resulting in 12.5 µl total volume per well. Standard curves were generated from pooled ovary samples serially diluted to 5, 1, 0.25 and 0.05 ng cDNA and run in triplicate. Assays were run on a 7900HT Fast Real-Time PCR System (Life Technologies) with standard cycling conditions. Dissociation curves were included in each run to confirm that only one product amplified in the reactions. In addition to NACs, no template controls (NTCs), which contained no cDNA template, were included in each assay. Quantitative PCR products from each assay were directly sequenced by MCLAB (South San Francisco, CA, USA) to further confirm that targeted cDNAs were amplified. This included confirmation that the *cyp19a1a* assay amplified *cyp19a1a* and not the paralog, *cyp19a1b*, which is primarily expressed in the sablefish pituitary and brain (Guzmán et al., 2018).

Following methods outlined in Vandesompele et al. (2002), geNorm software was used to measure the stability of five candidate reference genes: *btf3 (basic transcription factor 3)*, *eef1a (elongation factor 1 alpha)*, *rpl4 (ribosomal protein L4)*, *actb (actin beta)*, and *18s.* The three most stable genes with pairwise variation ≤0.15 were *btf3*, *eef1a, and actb*. Therefore, the geometric mean of these genes was used for normalization of qRT-PCR data.

2.5.4. RT-PCR and gel electrophoresis

RT-PCR was used to assess gene expression patterns in gonads of selected fish one year post-treatment using methods described by Smith et al. (2013). Complementary DNA samples were diluted to 0.5 ng/µl prior to PCR using the following conditions: 1 cycle for 3 min at 94 °C, 32 cycles for 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C and 1 cycle for 7 min at 72 °C. Products were electrophoresed on 1.5% agarose gels with 0.1% volume of 1X GelRed DNA stain (Biotium, Hayward, CA, USA) and photographed with camera settings held constant. An NTC was also included in each assay.

The gene transcripts *cyp19a1a* and *cyp11b* (*cytochrome P450 family 11 subfamily B*) were assessed to determine whether sex reversal had occurred one year post-treatment, *vasa* to confirm the presence of germ cells, and *eef1a* as a loading control. The genetic sex of fish found to have undergone phenotypic sex reversal (i.e., XX-genotype, neomales) was verified using a PCR assay that targets a sex-specific insert in the sablefish *gsdf* (*gonadal soma derived factor*) promoter (Luckenbach and Fairgrieve, 2016; Rondeau et al., 2013).

2.6. Plasma 17β-estradiol analysis

Plasma was obtained by centrifugation of whole blood (3000 × *g*, 15 min, 4 °C) and stored at −20 °C for E2 analysis. Plasma E2 levels were quantified by enzyme-linked immunosorbent assay (ELISA) using a protocol previously validated for sablefish (Guzmán et al., 2015). Sensitivity, calculated from maximum binding minus two standard deviations was 12.6 pg/ml; intra- and inter-assay coefficients of variation were 1.2% and 8.6%.

2.7. Statistical analyses

Statistical analyses were conducted using Prism 7 software (Graphpad, La Jolla, CA, USA). Data for oocyte measures, gonadal gene expression, plasma E2, and fish size (FL and BW) among groups within each time point were tested for normality with the D'Agostino-Pearson test and log transformed when necessary. Normally distributed data were then checked for outliers using Grubb's test and one-way ANOVA followed by Tukey multiple comparisons post-hoc tests when ANOVA indicated overall significant differences. In cases where data were not normally distributed after log transformation, Kruskal-Wallis test was conducted, followed by Dunn's multiple comparison (non-parametric) post-hoc test. Fisher's exact test was conducted for each treatment group to determine if temperature affected the presence/absence of morphological deformities. Results for all tests were considered significant when $P < 0.05$.

3. Results

3.1. Treatment period

3.1.1. Gonadal morphology and metrics

Fish analyzed from the control, moderate and high treatment group at 4 weeks averaged 79.3 ± 10.1 mm FL (4.4 ± 1.7 g BW), 90.5 ± 12.8 mm (6.7 ± 2.8 g BW), and 66.5 ± 12.3 mm (2.7 ± 1.6 g BW), respectively, and were significantly different in FL and in some cases BW as well; fish from the control and high group were not significantly different, but both were different than fish from the moderate group. Fish selected for analysis at 12 weeks in the control, moderate, and high treatment group averaged 126.4 ± 6.7 mm FL (17.0 ± 3.2 g BW), 127.2 ± 14.8 mm (18.4 ± 8.0 g BW), and 122.8 ± 17.5 mm (16.8 ± 7.0 g BW), respectively, and were not significantly different in FL or BW. Fish selected for analysis at 19 weeks in the control, moderate, and high treatment group averaged 171.6 ± 11.9 mm FL (39.1 ± 7.6 g BW), 157.6 ± 14.7 mm (39.7 ± 8.1 g BW), and 163.0 ± 12.3 mm (33.34 ± 8.98 g BW), respectively, and were not significantly different in BW among groups, however control fish FL was significantly higher than that of the moderate fish.

Histological sections of monosex female gonads sampled during the treatment period showed neither discernible signs of testicular development that would indicate sex reversal nor complete absence of germ cells that would indicate sterility. Instead we found that exposure to

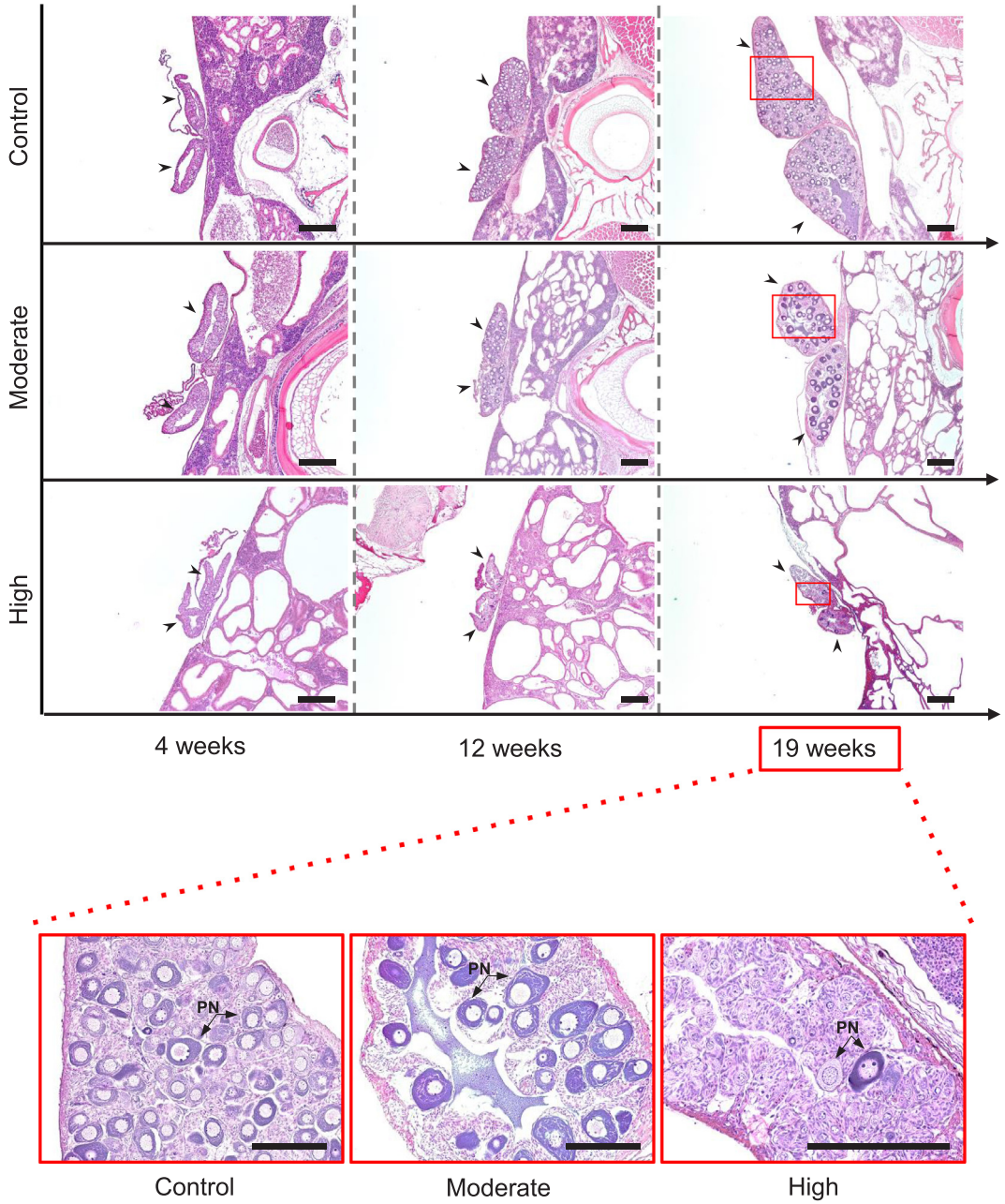

Fig. 1. Comparative ovarian development in monosex female sablefish after 4, 12, and 19 weeks of continuous exposure to control (15.6 °C ± 0.8 °C), moderate (20.4 °C ± 0.5 °C), or high (21.7 °C ± 0.5 °C) water temperature. All photomicrographs represent transverse histological sections of the gonads. Arrowheads (➤) denote the dual ovary lobes. Perinucleolar oocytes, PN. Scale bars = 100 µm.

moderately elevated temperatures at first slightly accelerated some aspects of ovarian differentiation and development relative to controls (Fig. 1). At 4 weeks, 70% of fish from the moderate treatment group displayed fusion of the distal ends of the ovary lobes to form ovarian cavities compared to only 22% and 10% of fish from the control and high treatment group, respectively. Fish from the moderate treatment group also had significantly larger ovaries (based on cross-sectional area), average PN oocyte area, and PN oocyte density compared to controls, while fish from the high treatment group generally had measures similar to those of the control group (Fig. 2A–C).

By 12 weeks, 70% of the fish from the high treatment group exhibited fused ovarian cavities compared to 100% of fish from the control and moderate treatment group. Fish from the moderate and high treatment groups exhibited significantly smaller ovaries compared to control fish and those from the high treatment group had significantly smaller PN oocytes compared to those of the control and moderate groups (Figs. 1, 2A–B). PN oocyte density was also significantly lower in moderate and high treatment group fish relative to control fish at 12 weeks (Fig. 2C).

By 19 weeks, 100% of the fish from each treatment group exhibited fused ovarian cavities. However, differences in ovary size among treatment groups were striking (Fig. 1), with significantly smaller ovaries observed in the moderate and high groups compared to control (Fig. 2A). Average PN oocyte size was significantly different between the control and high treatment groups, while the moderate group showed high variance and was not different from the other groups (Fig. 2B). In agreement with data for ovary size, PN oocyte density was significantly lower in the moderate and high treatment groups compared to the control (Fig. 2C). Over the entire treatment period, the density/number of PN oocytes showed little increase in fish from the

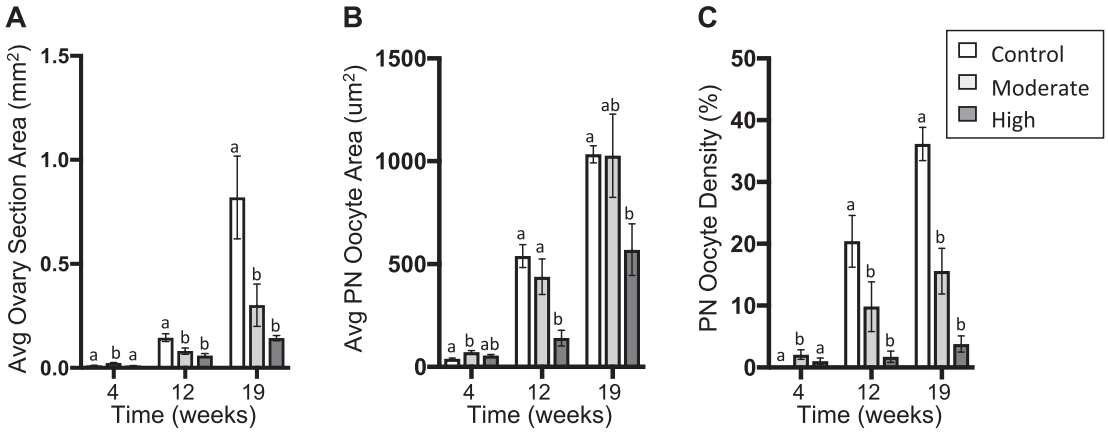

Fig. 2. Quantitative analysis of ovarian development in monosex female sablefish after 4, 12, and 19 weeks of continuous exposure to control (15.6 °C ± 0.8 °C), moderate (20.4 °C ± 0.5 °C), or high (21.7 °C ± 0.5 °C) water temperature. (A) Average cross-sectional area of the ovaries, (B) Average perinucleolar (PN) oocyte area, and (C) PN oocyte density. Data shown represent means ± SEM of n = 10 fish per treated group at each time point. Shared lettering within a time point indicates no significant difference ($p > 0.05$) between treatment groups.

high treatment group relative to fish from the moderate and control groups (Figs. 1, 2C).

3.1.2. Somatic and germ cell-specific gonadal genes

Targeted somatic cell genes associated with ovarian development included *cyp19a1a* and *foxl2a*, which exhibited similar patterns of expression across treatment groups at 12 and 19 weeks (Fig. 3). Levels of both *cyp19a1a* and *foxl2a* were significantly elevated in the moderate and high treatment group compared to the control group at 12 weeks. At 19 weeks, this pattern was maintained for *cyp19a1a* and *foxl2a* in the high treatment group, whereas the moderate treatment group had intermediate levels not significantly different from those of the control or high treatment group (Fig. 3).

Targeted somatic cell genes associated with testicular development included *dmrt1* and *amh*. For *dmrt1*, although transcript levels in the moderate and high treatment group trended higher than control at 12 and 19 weeks, there were no significant differences among groups (Fig. 3). As for *amh*, transcript levels in the high treatment group were significantly higher than those of controls at 12 and 19 weeks, while levels in the moderate group were intermediate and only significantly elevated relative to the control group at 19 weeks (Fig. 3).

Targeted germ cell/oocyte genes included *zpc* and *vasa*. Transcript levels for *zpc* were not significantly different between the control and moderate treatment group at 12 or 19 weeks, however *zpc* levels in the high treatment group were significantly lower than control at both 12 and 19 weeks (Fig. 3). Levels of *zpc* also increased about two times in the control and moderate groups between 12 and 19 weeks but showed little change in the high treatment group. For *vasa*, transcript levels at 12 weeks in the control group were markedly elevated compared to the moderate and high treatment groups (Fig. 3). Similarly, at 19 weeks, transcript levels in the control group were significantly higher than those of the high treatment group, while levels in the moderate group were not different from control (Fig. 3).

3.1.3. Apoptosis-associated genes

Caspase 8 and 3 represent cysteine proteases associated with the extrinsic apoptotic pathway. At 12 and 19 weeks, transcript levels for *casp8* in the moderate treatment group were significantly higher than control, while levels in the high treatment group were higher than control at 12 weeks and comparable to both the control and moderate treatment group at 19 weeks (Fig. 4). Transcript levels for *casp3* exhibited a declining pattern with exposure to elevated temperatures at both 12 and 19 weeks (Fig. 4); levels in the high treatment group were significantly lower than the control group, while the moderate group was intermediate. For *casp9*, no significant differences were observed between treatment groups at either time point, though an increasing trend with temperature was noted at 19 weeks (Fig. 4). Lastly, transcript levels of the tumor suppressor *p53* were significantly higher in the moderate and high treatment groups relative to control at both 12 and 19 weeks (Fig. 4).

3.1.4. Plasma 17β-estradiol levels

At week 12 of the treatment period, plasma E2 levels in fish from the high treatment group were significantly lower than those of control fish, while those of fish from the moderate treatment group were intermediate and not significantly different than the control or high treatment group (Fig. 5). By 19 weeks, mean plasma E2 levels had declined for all groups and no significant differences were found among treatments (Fig. 5).

3.2. One year post-treatment

3.2.1. Gonadal morphology and metrics

Fish analyzed from the control, moderate and high treatment group at one year post-treatment averaged 470.0 ± 32.4 mm FL (1168.3 ± 264.3 g BW), 454 ± 30.8 mm (1012.1 ± 261.0 g BW), and 394.4 ± 38.0 mm (647.8 ± 156.9 g BW), respectively, and were significantly different in FL and BW in one case; fish from the control group were significantly larger than those from the high treatment group. Rates of morphological deformities were 40, 83, and 91% for fish from the control, moderate and high treatment groups, respectively, and significantly elevated in fish from the moderate ($p = 0.0005$) and high treatment ($p < 0.0001$) groups compared to the control group.

Histology of monosex female gonads after one year in ambient seawater generally (see exceptions below) indicated that temperature effects on ovarian development were not permanent, and that development, which was severely inhibited during the treatment period, fully recovered by one year post-treatment (Fig. 6A). Consistent with these histological observations, average PN oocyte size and density were not different across treatment groups (Fig. 6B–C). Furthermore, the gonadosomatic index (GSI; (gonad weight/body weight) × 100)) was approximately 0.3% for all treatment groups and not significantly different among groups (data not shown).

3.2.2. Plasma 17β-estradiol

One year post-treatment, no significant differences in plasma E2 levels were found among treatment groups (Fig. 6D). There was however a trend of higher levels in controls, followed by the moderate and high treatment groups, respectively ($p = 0.06$ for high compared to

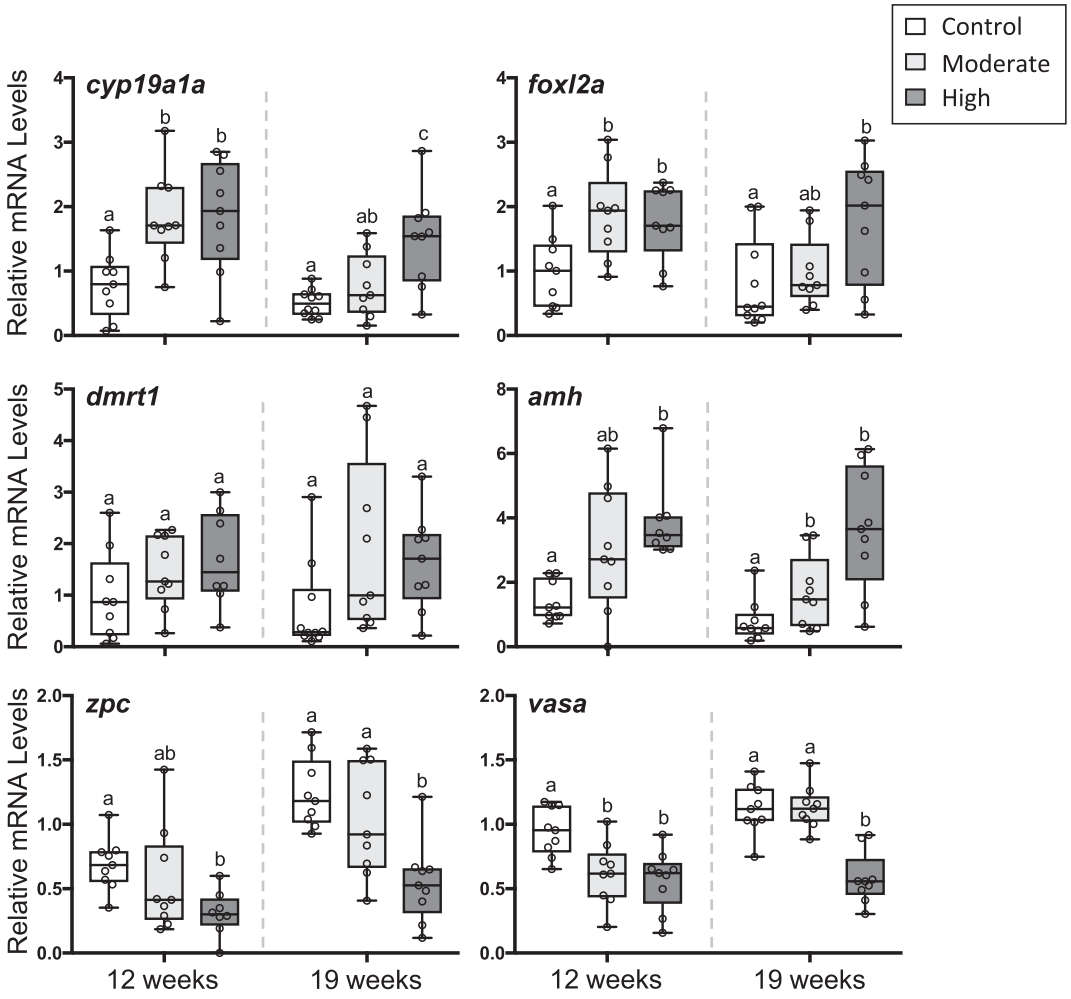

Fig. 3. Relative mRNA levels for markers of ovarian and testicular differentiation and germ cell development in monosex female sablefish after 12 and 19 weeks of continuous exposure to control (15.6 °C ± 0.8 °C), moderate (20.4 °C ± 0.5 °C), or high (21.7 °C ± 0.5 °C) water temperature. Box plots represent the median and interquartile range (IQR); whiskers extend to the minimum and maximum observed values with n ≥ 8 per treatment group at each time point. Shared lettering within a time point indicates no significant difference ($p > 0.05$) between treatment groups.

control).

3.2.3. Instances of sex reversal

Phenotypic sex reversal was not observed in any fish sampled from the control or moderate treatment group. However, 6% of the fish (2/35) sampled from the high treatment group appeared to be sex reversed and possessed putative non-meiotic testes with no ovarian features (Fig. 7A). Gonads of the two high temperature-generated neomales had undetectable expression of the ovarian marker *cyp19a1a* and elevated expression of the testis marker *cyp11b* (Fig. 7B). This corresponded well with results for MT-generated neomale and control male sablefish from a previous study (Luckenbach et al., 2017) analyzed in parallel with samples from this study (Fig. 7B). Control females on the other hand exhibited the opposite pattern, having discernable expression of *cyp19a1a* and no detection of *cyp11b* (Fig. 7B). The germ cell marker *vasa* was detected in gonads of all individuals, indicating that the fish possessed germ cells and thus were not reproductively sterile. Designation of the high temperature-treated fish as neomales was further verified by confirming that they were indeed genotypic females using the *gsdf* genetic sex marker (Fig. 7C).

4. Discussion

This study with monosex female sablefish evaluated the effects of elevated temperature on early reproductive development and potential induction of sex reversal or sterility. Most studies of temperature effects on sex determination/differentiation have been conducted with species that exhibit a high degree of sexual plasticity in response to environmental conditions. Sablefish on the other hand appear to possess a strict GSD mechanism not easily disrupted by standard rearing procedures. At week 4 of the treatment period, we found only minor differences in ovarian differentiation and development in response to elevated temperatures, which may have been influenced by differences in body size. However, by 12 weeks, morphological, endocrine and molecular analyses indicated significant inhibition of female reproductive development, including reduced ovarian growth and E2 production, and increased expression of the testis marker *amh* and some apoptotic genes, particularly in fish from the high treatment group. By the end of the treatment period (week 19), inhibitory effects of elevated temperature were even more pronounced with fish from the high treatment group exhibiting significantly reduced ovary size, PN oocyte size and density, and germ cell marker (*vasa*, *zpc*) gene expression compared to controls, suggesting germ cell loss or stasis.

Despite the significant developmental impairment observed during the treatment period, these effects were not permanent. After all fish were transferred to ambient seawater for one year, oocyte development resumed and fish from the moderate and high treatment groups, with some exceptions, developed ovaries indistinguishable from those of controls. Treated females therefore demonstrated full compensatory recovery of ovarian development one year post-treatment. Although

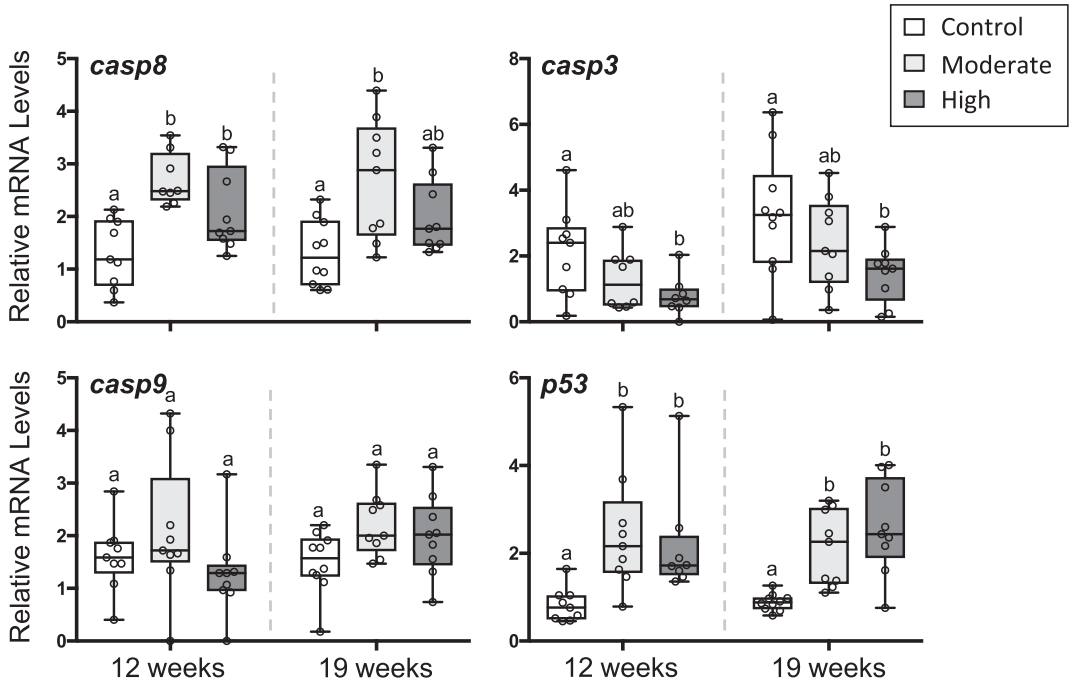

Fig. 4. Relative mRNA levels for apoptosis-associated genes in gonads of monosex female sablefish after 12 and 19 weeks of continuous exposure to control (15.6 °C ± 0.8 °C), moderate (20.4 °C ± 0.5 °C), or high (21.7 °C ± 0.5 °C) water temperature. Genes represent cysteine proteases in the extrinsic and intrinsic apoptotic pathway, along with a tumor suppressor. Box plots represent the median and interquartile range (IQR); whiskers extend to the minimum and maximum observed values with n ≥ 8 per treated group at each time point. Shared lettering within a time point indicates no significant difference ($p > 0.05$) between treatment groups.

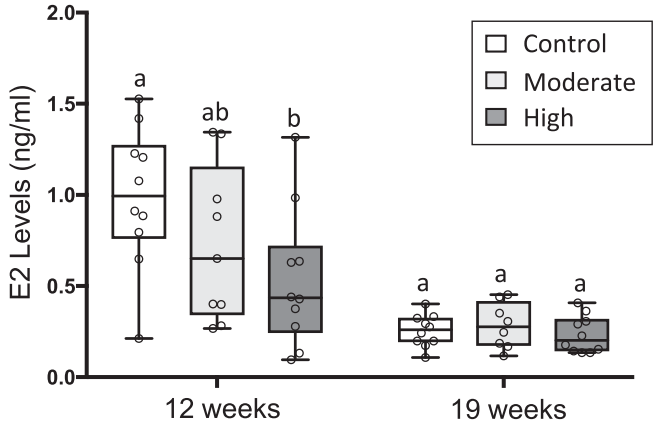

Fig. 5. Plasma 17β-estradiol (E2) levels of monosex female sablefish sampled after 12 and 19 weeks of continuous exposure to control (15.6 °C ± 0.8 °C), moderate (20.4 °C ± 0.5 °C), or high (21.7 °C ± 0.5 °C) water temperature. Box plots represent the median and interquartile range (IQR); whiskers extend to the minimum and maximum observed values with n ≥ 8 per treated group at each time point. Shared lettering within a time point indicates no significant difference ($p > 0.05$) between treatment groups.

reproductively sterile (germ-cell free) fish were not observed, 6% of the fish from the high treatment group were sex reversed by temperature and possessed testes that expressed the testis marker *cyp11b* and had no discernable expression of the ovarian marker *cyp19a1a*. These represent the first documented instances of temperature-induced masculinization in this species.

High temperature exposure near the time of gonadal sex differentiation has been shown to induce masculinization or reproductive sterilization in several fish species (Guiguen et al., 2010), and the switch between masculinization and sterilization may be attributable to the interactive effects of selected temperature and duration of exposure. In tilapias (genus *Oreochromis*), female-to-male sex reversal has been documented at temperatures > 32 °C with relatively short durations of exposure (Baroiller et al., 2009a), whereas irreversible sterilization was recently documented in Nile and Mozambique tilapia (*O. niloticus* and *O. mossambicus*) exposed to ~37 °C for longer durations (Nakamura et al., 2015; Pandit et al., 2015). Because we observed a 6% rate of sex reversal of sablefish in the high treatment group alone and no instances of sterility, a prolonged treatment duration would be worth testing in the future to potentially achieve higher rates of sex reversal or sterilization. This is supported by other work in our lab which indicated that dietary MT treatment needed to extend well beyond the period of morphological sex differentiation (70–150 mm FL) to induce masculinization of XX-genotype sablefish and that further masculinization may occur post-treatment (Luckenbach et al., 2017; Luckenbach and Fairgrieve, 2016). Importantly, since the high temperature treatment approached the lethal level for juvenile sablefish (24 °C; Sogard and Olla, 2001), it is unlikely that higher temperatures could be tested without detrimental effects to the fish.

During the process of sexual differentiation, female- and male-determining factors expressed in the gonads have antagonistic roles, with their interplay ultimately tipping the balance toward one sex versus the other (e.g., Kim et al., 2006; Li et al., 2013; Siegfried, 2010). Two of the most important genes to E2 biosynthesis and hence ovarian differentiation and maintenance in fishes are the steroidogenic enzyme *cyp19a1a* and the female predominant transcription factor *foxl2a* (Baroiller et al., 2009b; Guiguen et al., 2010; Shen and Wang, 2014; Siegfried, 2010). Foxl2a regulates the expression of *cyp19a1a* and up-regulation of *cyp19a1a* is necessary to trigger and maintain ovarian differentiation, while suppression of *cyp19a1a* can lead to masculinization. In several studies, high temperature has been shown to suppress gonadal *foxl2a* and *cyp19a1a* expression, and thermal application particularly during the sexually labile period can override sex differentiation of genotypic females, resulting in female-to-male sex reversal (Baroiller et al., 2009b; D'Cotta et al., 2001; Kitano et al., 1999; Penman and Piferrer, 2008; Shen et al., 2018). Previous work in sablefish has demonstrated that *foxl2a* and *cyp19a1a* are both highly

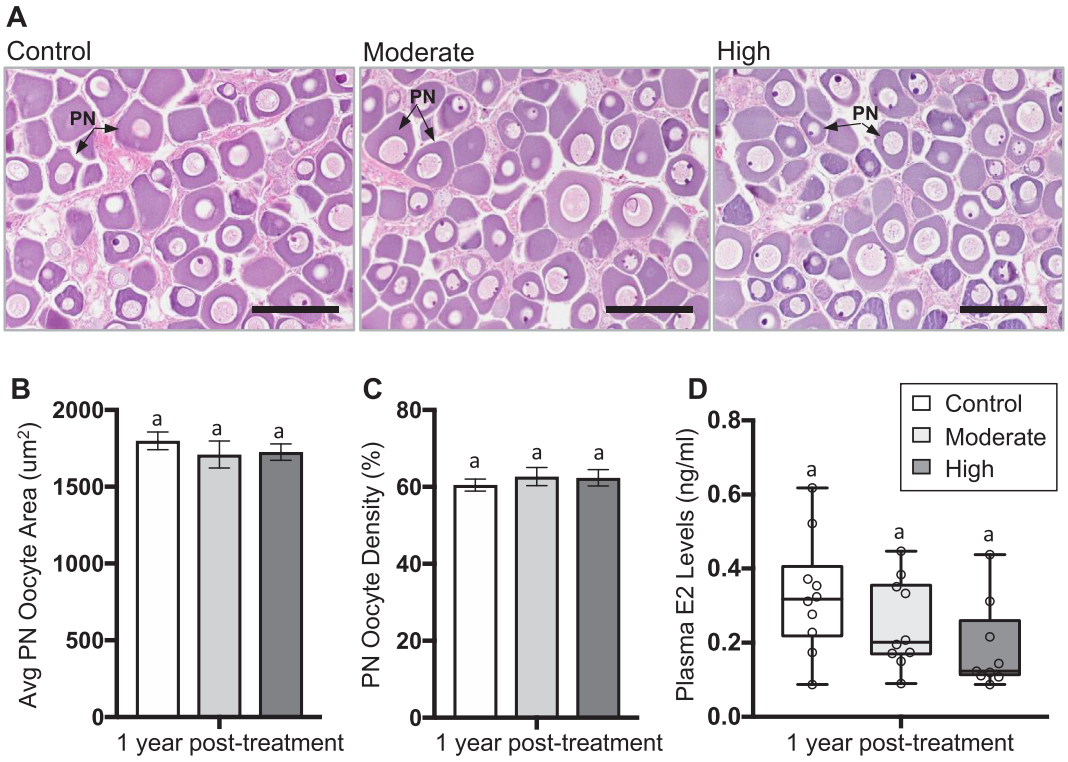

Fig. 6. Comparative ovarian development in yearling sablefish continuously exposed to control (15.6 °C ± 0.8 °C), moderate (20.4 °C ± 0.5 °C), or high (21.7 °C ± 0.5 °C) water temperature for 19 weeks followed by one year at ambient temperature (11.2 °C ± 2.3 °C). (A) Representative photomicrographs of ovarian histological sections (Scale bars = 100 μm), (B) Average perinucleolar (PN) oocyte area, (C) PN oocytes density, and (D) plasma 17β-estradiol (E2) levels. Data shown represent means ± SEM of n = 10 per treatment. Shared lettering indicates no significant difference ($p > 0.05$) between treatment groups.

expressed during ovarian differentiation and low during testicular differentiation (Smith et al., 2013). Interestingly, in the present study, levels of these mRNAs increased in response to exposure to elevated temperatures. Upregulation of these genes was not anticipated based on studies in other species and may indicate resilience of the endogenous female program in sablefish. This is also supported by the relatively low rate of sex reversal observed.

On the other hand, plasma E2 levels were significantly lower in fish from the high treatment group compared to controls at 12 weeks. This occurred concomitant with higher gonadal expression of *foxl2a* and *cyp19a1a*, which again are essential to E2 biosynthesis. It is difficult to reconcile this contradiction, but one must consider that although gonadal *foxl2a* and *cyp19a1a* mRNAs were significantly elevated by temperature, ovaries of fish from the high treatment group were significantly smaller than those of control fish. Therefore, total capacity to produce E2 should be reduced proportionally.

For sex reversal to occur, it may have been essential that E2 levels were reduced at 12 weeks (and possibly earlier in development). Lower levels of E2 have been shown to upregulate gonadal expression of the transforming growth factor gene *amh* and thus drive sex reversal in genotypic female fish (Fernandino et al., 2008). A number of studies have also documented upregulation of *amh* and the male-predominant transcription factor *dmrt1* during high temperature treatment (Baroiller et al., 2009b; Piferrer and Guiguen, 2008; Poonlaphdecha et al., 2013; Wang et al., 2010). We found that sablefish *dmrt1* mRNA levels were not significantly different among groups at any time point, but consistently trended higher at more elevated temperatures. Gonadal levels of *amh* on the other hand were significantly upregulated at elevated temperatures relative to controls. These results together suggest that male-determining factors were generally activated in gonads of the monosex female sablefish exposed to elevated temperatures and likely played a role in 'tipping the balance' toward testicular differentiation in some individuals.

In addition to the above genes associated with sex differentiation, several apoptosis- and germ cell-associated genes were assessed during the treatment period to determine whether apoptotic mechanisms could have played a part in the observed inhibition of ovarian development or sex reversal. Gonadal apoptosis/atresia is a common process that maintains ovarian integrity by eliminating non-viable or excess germ cells and their surrounding somatic cells (Saidapur, 1978). Rates of apoptosis and atresia may also increase with exposure to environmental stressors like high temperature (Saidapur, 1978; Uchida et al., 2004; Yamamoto et al., 2011). Caspases are key factors in the apoptotic pathway that can be largely categorized as either initiator caspases (e.g., *casp8*, *casp9*), responsible for activating effector caspases, or effector caspases (e.g., *casp3*), responsible for cleaving cellular targets, resulting in cell death (Johnson and Bridgham, 2002; Takle and Andersen, 2007). In general, we found that gonadal *casp8* mRNA levels were significantly elevated in fish from the moderate and high treatment groups and *casp9* levels were not different among groups. Increases in gonadal *casp8* expression occurred concomitant with decreases in germ-cell markers *zpc* and *vasa*, which were consistently downregulated in the high treatment group. Interestingly, the effector caspase, *casp3*, exhibited a downward-stepping pattern with gonads of control fish having the highest mRNA levels and fish from the high treatment group having the lowest levels. Caspase 3 is known to be heavily post-transcriptionally regulated however to avoid unwanted cell death (Ruest et al., 2002), so this could potentially explain the discordance in expression patterns between some of the caspase genes.

In addition to caspases, we investigated *p53*, a tumor suppressor responsible for preventing unwanted cell growth, typically expressed at low levels under normal conditions (Fridman and Lowe, 2003). Under different types of cellular stress, the *p53* apoptotic pathway may be activated to suppress proliferation and development of damaged cells (Fridman and Lowe, 2003). We found that the expression pattern for *p53* resembled *casp8* with significantly higher levels in the moderate

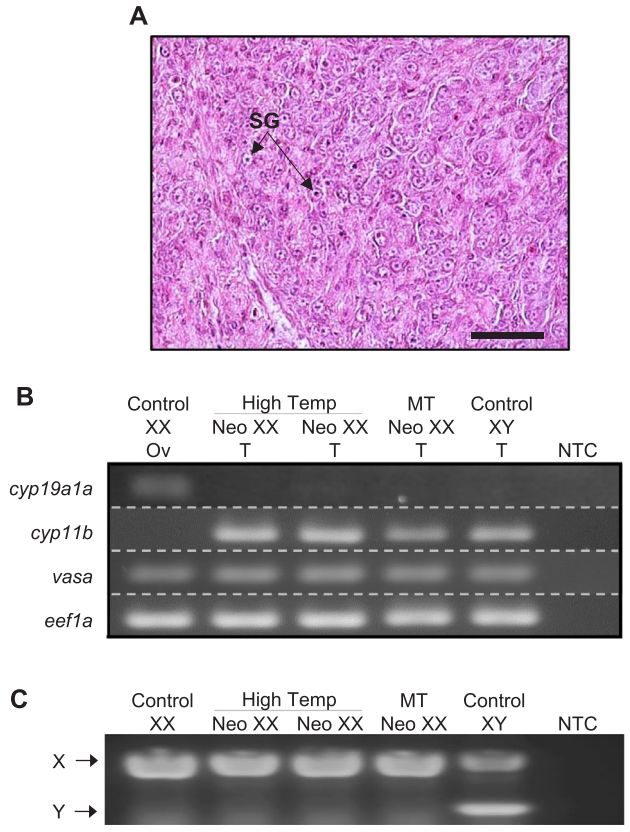

Fig. 7. Analysis of sex-reversed, genotypic-female sablefish continuously exposed to high (21.7 °C ± 0.5 °C) water temperature for 19 weeks followed by one year at ambient temperature (11.2 °C ± 2.3 °C). (A) Representative histological section of a putative non-meiotic testis composed of type-A spermatogonia (SG, Scale bar = 25 mm); (B) RT-PCR results for ovarian (*cyp19a1a*), testicular (*cyp11b*) and germ cell (*vasa*) markers assessed in a control female (XX genotype) and two high temperature-induced neomales (XX) from the present study, as well as a methyltestosterone (MT)-induced neomale (XX) and control male (XY) from Luckenbach et al. (2017); (C) PCR results for the sablefish genetic sex marker, *gsdf*, using genomic DNA isolated from the same individuals. A positive control RT-PCR targeting *eef1a* and no template controls (NTC) were included where appropriate. All PCRs were 32 cycles and electrophoresed on 1.5% agarose gels. Ovaries, Ov; Testes, T.

and high treatment groups at both 12 and 19 weeks, suggesting these genes together could have played a role in the observed ovarian degeneration and/or sex reversal. Previous studies in zebrafish (*Danio rerio*) and rice eel (*Monopterus albus*) have suggested that *p53*-mediated gonadal apoptosis may be involved in the process of sex reversal (He et al., 2010; Rodríguez-Marí et al., 2010). Furthermore, in coho salmon (*Oncorhynchus kisutch*), *casp8*, *casp9*, and *p53* were some of the strongest markers of fasting-induced ovarian atresia (Yamamoto et al., 2011).

Another mechanism worth exploring in future temperature experiments with sablefish would be the potential role of the stress-associated glucocorticoid hormone, cortisol. Temperature-induced sex reversal was found to be mediated by elevated cortisol production in several fish species (Baroiller and D'Cotta, 2016; Hattori et al., 2009; Hayashi et al., 2010; van Den Hurk and van Oordt, 1985) and could have a role in the ovarian inhibition and/or sex reversal we observed.

From an applied perspective, neomale broodstock are a critical component to monosex female production of sablefish, which capitalizes on the superior growth of females relative to males (Luckenbach et al., 2017). Based on methods tested in this study, we now know that neomale sablefish can be generated via high temperature treatment, which is a chemical-free and more eco-friendly approach compared to dietary MT treatment. Given the relatively low rate of sex reversal in this study, optimization of temperature treatment would be necessary to increase proportions of sex-reversed individuals. As mentioned above, modifying the duration of treatment and/or developmental timing of thermal application (e.g., earlier developmental exposure to high temperature) may increase neomale proportions. Longer-term studies are also needed to assess the ultimate reproductive performance of neomale sablefish generated via different approaches (i.e., temperature as opposed to MT) and potential deleterious morphological deformities that may arise from high temperature exposure.

In conclusion, continuous exposure to extreme elevated temperatures markedly inhibited early ovarian development in sablefish, reducing ovarian growth, PN oocyte density and size, plasma E2 levels, and germ-cell marker gene expression. Meanwhile, gonadal expression of key female- and male-determining factors and some cell death-associated genes were stimulated. Following transfer to cooler, ambient temperatures, sablefish exhibited striking compensatory recovery of ovarian development. The exception to this was sex reversal of 6% of the individuals exposed to the highest temperature. We posit that the reduction in E2 production and upregulation of expression of *amh* and apoptotic factors led to the observed instances of sex reversal. To some degree, the low percentage of sex reversal and lack of sterile individuals lends further support to prior research suggesting sablefish are a strongly GSD species with relatively low sexual plasticity. Sex-reversed sablefish have only previously been produced by dietary exposure to sex steroids, therefore this is the first demonstration of sex reversal by rearing conditions alone. These results may have positive implications for neomale broodstock production for aquaculture.

Declarations of interest

None.

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