Effects of high temperature exposure on early reproductive development in monosex female sablefish (*Anoplopoma fimbria*)

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Abstract

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Aquaculture supplies over half of all seafood produced for human consumption. However, production processes are raising environmental concerns, and escapement of farmed fish is a prominent issue. A viable solution is to produce reproductively sterile fish so escapees cannot genetically contaminate wild populations. For this study, we examine high temperature treatment as a method for achieving sterility since it is shown to induce germ cell loss in some female fishes. Monosex female sablefish larvae (~30 mm in fork length; ~90 days post-fertilization) were randomly divided into three groups and exposed to different temperatures, control (15°C), moderate (20°C), or high (22°C), for 19 weeks. During this time, larvae were periodically sampled for paraffin histology and gene expression analysis from each treatment group to assess gonadal development. After the treatment period, remaining fish were tagged and transferred to ambient temperatures for one year to determine whether temperature effects were maintained post-treatment. We found that exposure to elevated

temperatures induced severe impairment of early ovarian development. Sablefish at moderate and high temperatures had significantly less developed oocytes relative to controls, and germ cell-specific genes and apoptosis-related genes indicated germ cell degeneration. However, after one year at ambient temperatures, impaired gonads were no longer observed and most high-temperature treated fish had ovaries similar to those of controls. We also observed two sex-reversed females (female genotype but male phenotype), referred to as neomales, from the high temperature group which opens the possibility of using high temperature for future neomale broodstock production. Neomale broodstock are a key component for monosex sablefish production, and being able to generate them via high temperature may represent an improvement upon current methodology. Based on these results, we conclude that high temperature is likely not an effective method for inducing reproductive sterility but may be a preferable method for neomale production in sablefish.

Preface:

Currently, the world supports 7 billion people and is expected to host an additional 2 billion by 2050[1]. As such, the demand for seafood is increasing, placing further stress on wild stocks, of which 30% are already overfished and 60% percent fully exploited, with no possibility of further increase in production[2]. In the mid-80s, capture fisheries production reached a capacity of 80 million metric tons per year and has since leveled out[3]. To ensure continued availability of seafood in the face of a rising population and increasing per capita demand, aquaculture is stepping in to fill the demands that otherwise would not be met by capture fisheries alone. At present, aquaculture provides over 50% of all fish directly consumed by people worldwide, and reliance on this industry will increase as research development improves farming capacity[4].

The US can be a global leader of aquaculture, having the resources and capacity to develop in a sustainable and responsible manner. However, the US is currently ranked 11th in global aquaculture production, contributing only 1.1% of total seafood by weight and 1.6% by value[5]. Meanwhile, the US imports over 90% of its seafood from overseas, mainly from Asia where poor regulation sometimes results in environmentally- and socially- damaging aquaculture practices[5][6]. Furthermore, the US seafood trade deficit has increased to 14 billion annually due to the volume of imports[4]. Considering the US has the world's largest exclusive economic zone (EEZ), a sovereign zone for economic utilization, there is potential in both state and federal waters to increase aquaculture production for domestic consumption and export[5].

Given the capacity for, and potential benefits of, domestic aquaculture, there is much public concern about the risks of farmed fish in the US[6], more so compared to other developed countries with well-established industries, such as Australia, Sweden, and Norway[7][8][9]. Reluctance primarily stems from beliefs that 1) aquaculture degrades the environment and 2) displaces wild fisheries[9]. Concurrently though, support for aquaculture can be garnered if positive impacts are made clear[9]. For instance, increased production can stimulate economic development, supply high-quality

protein, reduce fishing pressure, and restore and enhance commercial and recreational species[6][9]. Overall, education, awareness, and transparency of practice can shift current perceptions of and build support for aquaculture.

Although support at the individual level is still being cultivated, there is advocacy for fish farming at the institutional level in the US. The National Science and Technology Council (NSTC), National Oceanic and Atmospheric Administration (NOAA), and National Sea Grant College Program have recently published strategic plans, expressing an interest to not only build a competitive aquaculture sector but to do so in an environmentally and socially responsible manner[5][10][11]. Currently, the US is striving to increase production by 50% (by volume) by 2020[5], and this percentage is anticipated to rise over the following decades.

Toward this end, the research presented in this thesis introduces sablefish (i.e. black cod; *Anoplopoma fimbria*), a marine groundfish of high value and demand[12], as an emerging species for production in the Pacific Northwest of the USA. To improve the sustainability of sablefish farming and particularly address escapement concerns, we will tested high temperature exposure as a method for reproductive sterilization. High temperature is generally considered an eco-friendly, consumer-friendly, and chemical-free method of sterilization that has been effectively applied to other prominent aquaculture species[13][14]. If successful in our study, this will ensure bio-containment of sablefish and prevent genetic pollution in an escapement scenario.

Ultimately, we conclude that there is an emerging opportunity to invest in domestic aquaculture to reduce fishing pressure and promote economic growth, and sablefish is an ideal candidate given its high value and demand. Although sablefish are not yet commercially grown in the US, current research and development (R&D) on the reproduction and growth of these fish will make farming more feasible in the near future. Furthermore, we hope these techniques developed for sablefish can be widely applied to other candidate species as well.

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Introduction:

Temperature is a natural environmental factor that can influence, redirect, or even block early reproductive development in some species. For example, some reptiles are well-documented as being thermosensitive with their sex directly determined by temperature (temperature-dependent sex determination, TSD; Pieau, et al., 1999). In contrast, species such as 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. However, even among the spectrum of GSD species, sexual plasticity is often observed under applications of temperature (GSD with thermal effects, GSD+TE), particularly in teleost fish, where sex differentiation is more plastic compared to higher vertebrates (Ospina-Álvarez et al., 2008; Baroiller and D'Cotta, 2016; Piferrer, 2001; Strüssmann and Nakamura, 2002).

At the molecular level, temperature effects can be observed in a suite of genes commonly associated with sex differentiation. To provide an example, *cyp19a1a*, or P450 aromatase, is considered a key player in female sex differentiation (Piferrer and Guiguen, 2008), and elevated temperatures have been shown to suppress *cyp19a1a* expression in gonadal somatic cells, causing masculinization in genotypic female fish (Kitano et al., 1999; Van Nes and Andersen, 2006; Karube et al., 2007). In addition to genes regulating sex determination/differentiation, elevated temperatures have been shown to upregulate apoptosis-related genes such as caspases (i.e. cysteine proteases) and to promote follicular atresia, which may have reproductively sterilizing or masculinizing effects (Guraya, 1986; Linares-Casenave et al., 2002; Takle and Andersen, 2007).

Despite the abundance of temperature-related research, studies documenting temperature effects on reproductive development of marine teleosts have not been widely conducted. Thus, this study tests effects of high temperature in sablefish (i.e. black cod; *Anoplopoma fimbria*). Sablefish is an economically important groundfish in

commercial fisheries of the Pacific Northwest (USA) (NMFS, 2016), and an emerging aquaculture species in the United States and Canada. Naturally, sablefish range from northern Mexico to the Bering Sea and Japan and can live up to 100 years (NOAA, 2007). In terms of reproductive development, sablefish has been established as a gonochoristic species that utilizes an XX-XY GSD system (Luckenbach and Fairgrieve, 2016; Luckenbach et al., 2017), with gonadal sex differentiation typically occurring when juveniles are ~75-150 mm fork length (FL) (Luckenbach and Fairgrieve, 2016). This serves as important information to the development of techniques for sex control and bio-containment of sablefish in aquaculture.

Monosex production is primarily important for commercial aquaculture due to sexually dimorphic growth commonly observed in fish, where some species exhibit superior female growth while others superior male growth (Parker, 1992). For sablefish, females have been documented to attain larger sizes than males (Echave et al., 2012; Luckenbach et al., 2017). To attain all-female sablefish stocks for aquaculture, a study conducted by Luckenbach et al. (2017) treated juvenile female sablefish with 17α-methyltestosterone (MT), inducing female to male sex reversal. The resulting fish, referred to as neomales (i.e. genotypically female but phenotypically male), can then be crossed with wild-caught females to generate all-female progeny. Given that high temperature treatment has been observed to induce sex reversal in a number of important aquaculture species as well as other teleost fish, temperature may be a more eco- and consumer-friendly method to generate monosex female sablefish (Pandit et al., 2015; D'Cotta et al., 2001; Shen and Wang, 2014).

In conjunction with monosex production, generating reproductively sterile fish is also important to address escapement and genetic pollution commonly associated with finfish aquaculture (Crozier, 1993; Skaala, 2006). There are several studies linking high temperature exposure to follicular atresia and germ cell degeneration, which ultimately leads to reproductive sterilization in several species (Pandit et al., 2015, Nakamura et

al., 2015, Lee et al., 2009; Byerly et al., 2005; Linares-Casenave et al., 2002; Strüssmann et al., 1998). Although the exact mechanisms linking temperature to gonadal apoptosis are not yet clearly understood, the suppression of aromatase and activation of apoptotic pathways are commonly observed in these studies. Once again though, high temperature may hold promise as a chemical-free method to achieve bio-containment for sablefish aquaculture.

The objective of this study was to document effects of high temperature treatment on the morphological and molecular reproductive development of female sablefish. Furthermore, we aimed to determine the utility of high temperature treatment for inducing either sex reversal or reproductive sterility. Through this study we sought to provide greater insight into early reproductive mechanisms that regulate sex differentiation and development, including temperature effects on a suite of gonadal sex differentiation- and apoptosis-related genes. Successful application would open possibilities for developing commercial sablefish aquaculture in the Pacific Northwest (USA) and could be more widely applied to other marine species.

Materials and Methods:

Experimental Animals and General Rearing Conditions

Monosex 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 female 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 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 (0.618m^3) continuously supplied with heated (~15°C) hatchery water in a flow-through system until they were 0.2-0.5g and fully weaned onto dry hatchery feed (BioVita, BioOregon Inc., Longview, WA).

Experimental Conditions

Fish near 30 mm fork length (FL) (30.54 ± 3.73 mm FL; 0.3 ± 0.08 g BW; ~90 days post-fertilization (mean ± SD)) were targeted for experimentation since they are more robust (i.e. mortality rates were low) and 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-hour 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 (15.58 °C ± 0.77 °C), moderate at 20.5 °C (20.76 °C ± 0.12 °C), and high at 22.5 °C (22.45 °C ± 0.25 °C) (**Figure 1**). Hatchery water (14.6 °C ± 0.12 °C) was heated to the target temperatures using digitally-controlled immersion

heaters (Finnex, Amazon, Seattle, WA, USA). To acclimate fish, water temperature was increased by approximately 2 °C each day until target temperatures were reached (**Figure 1**). Targeted temperatures were determined based on previous studies with juvenile sablefish, which identified 15 °C as an optimal temperature for survival and growth and 24 °C as lethal. (Lee et al., 2017; Sogard and Olla, 2001). Furthermore, sablefish reared at 18 °C did not show signs of sterilization or masculinization (Lee et al., 2017), and thus 22.5 °C and 20.5 °C were chosen for this study.

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-hour 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 level control switches (FloTec, Delavan, WI, USA). Water temperature was monitored twice daily with a hand-held digital thermometer (Bel-Art - SP Scienceware, Wayne, NJ, USA) and recorded at 15-minute intervals using Hobo Pendant temperature loggers (Onset Computer Corporation, Bourne, MA, USA). Mortalities were removed and recorded daily.

Fish were exposed to treatment temperatures for approximately 19 weeks (136 days). During the treatment phase they were fed a commercial salmon diet (BioVita, BioOregon Inc., Longview, WA, USA) to apparent satiation by hand 3-4 times daily, except on sampling days, when feed was withheld. Overall, mortality rate for the high treatment group was 43.7% (142 fish) compared to 26.5% (86 fish) for the moderate treatment group and 30.2% (98 fish) for control. In an effort to reduce mortality and preserve fish for sampling, the targeted high temperature was lowered from 22.5 °C to 21.5 °C (21.42 °C ± 0.21 °C) and targeted moderate temperature from 20.5 °C to 20 °C (19.80 °C ± 0.44) (**Figure 1**).

After 19 weeks of exposure to treatment temperatures, remaining fish from each group (n = 43, 44, and 41 fish in control, moderate and high, respectively) were 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 per minute) ambient seawater (11.15 °C ± 2.31 °C) located outdoors and covered with shade cloth. During the post-treatment period, the fish were fed 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 hours per day. Feeding levels were calculated using a growth model developed in previous studies (Luckenbach and Fairgrieve, 2016; Buterbaugh and Willoughby, 1967).

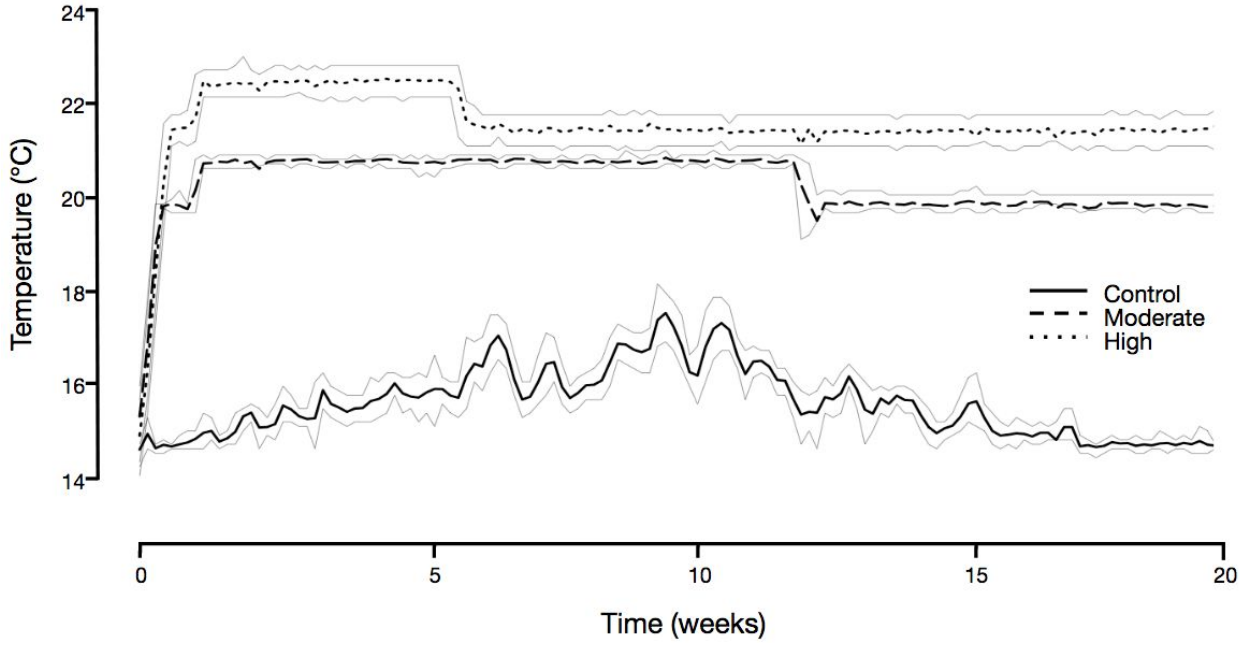


Figure 1. Temperature profiles during the sablefish high temperature study. Bolded lines indicate daily average temperatures while lighter lines indicate daily maximum and minimum temperatures.

Sample Collection

Fish were selectively size-matched during sampling at 4, 12, and 19 weeks for gonadal assessment (**Figure 2**). Fish sampled at 4 weeks in the control, moderate and high treatment group had an average FL of 79.28 ± 10.06 mm (4.42 ± 1.70 g BW), 90.53 ± 12.78 mm (6.70 ± 2.83 g BW), and 66.50 ± 12.26 mm (2.68 ± 1.63 g BW), respectively. Fish sampled at 12 weeks in the control, moderate, and high treatment group had an average FL of 126.36 ± 6.69 mm (17.02 ± 3.24 g BW), 127.20 ± 14.81 mm (18.42 ± 8.00 g BW), and 122.76 ± 17.54 mm (16.81 ± 7.02 g BW), respectively. Fish sampled at 19 weeks in the control, moderate, and high treatment group had an average FL of 171.56 ± 11.85 mm (39.14 ± 7.63 g BW), 157.60 ± 14.69 mm (39.70 ± 8.13 g BW), and 163.00 ± 12.26 mm (33.34 ± 8.98 g BW), respectively.

At each time point (i.e. 4, 12, and 19 weeks), 10 fish were sacrificed for paraffin histology and 10-15 fish for quantitative reverse transcription-PCR (qRT-PCR) from each treatment group. Overall, these sampling time points targeted body sizes between 60-180 mm FL, capturing the periods of molecular and histological sex differentiation which could reflect sex reversal or sterility (Luckenbach and Fairgrieve, 2016; Hayman et al., in preparation).

One-year post-treatment, 35 randomly selected fish from each treatment group were sampled for gonadal histology and RT-PCR in order to assess whether temperature effects on reproductive development were permanent. Fish sampled from the control, moderate, and high treatment group had an average FL of 470 ± 32.38 mm (1168.26 ± 264.25 g BW), 454 ± 30.80 mm (1012.06 ± 260.98 g BW), and 394.39 ± 37.99 mm (647.76 ± 156.85 g BW), respectively.

During sampling, fish were first euthanized using a lethal dose of Tricaine-S (200 ml/L; Western Chemical, Ferndale, WA, USA) then severed at the spinal cord. All fish were humanely handled by NOAA NWFSC staff during experimentation in accordance with

the National Research Council for aquatic animals (NRC, 2011) and American Veterinary Medical Association (AVMA, 2007).

During the treatment period, gonads were isolated from fish via one of two dissection methods depending on the type of analysis. For gonadal histology, gonads were dissected along with residual kidney and muscle tissue (i.e. trunk sections of the body). For gene expression analysis, gonads were directly extracted from the coelomic cavity. To locate gonads in either dissection method, the head was severed at the insertion of the dorsal fin followed by the tail anterior of the anus. An incision was made parallel to the coelomic cavity and viscera were removed, exposing the gonads. Gonadal tissues were either fixed in Bouin's solution for paraffin histology or preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA) for molecular analysis. One-year post-treatment, the second dissection method was used exclusively for fish sampled, and portions of the same gonad were either preserved Bouin's solution or RNAlater.

Gonadal Histology

Methods for sablefish gonadal histology and staging are further outlined in Luckenbach and Fairgrieve (2016). In brief, gonads were fixed in Bouin's solution for at least 48 hours, 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. Microscope images were captured with a Nikon digital camera (Melville, NY, USA) and analyzed with NIS Element image software version 4.0 (Nikon).

To quantify histological characteristics, the area of perinucleolar (PN) oocytes (the most advanced oocytes observed, discernible based on their large grainy nuclei outlined with nucleoli and surrounding cytoplasm), PN oocyte count, cross-sectional area of the ovary, and percentage of ovary composed of PN oocytes were measured for samples during the treatment period. All measurements were conducted in triplicate for ten fish from each treatment group, and the average and standard error of the mean (SEM)

were calculated. To measure PN oocyte area (μm^2), ten centrally cross-sectioned PN oocytes (in which the oocyte nucleus was visible) were targeted in each histological section, resulting in at least 30 measurements per fish. For PN oocyte count, all PN oocytes identified within the area of the ovary were counted. The cross-sectional area of the ovary (μm^2) was measured using a trace function in the NIS Element image software. The percentage of the ovary composed of PN oocytes was calculated based on measured values of the PN oocyte area, PN oocyte count, and area of the ovary. The PN oocyte area and PN oocyte count were first multiplied then divided by the area of the ovary. This value was then multiplied by 100 to acquire a percentage.

For fish sampled one-year post-treatment, the PN oocyte area and count were measured using the same prescribed method as above. Since histological samples consisted of partial gonadal tissue (not trunk sections of the body as with fish during the treatment period), the cross-sectional area of the ovary could not be measured. Thus, to find the percentage of the ovary composed of PN oocytes, the area of all PN oocytes present within a 20X frame was measured, summed, divided by the area of the frame (~150,000 μm^2), and then multiplied by 100. Again, all measurements were conducted in triplicate for ten fish from each treatment group, and the average and SEM were calculated.

Gene Expression Analysis

RNA isolation and reverse transcription

Methods for RNA isolation and RT followed Luckenbach et al. (2011). Briefly, ovarian tissues were homogenized using a TissueLyser II (Qiagen, Germantown, MD, USA), and total RNA was 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 reverse transcriptase enzyme, to confirm that genomic DNA had been eliminated by DNase treatment.

Primer Design

PCR primers were designed using Primer3 in MacVector software (Accelrys, San Diego, CA, USA). Primers were redesigned if an assay displayed multiple products in melt curve analyses.

Quantitative PCR

Quantitative PCR was used to determine steady-state mRNA levels for targeted genes in gonadal samples collected at week 12 and 19. Methods for qPCR are further outlined in Luckenbach et al. (2011). Briefly, qPCRs 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 the following concentrations and run in triplicate: 5, 1, 0.25 and 0.05 ng. Assays were run on a 7900HT Fast Real-Time PCR System (Life Technologies) with standard cycling conditions. To confirm that only one product amplified in the reactions, dissociation curves were included in each run. In addition to NACs, no template controls (NTCs), which contained no cDNA template, were included in each assay.

RT-PCR and gel electrophoresis

RT-PCR was used to assess gene expression patterns in gonads of selected fish one-year post-treatment. Methods are further outlined 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 minutes at 94 °C, 32 cycles for 30 seconds at 94 °C, 30 seconds at 60 °C, 60 seconds 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) and photographed with camera settings held constant. A negative control, which contained no cDNA template, was included in each assay.

Target genes

Several female and male-associated gene markers were selected to assess phenotypic sex and gonadal status. 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* and *zpc (zona pellucida protein c)* (**Table 1**). Apoptosis-associated genes were also targeted because signs of follicular atresia 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. For RT-PCR and gel electrophoresis, *cyp19a1a* and *cyp11b* (*cytochrome P450 family 11 subfamily B*) were assessed to determine whether sex reversal had occurred one-year post-treatment.

Following methods outlined in Vandesompele et al. (2002), geNorm software was used to measure the stability of 5 candidate reference genes: *btf3*, *eef1a*, *rpl4*, and *actb*, and *18s.* All genes had an acceptable M-value (i.e. $M < 1.5$), and *btf3*, *eef1a, and actb* showed the greatest stability. Therefore, the geometric mean of *btf3*, *eef1a, and actb* was used for qPCR data normalization.

Table 1

Primer sequences for real-time qPCR

Transcript	Sequence (5'-3')	Order	Product size (bp)
cyp19a1a	CATCTGGACTGGTATAGGCACA	Forward	136
	TCCATTCTTCAGTACATGGTGC	Reverse	
foxl2a	CCTACTCCTACGTCGCTCTCAT	Forward	220
	GTCCAGTAGTTCCCCTTCCTCT	Reverse	
dmrt1	CTGAGGTGATGGTGAAGAATGA	Forward	183
	AATAGGAAGTTTCCAGCAGCAG	Reverse	
amh	TACATATTGCTGACGGGACAAG	Forward	181
	TATCAGTTCCTCTTTCCCCAGA	Reverse	
vasa	TCCATTTTTGCCCACTACGAGA	Forward	138
	GTTTCTTTTCAGGGACTCGCAC	Reverse	
zpc	ATCTGGTACTGTTTGGTGCTGT	Forward	154
	GATGTCATGGGTAGCCAGGTAG	Reverse	
casp8	GAAACGACTTGCACTCTGACAC	Forward	196
	CTGTTCTTCATCCAAGCATGTC	Reverse	
casp3	ACAGGCATGAATCAACGAAACG	Forward	194
	TCTCCGTGACTCAACAGAACAC	Reverse	
casp9	TACCGATGACTTGGTCAGAATG	Forward	131
	CTTGGGTTTGAAAGTGGAAAG	Reverse	
p53	TGCCATTCGTTTAGATTCACAC	Forward	115
	CGATTGACAGCCTAAAGGAGAG	Reverse	

Statistical Analysis

Analyses were conducted in R software version 3.3.2 (SAS Institute, Cary, NC, USA). Data between treatment groups (i.e. control, moderate, and high) within each time point were compared using one-way analysis of variance (ANOVA) followed by Tukey post-hoc tests where appropriate. The minimum level of statistical significance was $p < 0.05$.

Results:

Treatment Period

Gonadal histology and oocyte measurements

Histological sections of monosex female gonads indicated that elevated temperatures impaired ovarian development after prolonged exposure (**Figure 2**). At 4 weeks, fish from the moderate and high treatment groups demonstrated accelerated ovarian development relative to control. Eighty percent of the fish from the moderate treatment group and 55% from the high treatment group displayed either signs of fusing or had fused distal ends of their ovaries (to form ovarian cavities) compared to only 11% of samples in the control. At 12 and 19 weeks, fish from the moderate and high treatment group exhibited ovaries that were discernibly inhibited in size and oocyte stage relative to control (**Figure 2**). The average cross-sectional area of ovaries at 19 weeks was 41% (296,490 ± 87,946 μm^2) smaller in the moderate treatment group and 72% (139,813 ± 1,171 μm^2) smaller in the high treatment group compared to control (501,940 ± 104,080 μm^2). Furthermore, the average PN oocyte count at 19 weeks was 82% (55 ± 4 PN oocytes) less in the moderate treatment group and 95% (13 ± 4 PN oocytes) less in the high treatment group compared to control (312 ± 65 PN oocytes).

Measurement of PN oocyte area in histological sections demonstrated that average PN oocyte area in the moderate and high treatment group was significantly greater than that of control at 4 weeks (**Figure 3A**), which was consistent with other signs of accelerated ovarian development at this time point. At 12 and 19 weeks, the average PN oocyte area in the control and moderate treatment group was not different while the average PN oocyte size in the high treatment group was significantly lower.
The percentage of ovary composed of PN oocytes in the moderate and high treatment group at 4 weeks was significantly greater than control (**Figure 3B**). However, by 12 weeks, the control group showed a markedly higher percentage than that of the moderate and high treatment groups. By 19 weeks, this pattern was further exaggerated with significant differences displayed between all treatment groups; the

control group having the highest percentage (~60% of the ovary composed of PN oocytes), moderate being intermediate (~20%) and high being the lowest (~5%).

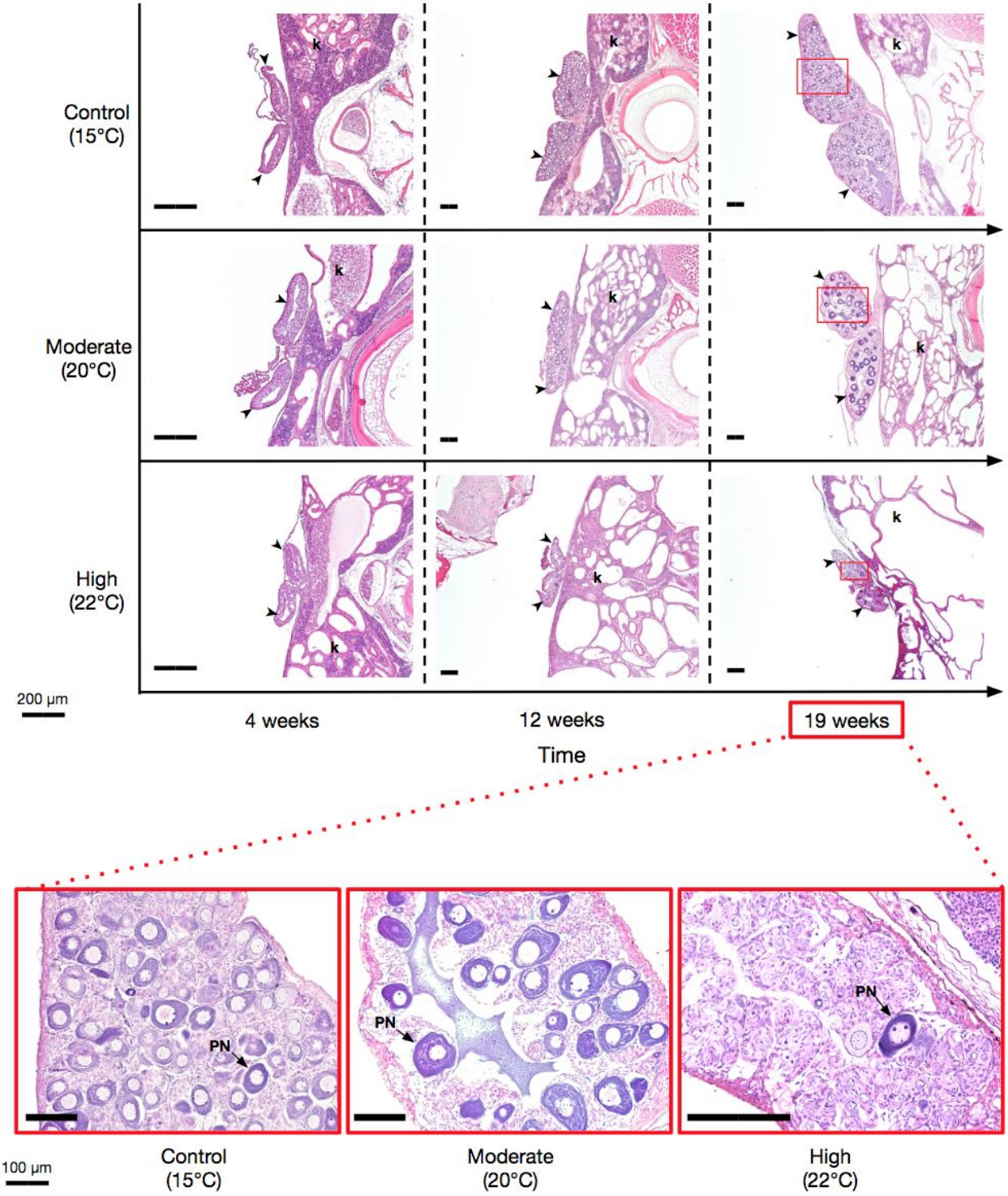


Figure 2. Representative histological sections of gonads of monosex female sablefish sampled at 4, 12 and 19 weeks during the high temperature study. All photomicrographs represent transverse sections of the gonads. Arrowheads (➤) denote the dual ovary lobes. Abbreviations: k, trunk kidney; PN, perinucleolar oocyte

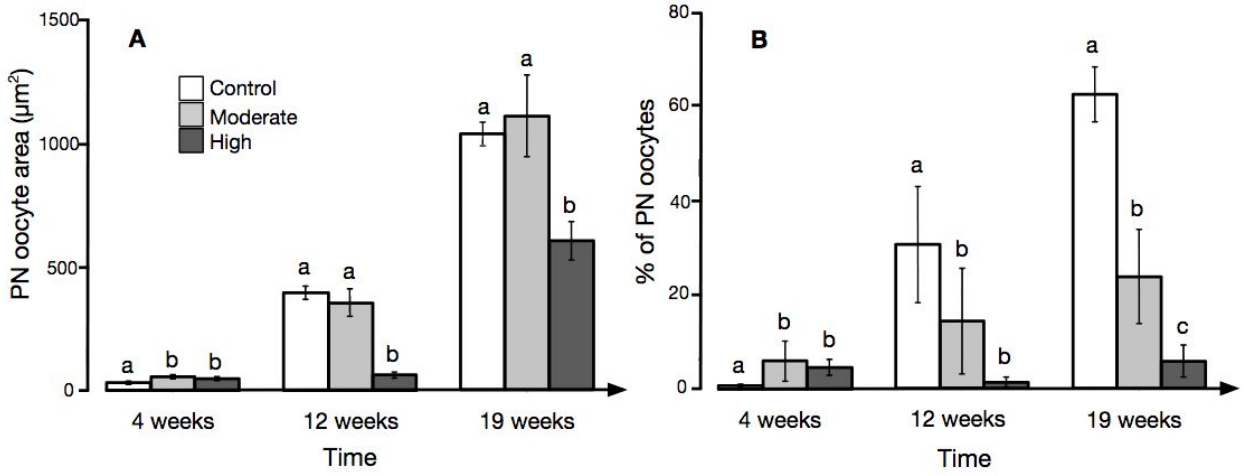


Figure 3. Quantitative analysis of ovarian development in monosex female sablefish sampled at 4, 12, and 19 weeks during the high temperature study. A) Average perinucleolar (PN) oocyte area (μm^2), B) Percentage of ovary composed of PN oocytes (%). Data represent mean ± SEM (n = 10 fish per group per time point). Shared lettering within a time point indicates no significant difference between treatment groups.

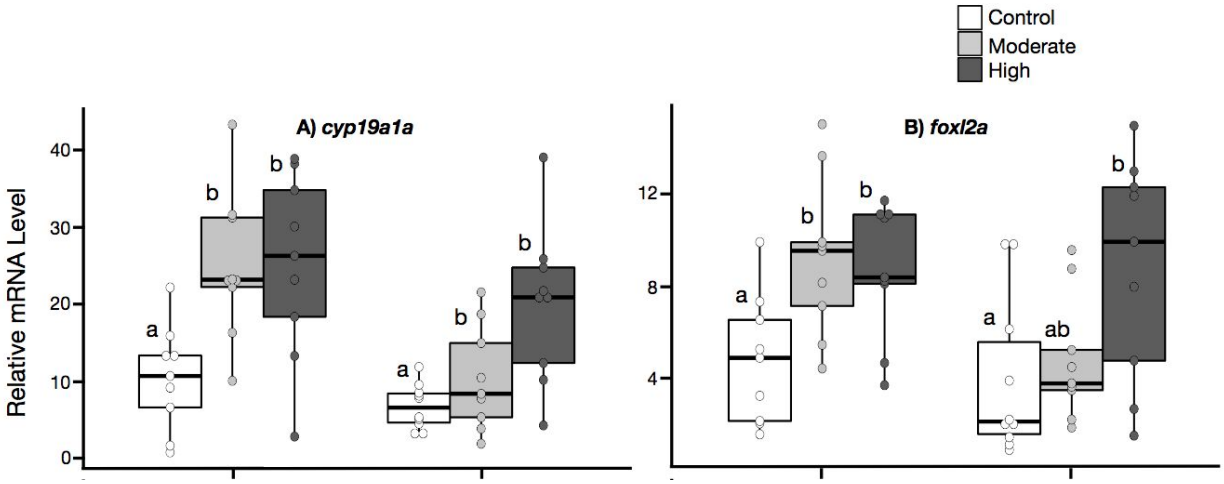
Somatic- and Germ Cell-Specific Gonadal Genes

Targeted somatic cell genes associated with ovarian development included *cyp19a1a* and *foxl2a*, and both exhibited similar patterns of expression across treatment groups at 12 and 19 weeks (**Figure 4A, B**). Levels of both *cyp19a1a* and *foxl2a* were significantly elevated in the moderate and high treatment group compared to control at 12 weeks. At 19 weeks, this pattern was maintained for *cyp19a1a*, while for *foxl2a*, the high treatment group showed significantly higher levels than control and the moderate treatment group had intermediate levels, not significantly different than control or the high treatment group (**Figure 4B**).

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 (**Figure 4C**). As for *amh*, transcript levels in the high treatment group were significantly higher than control at 12 and 19 weeks, while levels in the moderate treatment were intermediate and not significantly different than those of the control or high treatment group (**Figure 4D**).

Targeted germ cell genes include *zpc* and *vasa*. For *zpc*, transcript levels at 12 weeks were not significantly different between treatment groups, although the trend showed control was greater than moderate, and moderate was greater than high (**Figure 4E**). By 19 weeks this pattern was more exaggerated, with the control and moderate treatment group having *zpc* levels that were not significantly different and the high treatment group having notably lower levels than the control and moderate groups (**Figure 4E**). For *vasa*, transcript levels at 12 weeks in control were markedly elevated compared to the moderate and high treatment group (**Figure 4F**). Similarly, at 19 weeks transcript levels in control were significantly higher than the high treatment group, while transcript levels in the moderate treatment group were not different than control (**Figure 4F**).



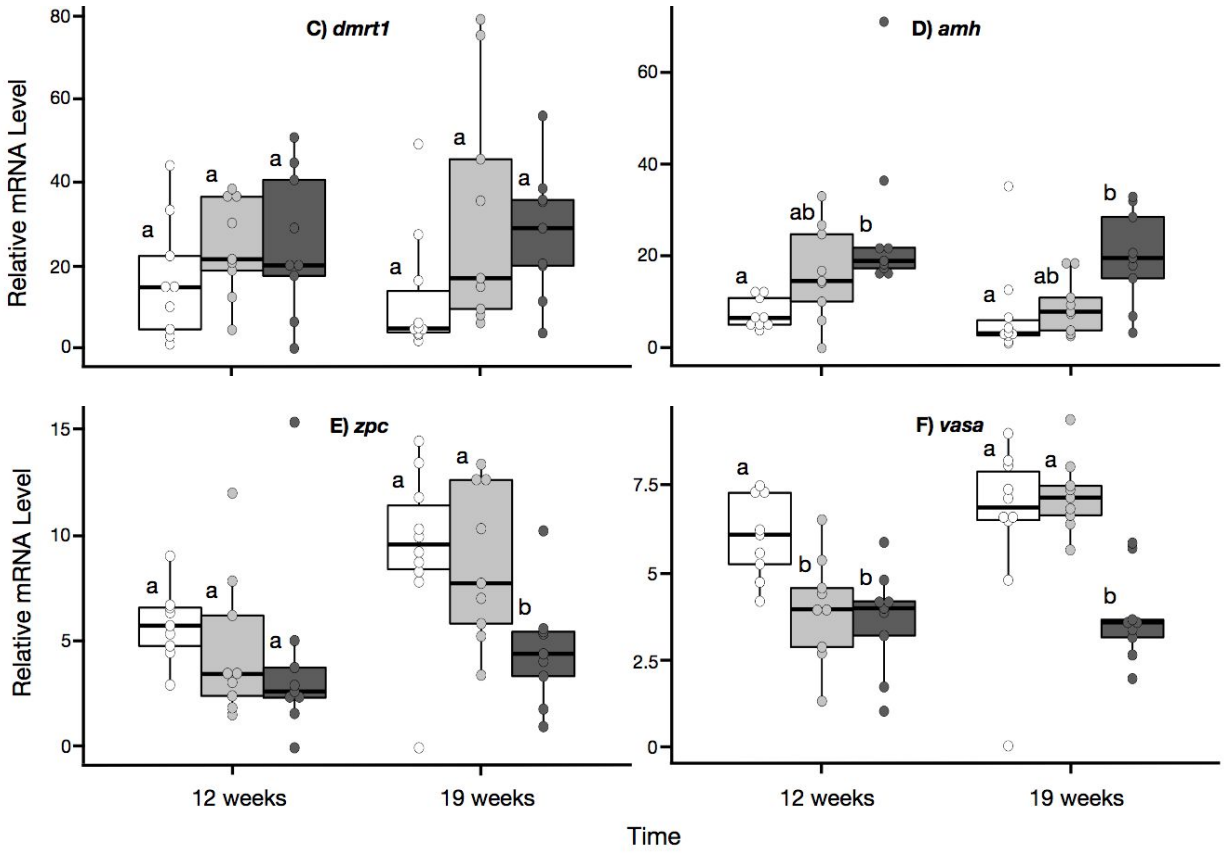


Figure 4. Relative mRNA levels for a suite of gonadal genes in monosex female sablefish sampled at 12 and 19 weeks during the high temperature study. A-B represent somatic cell genes associated with ovarian development, C-D represent somatic cell genes associated with testicular development, and E-F represent germ cell-specific genes. Box plots represent the median and interquartile range (IQR). Whiskers represent ± 1.5 * IQR (n ≥ 8 per group per time point). Shared lettering within a time point indicates no significant difference between treatment groups.

Apoptosis-Related 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 greater relative to control, and transcript levels in the high treatment group were comparable to both control and the moderate treatment group (**Figure 5A**). Transcript levels for *casp3* were not markedly different between treatment groups at 12 weeks, although the trend showed control was greater than moderate, and moderate was greater than high (**Figure 5B**). At 19 weeks, transcript levels in the

control and high treatment group significantly differed, with high levels in control and lower levels in high, while the moderate treatment group was comparable to both at 19 weeks (**Figure 5B**). For *casp9* (associated with the intrinsic apoptotic pathway), there were no notable differences between treatment groups at either time point (**Figure 5C**). Lastly, for *p53*, a tumor suppressor, transcript levels in the moderate treatment group were markedly higher relative to control, while the high treatment group had comparable levels to both at 12 weeks (**Figure 5D**). At 19 weeks, both the moderate and high treatment group had notably elevated levels compared to control (**Figure 5D**).

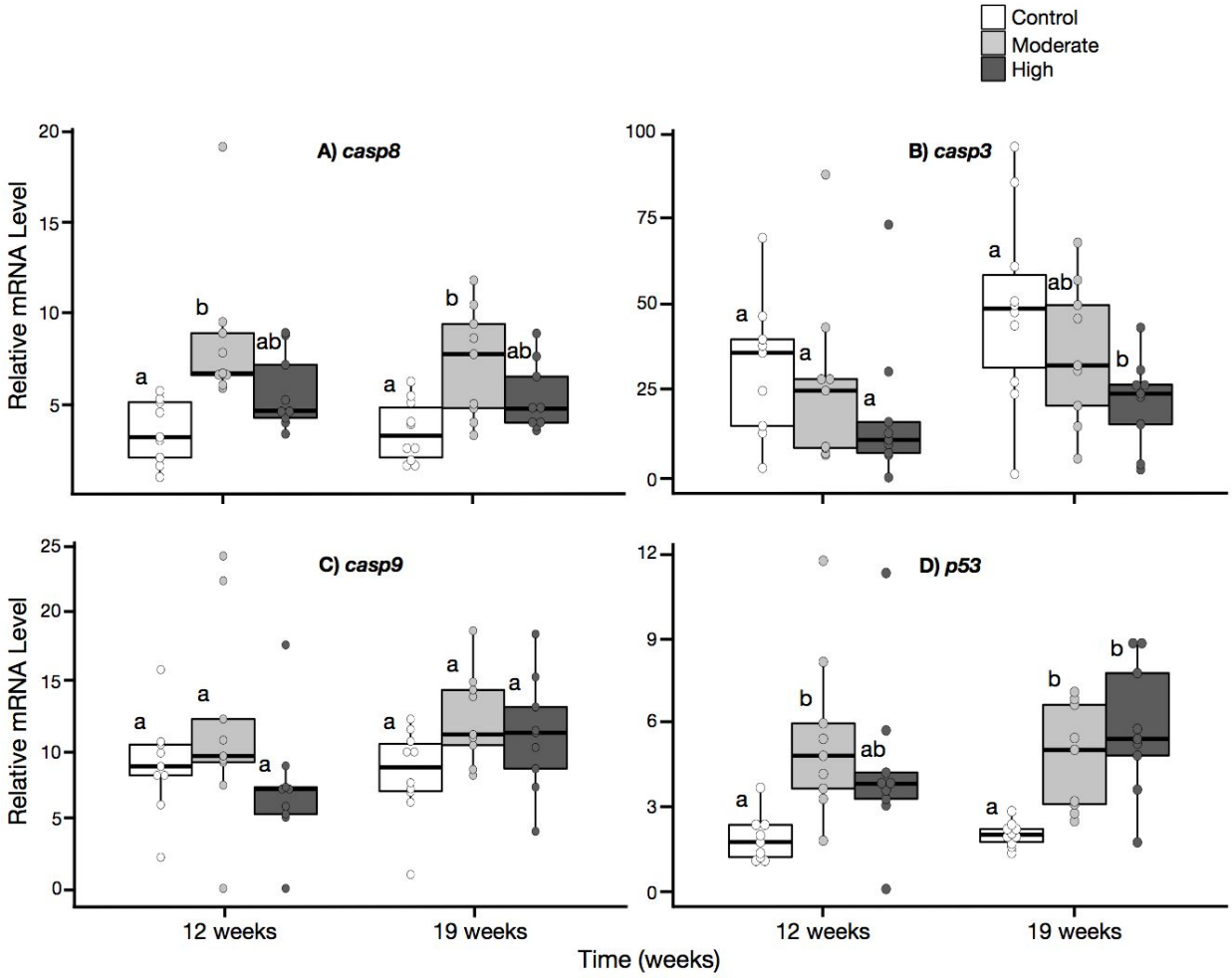


Figure 5. Relative mRNA levels for apoptosis-related genes in gonads of monosex female sablefish sampled at 12 and 19 weeks during the high temperature study. A-B represent

cysteine proteases in the extrinsic apoptotic pathway, C represents a cysteine protease in the intrinsic apoptotic pathway, and D represents a tumor suppressor gene. Box plots represent the median and interquartile range (IQR). Whiskers represent ± 1.5 * IQR (n ≥ 8 per group per time point). Shared lettering within a time point indicates no significant difference between treatment groups.

One Year Post-treatment

Gonadal histology and oocyte measurements

Histological sections 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 impaired during the treatment period, fully recovered by one year post-treatment (**Figure 6**). Consistent with histological observations, average PN oocyte size and the percentage of ovary composed of PN oocytes were not different across treatment groups (**Figure 7A and 7B**). Additionally, the control, moderate, and high treatment group had an average PN oocyte count of 77 ± 2, 78 ± 5, and 82 ± 5 PN oocytes, respectively.

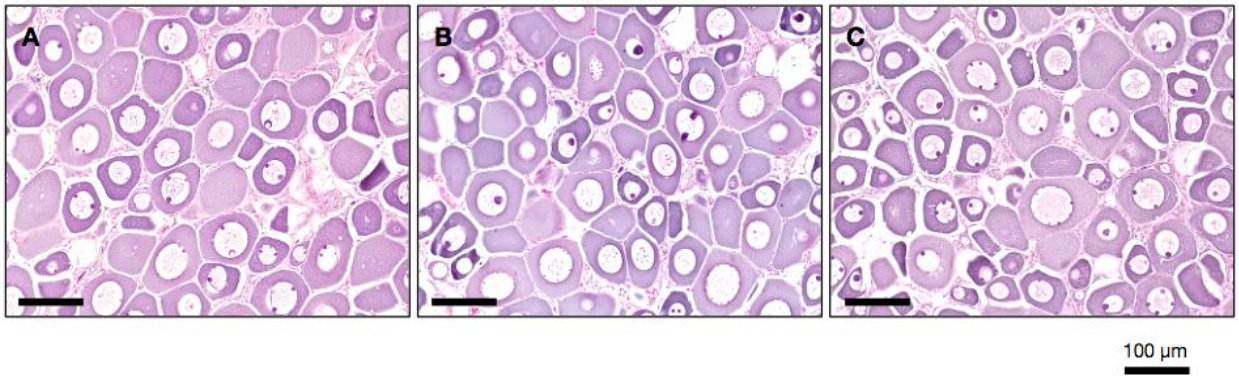


Figure 6. Representative histological sections of gonads of monosex female sablefish from the control (A), moderate (B), and high temperature group (C) sampled one year post-treatment following rearing in ambient temperature seawater.

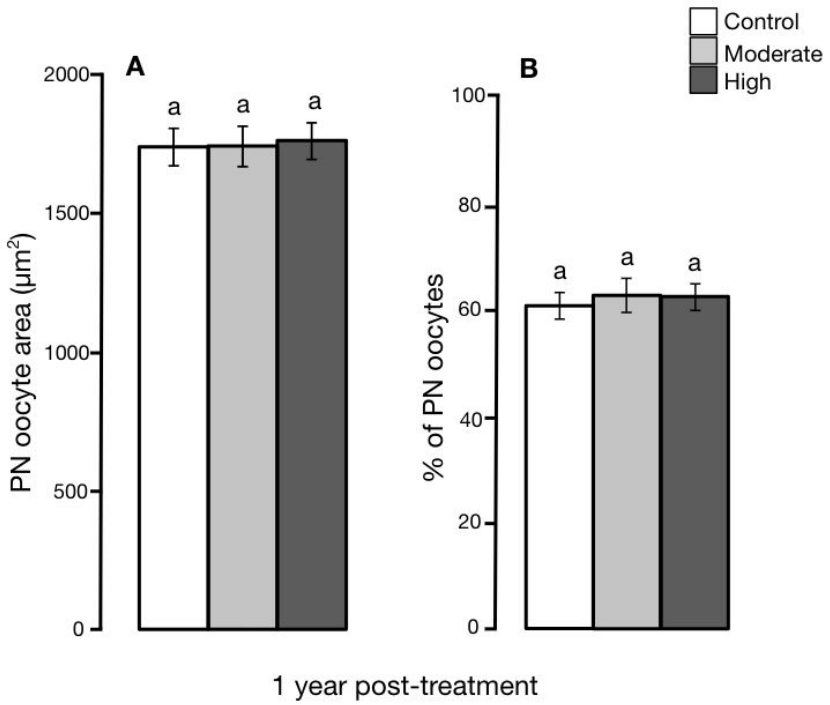


Figure 7. Quantitative analysis of ovarian sections of monosex female sablefish from the control, moderate, and high temperature group sampled one year post-treatment. A) Average perinucleolar (PN) oocyte area (μm^2), B) Percentage of ovary composed of PN oocytes (%). Data represent mean ± SEM (n = 10 fish per group per time point). Shared letterings within a time point indicate no significant differences between treatment groups.

Incidences of Sex Reversal

No incidences of sex reversal were observed in fish sampled from the control or moderate treatment group. However, 6% of the fish (n=2) sampled from high treatment appeared to be completely sex reversed (i.e. neomales) and possessed putative non-meiotic testes with no ovarian features observed (**Figure 8**). Designation of these fish as neomales was further verified by RT-PCR for established testicular and ovarian molecular markers. Gonads of high temperature-generated neomales had high expression of the testis marker, *cyp11b*, and undetectable expression of the ovarian marker, *cyp19a1a* (**Figure 9**). This corresponded well with results for MT-generated neomale and control male sablefish from previous work (Luckenbach et al., 2017). Control females displayed the opposite pattern, having non-detectable *cyp11b* expression and discernable expression of *cyp19a1a* (**Figure 9**). The germ cell marker

vasa was detected in the control female, control male, and both types of generated neomales, which indicated that no fish were reproductively sterile (**Figure 9**).

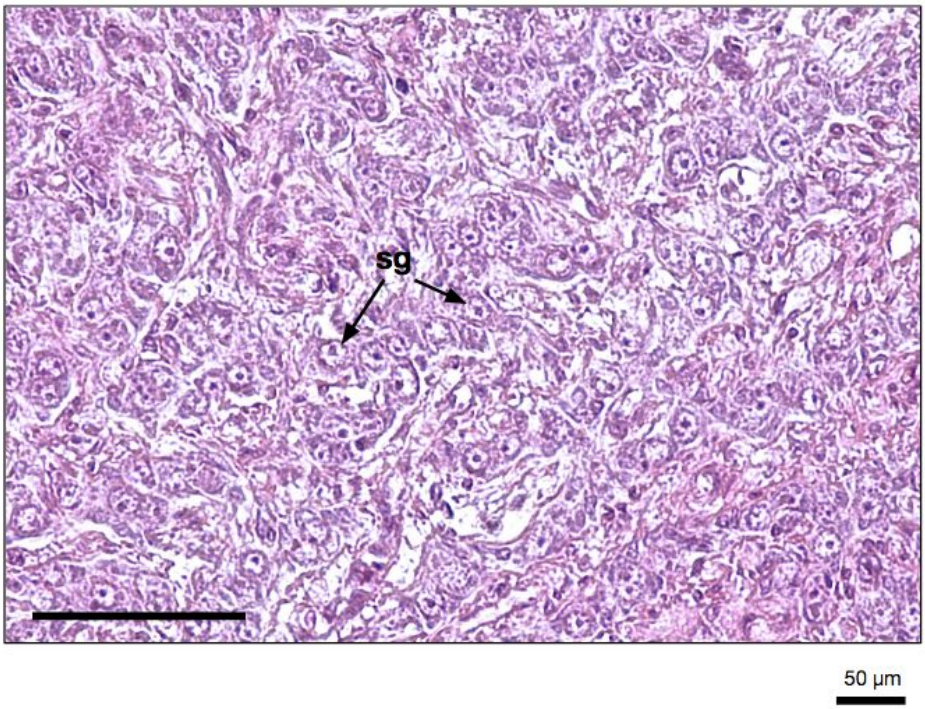


Figure 8: Representative histological section of a putative non-meiotic testis of a sex-reversed female (neomale) from the high temperature treatment group sampled one-year post-treatment. Abbreviations: sg, spermatogonia.

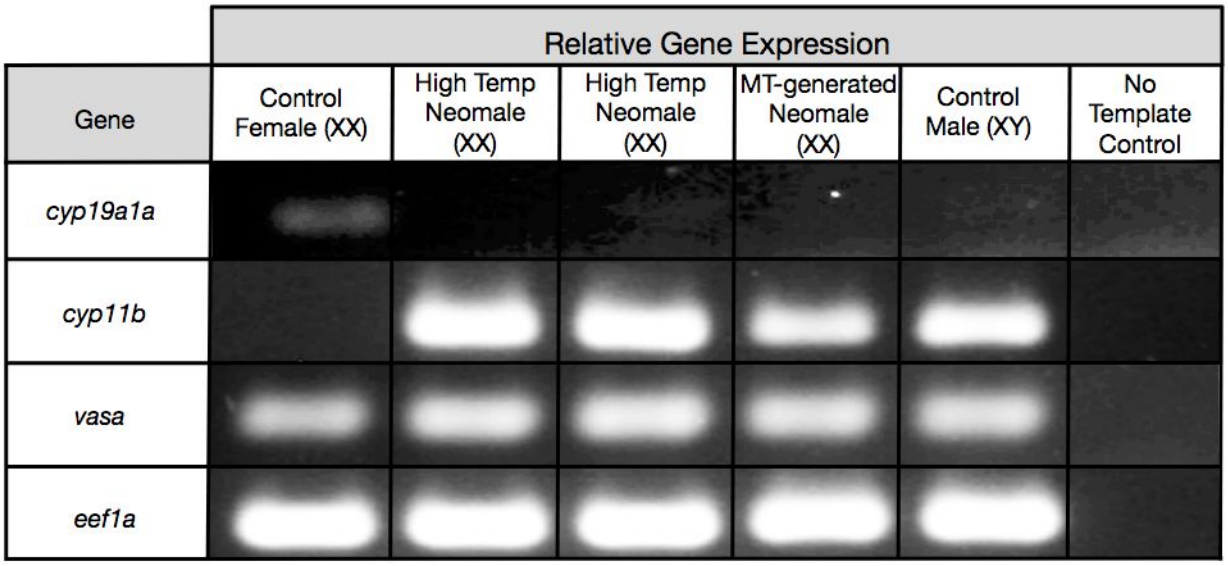


Figure 9: Representative RT-PCR results for molecular markers *cyp11b* and *cyp19a1a*, steroidogenesis-related male and female genes, respectively. High temperature- and MT-generated neomales from a previous study (Luckenbach et al., 2017) display detectable expressions of *cyp11b*, a testis marker, while a control female displays expression of *cyp19a1a*, an ovarian marker. Detectable expression is indicated by the presence of a fluorescent band. A loading control (LC) (i.e. *eef1a*) and no template control (NTC) were included. All PCRs were 32 cycles and visualized on a 1.5% agarose gel.

Discussion:

This study sought to determine effects of high temperature exposure on early reproductive development of juvenile female sablefish and its potential use for induction of sex reversal or sterility for aquaculture applications. Toward the beginning of our study, at week 4 of the 19-week treatment period, elevated temperatures (~20 °C and ~22 °C) initially stimulated ovarian development compared to control (~15 °C), based on advanced formation of the ovarian cavity and greater size and number of PN oocytes. At 12 weeks, however, both morphological and molecular characteristics (e.g. germ cell-specific genes and apoptosis-related genes) began to indicate that prolonged exposure to elevated temperatures was inhibitory to female reproductive development and caused germ cell degeneration. By the end of the treatment period (19 weeks), fish in the moderate and high treatment group had lower PN oocyte counts (82% and 95% lower, respectively) and smaller ovarian cavity area (41% and 72% smaller, respectively) compared to control. Particularly in the high treatment group, the average PN oocyte area, percentage of ovary composed of PN oocytes, and expression level of germ cell markers *vasa* and *zpc*, were significantly reduced compared to control. Altogether, there were clear morphological and molecular features indicating that prolonged exposure to elevated temperatures inhibited ovarian development and caused germ cell loss. Despite the significant impairment observed during the treatment period, however, these effects were not permanent. After all fish were transferred from treatment temperatures into ambient seawater for one year, germ cell proliferation resumed and fish from the moderate and high treatment group showed compensatory development of the gonads. Interestingly, although reproductively sterile fish were not observed, 6% of the genotypic-female sablefish from the high treatment group were sex-reversed by temperature and possessed putative non-meiotic testes. RT-PCR results confirmed that gonads of these neomales expressed *cyp11b*, a testis marker, and had no discernable levels of the ovarian marker *cyp19a1a*. Overall, in the following discussion, we will address in detail the morphological and molecular effects of

temperature on reproductive development of sablefish from this study and compare and contrast our results to those in other species.

It has been demonstrated that high temperature exposure near the time of morphological sex differentiation can induce masculinization and even sterilization in female teleosts (Guiguen et al., 2010). Differences between masculinization and sterilization can, in part, be ascribed to the selected temperature and duration of treatment. In Nile tilapia (*Oreochromis niloticus*) and Mozambique tilapia (*Oreochromis mossambicus*), sex reversal was generally achieved utilizing lower temperatures (~27 °C for these species) for a shorter duration, whereas sterilization required higher temperatures (~ 37 °C) for a longer duration (Pandit et al., 2015; Nakamura et al., 2015). In our study, since we had 2 individual from the high treatment group (6%) develop non-meiotic testes and no individuals that were completely devoid of germ cells. these results may suggest that duration can be prolonged in future optimization studies. Regarding temperature, we believe that utilizing anything higher than 22 °C could not have been possible due to high mortality rates observed in the high treatment group (43.7%) compared to control (30.2%). Moreover, findings from a previous study with sablefish established 24 °C as a negative threshold for growth and survival, and as little as two weeks of exposure proved lethal (73% mortality; Sogard and Olla, 2001). However, based on the individual cases of sex reversal in our study, it may still be plausible that an optimized temperature-treatment protocol (utilizing a longer duration) could induce higher rates of sex reversal or sterility in sablefish.

One interesting characteristic worth noting is that at 4 weeks, both the average area of PN oocytes and the percentage of PN oocytes were significantly greater in the moderate and high treatment group than control, and other histological features (e.g. higher percentage of ovary cavity formation) further illustrated that fish were in a more advanced stage of gonadal development relative to control. However, beyond 4 weeks, as FL and BW increased, prolonged exposure had a detrimental effect on gonadal

development, as seen at 12 and 19 weeks. In several studies with juvenile turbot (*Scophthalmus maximus*), Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*) and cobia (*Rachycentron canadum*), general findings suggested that optimal temperatures for growth and survival change over time, with younger, smaller fish preferring warmer temperatures and older, bigger fish cooler (Jonassen et al., 1999; Sun and Chen, 2014; Imsland et al., 2007; Imsland et al., 2005). Previous studies with juvenile sablefish also suggested that tolerance for elevated temperatures decreased over time as fish grew in size and age (Sogard and Olla, 2001; Lee et al., 2017). Pertaining to our study, elevated temperatures may have been optimal for juvenile sablefish at 4 weeks (~80 mm FL; ~5 g BW), as can be seen by advanced morphological characteristics in the ovaries, but thermal preferences likely shifted over time, and continued exposure through 12 and 19 weeks proved detrimental to ovarian development and survival. Overall, for the purposes of our study, this may serve as further reason to explore duration of treatment, as opposed to temperature, to achieve sterilization or masculinization for future studies.

Several apoptosis-related genes were assessed during the treatment period to better understand apoptotic mechanisms associated with germ cell degeneration observed at moderate and high temperatures. Generally, gonadal apoptosis is a common process that maintains ovarian integrity by eliminating non-viable or excess germ cells and their surrounding somatic cells. Rates of apoptosis may increase with exposure to environmental stressors such as high temperature. Among others, caspases are key factors in the apoptotic pathway that can be largely categorized as either initiator caspases, responsible for activating effector caspases, or effector caspases, responsible for cleaving cellular targets, resulting in cell death (Takle and Andersen, 2007; Johnson and Bridgham, 2002). Both caspase 8 and 9 are considered initiators, and the former is involved in death receptor-mediated apoptosis (i.e. extrinsic pathway) while the latter mitochondria-mediated apoptosis (i.e. intrinsic pathway) (Takle and Andersen, 2007). In our study, gonadal *casp8* and *casp9* mRNA levels were slightly

elevated at 19 weeks in the moderate and high treatment group but there were no significant patterns directly correlated to temperature. Caspase 8 is also known to initiate *casp3*, an effector involved in the extrinsic pathway (Takle and Andersen, 2007). Interestingly, *casp3* showed a downward-stepping pattern with gonads of control fish expressing the highest mRNA levels and those of the high treatment group expressing the least; differences were only significant in the high treatment group at 19 weeks. This is in direct contrast with anatomical features observed at 19 weeks. Caspase 3, however, is known to be heavily post-transcriptionally regulated (to avoid unwanted cell death), and this may, in part, explain discordances between mRNA levels of *casp8* and *casp3* and morphological characteristics (Ruest et al., 2003). Broadly, we may speculate that caspases are early indicators of apoptosis and thus are not associated in later stages of germ cell degeneration generally seen toward the end of our treatment period (Yamamoto et al., 2011). At the least, this is the first study to report caspase genes in sablefish, and to better determine their role in ovarian apoptosis, further analyses of other caspases and proteins in the apoptotic pathway are necessary.

In addition to caspase pathways, we investigated *p53*, a tumor suppressor responsible for suppressing unwanted cell growth, expressed at low levels under normal conditions (Fridman and Lowe, 2003). Under different forms of cellular stress, such as high temperature, the *p53* apoptotic pathway may be activated to suppress proliferation and development of damaged cells (Fridman and Lowe, 2003). For sablefish in this study, mRNA levels of *p53* were significantly higher in the moderate treatment group at 12 weeks and in both the moderate and high treatment group at 19 weeks. This indicated that *p53* was likely associated with germ cell degeneration observed at higher temperatures and involved in regulating gonadal apoptosis.

Regarding the observed female-to-male sex reversal in 6% of individuals from the high treatment group, aromatase/cyp19a1a plays a distinctly important and pivotal role in both female and male sex differentiation (Guiguen et al., 2010). In many fish species,

up-regulation of *cyp19a1a* is necessary for triggering and maintaining ovarian differentiation, while suppression or inhibition of *cyp19a1a* can lead toward masculinization (Guiguen et al., 2010). In addition to aromatase, *foxl2a*, a female predominant transcription factor, is a potent upstream regulator that can promote expression of *cyp19a1a* and further drive ovarian differentiation (Siegfried, 2010). In several studies involving fish, high temperature has been shown to suppress gonadal *cyp19a1a* expression, and thermal application particularly during the labile period can override sex determination/differentiation of genetic XX females, resulting in female-to-male sex reversal (Budd et al., 2015; Penman and Piferrer, 2008; D'Cotta et al., 2001; Kitano et al., 2000). Although the exact mechanisms linking temperature to sex reversal are still unclear, several studies documented upregulation of *dmrt1* (i.e. male transcription factor) and *amh* (i.e. transforming growth factor) during temperature treatment, both of which are known to inhibit aromatase expression and are key drivers in testicular development (Piferrer and Guiguen, 2008; Wang et al., 2010; Baroiller et al., 2009). Additionally, two recent studies with zebrafish (*Danio rerio*) and rice eel (*Monopterus albus*) also suggested that *p53*-mediated gonadal apoptosis may be involved in sex reversal as well (Rodríguez-Marí et al., 2010; He et al., 2010). In the present study, sablefish at 12 and 19 weeks displayed similar expression patterns of *cyp19a1a* and *foxl2a*, with higher levels generally observed in the moderate and high treatment group compared to control. Given documented suppressive effects of high temperature on *cyp19a1a* and *foxl2a*, these results were surprising since we expected to see lower levels of expression in both genes. Furthermore, *dmrt1* levels were not significantly higher at elevated temperatures. Although levels of *cyp19a1a*, *foxl2a*, and *dmrt1* mRNA were contrary to our expectations, at 19 weeks gonadal *amh* and *p53* levels in the high treatment group were significantly higher relative to control, and this may, in part, explain the observed sex-reversal one-year post-treatment in some individuals.

Other mechanisms potentially mediating temperature-induced sex reversal include epigenetic factors. In European sea bass, elevated temperatures were documented to increase DNA methylation of the aromatase promoter and suppress aromatase expression, causing masculinization (Navarro-Martín et al., 2011). Additionally, temperature-induced sex reversal was found to be mediated by high cortisol levels in medaka (*Oryzias latipes*) and rainbow trout (*Oncorhynchus mykiss*) (Hayashi et al., 2010; Van Den Hurk and Van Oordt, 1985). Future studies for sablefish should further investigate these mechanisms. To some degree, the low percentage of sex reversal, and even lack of sterile individuals, may suggest that sablefish are strict GSD species with highly reserved sex-determining mechanisms and low sexual plasticity. At least in this study, we were excited to document the first case of sex reversal due to rearing conditions alone, and these results may have positive implications for sex control during aquaculture production.

Neomale broodstock are a critical component to monosex female production of sablefish, which capitalizes on the superior growth of females compared to males (Luckenbach et al., 2017). In prospect, neomales generated from this study may be crossed with wild-caught females to generate all-female progeny, and this is favorably achieved through temperature, considered an eco-friendly, consumer-friendly, and chemical-free method. Optimization, however, is required. In addition to duration of treatment (mentioned previously) timing of treatment may be essential as well for sex reversal. Until recently, sex reversal treatments were typically applied prior to or during the window of morphological sex differentiation, as many believed phenotypic sex was irreversible in gonochoristic fish after molecular pathways for testicular or ovarian development have been established (Guiguen et al., 2010). Conversely, studies with tilapia, medaka, and carp show that functional sex reversal is possible after differentiation, ultimately suggesting that sexual plasticity may still be retained into adulthood (Paul-Prasanth et al., 2013; Ogawa et al., 2008; Bhandari et al., 2006). Thus,

pertaining to sablefish, future studies experimenting with not only duration of treatment but also timing may optimize the process of female-to-male sex reversal.

In conclusion, this study demonstrated that elevated temperatures had a degenerative effect on early reproductive development in juvenile female sablefish; morphological (e.g. PN oocyte size and number) and molecular (e.g. gonadal- and apoptosis- related genes) features at the time of temperature treatment suggested that masculinization or sterilization (via germ cell loss) may have been induced. One-year post-treatment, sablefish exhibited compensatory ovarian development, despite inhibition observed during exposure to high temperatures. Although this study did not yield reproductively sterile individuals, sex-reversal was observed in 6% of individuals in high temperature. Further investigation into underlying mechanisms regulating sex-reversal in sablefish should be conducted. Moreover, continued exploration of temperature as a potential method for masculinization or sterilization will be necessary for aquaculture production. Information obtained in this study will be valuable for understanding thermal effects on early reproductive development in teleost fish.

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