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

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23 **Abstract**

24 This study determined high temperature effects on ovarian development in a marine groundfish
25 species, sablefish (*Anoplopoma fimbria*), with potential application in sex reversal or sterilization
26 for aquaculture. Monosex female (XX-genotype) sablefish larvae (~30-mm) were randomly
27 divided into three groups and exposed to control (15.6°C ± 0.8°C), moderate (20.4°C ± 0.5°C),
28 or high (21.7°C ± 0.5°C) temperatures for 19 weeks. Treated fish were then tagged and
29 transferred to ambient seawater (11.2°C ± 2.3°C) for one year to determine whether temperature
30 effects on reproductive development were maintained post-treatment. Fish were periodically
31 sampled for gonadal histology, gene expression and plasma 17β-estradiol (E2) analyses to assess
32 gonadal development. Short-term (4-week) exposure to elevated temperatures had only minor
33 effects, whereas longer exposure (12-19 weeks) markedly inhibited ovarian development. Fish
34 from the moderate and high treatment groups had significantly less developed ovaries relative to
35 controls, and mRNA levels for germ cell (*vasa*, *zpc*) and apoptosis-associated genes (*p53*, *casp8*)
36 generally indicated gonadal degeneration. The high treatment group also had significantly
37 reduced plasma E2 levels and elevated gonadal *amh* gene expression. After one year at ambient
38 temperatures, however, ovaries of moderate and high treatment fish exhibited compensatory
39 recovery and were indistinguishable from controls. Two genotypic females possessing immature
40 testes (neomales) were observed in the high treatment group, indicating sex reversal had
41 occurred (6% rate). These results demonstrate that extreme elevated temperatures may inhibit
42 ovarian development or trigger sex reversal. High temperature treatment is likely not an effective
43 sterilization method but may be preferable for sablefish neomale broodstock production.
44

45 **Keywords**

46 Temperature; Sex differentiation; Sex reversal; Ovarian development; Germ cells; Sablefish

47 **1. Introduction**

48 Temperature is an environmental factor that can influence, redirect, or block early reproductive
49 development in some organisms. For example, sex in many reptiles is directly determined by
50 temperature (Temperature-dependent Sex Determination, TSD; Pieau, et al., 1999). In contrast,
51 mammals and birds display a pattern of strict genetic sex determination (GSD), where the sex of
52 an individual is fixed by the genes inherited at fertilization. Still, even among species with GSD,
53 sexual plasticity is often observed under application of high temperature (GSD with thermal
54 effects), particularly in teleost fishes, in which the process of gonadal sex differentiation may be
55 easily overridden by exogenous factors (Baroiller and D'Cotta, 2016; Devlin and Nagahama,
56 2002; Luckenbach and Yamamoto, 2018; Ospina-Álvarez et al., 2008).

57 At the molecular level, effects of temperature can be observed in a suite of genes commonly
58 associated with sexual differentiation. For instance, aromatase (Cyp19a1a) is principally
59 responsible for 17β-estradiol (E2) biosynthesis by the gonads and considered a key driver of
60 ovarian differentiation (Luckenbach et al., 2009; Piferrer and Guiguen, 2008; Shen and Wang,
61 2014). Elevated temperatures have been shown to suppress *cyp19a1a* expression in gonadal
62 somatic cells and in turn, E2 production, causing masculinization of genotypic female fish
63 (Karube et al., 2007; Kitano et al., 1999; van Nes and Andersen, 2006).

64 Elevated temperatures have also been shown to upregulate programmed cell death (apoptosis)-
65 associated genes such as caspases (i.e., cysteine proteases) and to promote germ cell
66 degeneration and follicular atresia, which may have masculinizing or sterilizing effects (Byerly
67 et al., 2005; Lee et al., 2009; Linares-Casenave et al., 2002; Pandit et al., 2015; Strüssmann et al.,
68 1998; Takle and Andersen, 2007; Uchida et al., 2004). Although the exact mechanisms linking
69 temperature to gonadal apoptosis are not clearly understood, suppression of *cyp19a1a* gene
70 expression and E2 production and activation of gonadal apoptotic pathways are signatures of this
71 process.

72 Temperature-induced sterility or sex reversal may be particularly beneficial in aquaculture
73 species. The utilization of reproductively sterile fish populations addresses many concerns of
74 escapement and genetic introgression associated with finfish aquaculture (Crozier, 1993; Skaala,
75 2006). Sex reversal is also widely used for commercial aquaculture purposes, typically to
76 capitalize on the superior performance (e.g., growth rate or morphology) of one sex relative to

77 the other (Baroiller and D'Cotta, 2016; Devlin and Nagahama, 2002). Sex reversal of
78 gonochoristic species which display sexually dimorphic growth may allow for the production of
79 monosex populations through specific broodstock crosses (e.g., sex reversed XX-genotype fish
80 [neomales] crossed with normal XX-genotype fish), ultimately maximizing efficiency and profit
81 for commercial aquaculture. The synthetic androgen 17α-methyltestosterone (MT) is widely used
82 to induce sex reversal in fishes for the generation of neomale broodstock (Devlin and Nagahama,
83 2002; Pandian and Sheela, 1995). However, exposure to elevated temperatures, which has been
84 shown to induce sex reversal in some fishes, may be a more eco-friendly and chemical-free
85 method to generate monosex or sterile fish (D'Cotta et al., 2001; Ospina-Álvarez et al., 2008;
86 Shen and Wang, 2014). Despite the abundance of temperature-related research, studies
87 documenting temperature effects on reproductive development of marine teleosts have not been
88 widely conducted.

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

105 The objective of this study was to determine effects of elevated water temperatures on early
106 gonadal development of genotypic female sablefish. We sought to gain greater insight into
107 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).

138 To acclimate the fish, the water temperature was increased by approximately 2°C each day until
139 target temperatures were reached. Targeted temperatures were based on previous studies with
140 larval and juvenile sablefish, which identified 15°C as an optimal temperature for survival and
141 growth and 24°C as lethal (Cook et al., 2018; Lee et al., 2017; Sogard and Olla, 2001).
142 Furthermore, sablefish larvae reared at temperatures up to 18°C did not show signs of sex
143 reversal or sterilization (Lee et al., 2017).

144 Fish were exposed to treatment temperatures for approximately 19 weeks (136 days, or nearly 5
145 months). During the treatment phase they were fed a commercial salmon diet (BioVita, Bio-
146 Oregon Inc., Longview, WA, USA) to apparent satiation by hand 3-4 times daily, except on
147 sampling days, when feed was withheld. Water in the tanks was continuously filtered (Cascade
148 1000, Penn-Plax Inc., Hauppauge, NY, USA) to remove suspended solids. Uneaten feed, feces
149 and other settleable solids were removed once daily by siphon. At 6-h intervals, 30-50% of the
150 water was drained from each treatment tank and replaced with preheated, filtered, and UV-
151 treated water from dedicated reservoirs by means of timer-controlled (Coleman Cable Inc.,
152 Waukegan, IL, USA) submersible pumps (TAAM Inc., Amazon, Seattle, WA, USA) equipped
153 with float switches (FloTec, Delavan, WI, USA). Water temperature was checked twice daily
154 with a hand-held digital thermometer (Bel-Art - SP Scienceware, Wayne, NJ, USA) and
155 continually recorded at 15 min intervals using Hobo Pendant temperature loggers (Onset
156 Computer Corporation, Bourne, MA, USA). During the course of the 19-week treatment period,
157 38.5% of the number stocked (325 fish per treatment) were lethally sampled. Mortality totaled
158 30.2%, 26.5% and 43.7% of the number stocked from the control, moderate and high
159 temperature groups, respectively.

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

168 *2.3. Sample Collection*

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

173 Fish were randomly sampled from each treatment group at week 4. However, because of the
174 known influence of body size on reproductive development and gonadal stage in this species
175 (Luckenbach and Fairgrieve, 2016), attempts were made to size-match fish for sample collection
176 and analysis at subsequent time points. At each time point (week 4, 12, and 19), 10 fish from
177 each group were sacrificed for paraffin histology and 10-15 fish for quantitative reverse
178 transcription-PCR (qRT-PCR). Overall, these sampling time points targeted body sizes between
179 60-180 mm FL, capturing critical developmental periods of molecular and morphological sex
180 differentiation that may reflect sex reversal or sterility (Hayman et al., in preparation;
181 Luckenbach and Fairgrieve, 2016; Smith et al., 2013). One year post-treatment, 35 fish from
182 each treatment group were sampled for gonadal histology and RT-PCR in order to assess
183 whether treatment effects on reproductive development were permanent. Morphological
184 deformities were recorded at one year post-treatment according to Lee et al. (2017).

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

195 *2.4. Gonadal Histology*

196 Methods for sablefish gonadal histology and staging followed Luckenbach and Fairgrieve
197 (2016). Briefly, gonads were fixed in Bouin's solution for at least 48 h, dehydrated in a series of

198 ethanol dilutions, cleared with xylene, embedded in paraffin, cross-sectioned at 5 µm thickness,
199 prepared onto slides, and stained with hematoxylin and eosin. Histological sections were first
200 examined for any signs of female-to-male sex reversal or sterility and notes recorded on gonadal
201 stages observed. Fish exhibiting fusion of the distal ends of the ovarian lobes to form ovarian
202 cavities were also noted. Sections were photographed with a Nikon digital camera (Melville, NY,
203 USA) and analyzed as follows using NIS Element image software version 4.0 (Nikon).

204 To compare ovary size across treatment groups, cross-sectional areas of the ovaries were
205 measured in triplicate photographed sections (anterior to posterior) for each fish and the average
206 calculated for each individual (n=10 fish/group; 30 total observations). To assess the degree of
207 ovarian differentiation/development, the proportion of fish with fused or unfused distal ends of
208 the ovarian lobes (to form ovarian cavities) was recorded. Cross-sectional areas of perinucleolus
209 stage oocytes (PN, the most advanced stage of oocytes observed) were measured across triplicate
210 sections for each fish (n=10 fish/group; 300 total observations) and individual means calculated.
211 Only centrally sectioned oocytes with the nucleus and surrounding nucleoli visible were
212 measured. Finally, PN oocyte density (i.e., percentage of the tissue composed of PN oocytes)
213 was determined for each fish by measuring the relative area of all the PN oocytes in triplicate
214 ovarian sections.

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

220 *2.5. Gene Expression Analysis*

221 *2.5.1. Targeted genes*

222 Ovarian and testicular-associated gene markers were selected to assess phenotypic sex and
223 gonadal status. Complementary DNA sequences for targeted genes were obtained from a 454-
224 pyrosequencing project focusing on differentiating ovaries and testes of sablefish (Hayman et al.,
225 in preparation) and some previously reported assays (Smith et al., 2013). Gonadal somatic cell
226 genes associated with ovarian development included *foxl2a (forkhead box L2a) and cyp19a1a*
227 *(cytochrome P450 family 19 subfamily A polypeptide 1a)*, somatic cell genes associated with

228 testicular development included *dmrt1 (doublesex and mab-3 related transcription factor 1)* and
229 *amh (anti-Mullerian hormone)*, and germ cell-specific genes included *vasa* (*DEAD (Asp-Glu-*
230 *Ala-Asp) box polypeptide 4*) and *zpc (zona pellucida protein c)*. Apoptosis-associated genes were
231 also targeted because signs of ovarian degeneration were observed in the moderate and high
232 treatment groups. These included *casp8 (caspase 8, apoptosis-related cysteine peptidase)*, *casp3*
233 *(caspase 3, apoptosis-related cysteine protease), casp9 (caspase 9, apoptosis-related cysteine*
234 *peptidase),* and *p53*, a tumor suppressor gene (Table 1).

235 *2.5.2. RNA isolation and reverse transcription*

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

247 *2.5.3. Quantitative PCR*

248 Quantitative PCR was used to determine steady-state mRNA levels for targeted genes in gonad
249 samples collected at weeks 12 and 19 using methods described in Luckenbach et al. (2011). PCR
250 primers were designed using Primer3 in MacVector software (Accelrys, San Diego, CA, USA)
251 and redesigned if an assay displayed multiple products in melt curve analyses. Quantitative PCRs
252 were conducted in 384-well plates using 2X Power SYBR Green Master Mix (Life
253 Technologies; final conc.), 150 nM of each primer, and 0.5 ng of cDNA, resulting in 12.5 µl total
254 volume per well. Standard curves were generated from pooled ovary samples serially diluted to
255 5, 1, 0.25 and 0.05 ng cDNA and run in triplicate. Assays were run on a 7900HT Fast Real-Time
256 PCR System (Life Technologies) with standard cycling conditions. Dissociation curves were
257 included in each run to confirm that only one product amplified in the reactions. In addition to

258 NACs, no template controls (NTCs), which contained no cDNA template, were included in each
259 assay. Quantitative PCR products from each assay were directly sequenced by MCLAB (South
260 San Francisco, CA, USA) to further confirm that targeted cDNAs were amplified. This included
261 confirmation that the *cyp19a1a* assay amplified *cyp19a1a* and not the paralog, *cyp19a1b*, which
262 is primarily expressed in the sablefish pituitary and brain (Guzmán et al., 2018).

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

268 *2.5.4. RT-PCR and gel electrophoresis*

269 RT-PCR was used to assess gene expression patterns in gonads of selected fish one year post-
270 treatment using methods described by Smith et al. (2013). Complementary DNA samples were
271 diluted to 0.5 ng/µl prior to PCR using the following conditions: 1 cycle for 3 min at 94°C, 32
272 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
273 electrophoresed on 1.5% agarose gels with 0.1% volume of 1X GelRed DNA stain (Biotium,
274 Hayward, CA, USA) and photographed with camera settings held constant. An NTC was also
275 included in each assay.

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

282 *2.6. Plasma 17β-estradiol analysis*

283 Plasma was obtained by centrifugation of whole blood (3000 x g, 15 min, 4˚C) and stored at
284 –20˚C for E2 analysis. Plasma E2 levels were quantified by enzyme-linked immunosorbent
285 assay (ELISA) using a protocol previously validated for sablefish (Guzmán et al., 2015).

286 Sensitivity, calculated from maximum binding minus two standard deviations was 12.6 pg/ml;
287 intra- and inter-assay coefficients of variation were 1.2% and 8.6%.

288 *2.7. Statistical analyses*

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

299 **3. Results**

300 *3.1. Treatment period*

301 *3.1.1. Gonadal morphology and metrics*

302 Fish analyzed from the control, moderate and high treatment group at 4 weeks averaged 79.3 ±
303 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
304 g BW), respectively, and were significantly different in FL and in some cases BW as well; fish
305 from the control and high group were not significantly different, but both were different than fish
306 from the moderate group. Fish selected for analysis at 12 weeks in the control, moderate, and
307 high treatment group averaged 126.4 ± 6.7 mm FL (17.0 ± 3.2 g BW), 127.2 ± 14.8 mm (18.4 ±
308 8.0 g BW), and 122.8 ± 17.5 mm (16.8 ± 7.0 g BW), respectively, and were not significantly
309 different in FL or BW. Fish selected for analysis at 19 weeks in the control, moderate, and high
310 treatment group averaged 171.6 ± 11.9 mm FL (39.1 ± 7.6 g BW), 157.6 ± 14.7 mm (39.7 ± 8.1
311 g BW), and 163.0 ± 12.3 mm (33.34 ± 8.98 g BW), respectively, and were not significantly
312 different in BW among groups, however control fish FL was significantly higher than that of the
313 moderate fish.

314 Histological sections of monosex female gonads sampled during the treatment period showed

315 neither discernible signs of testicular development that would indicate sex reversal nor complete
316 absence of germ cells that would indicate sterility. Instead we found that exposure to moderately
317 elevated temperatures at first slightly accelerated some aspects of ovarian differentiation and
318 development relative to controls (Fig. 1). At 4 weeks, 70% of fish from the moderate treatment
319 group displayed fusion of the distal ends of the ovary lobes to form ovarian cavities compared to
320 only 22% and 10% of fish from the control and high treatment group, respectively. Fish from the
321 moderate treatment group also had significantly larger ovaries (based on cross-sectional area),
322 average PN oocyte area, and PN oocyte density compared to controls, while fish from the high
323 treatment group generally had levels similar to the control group (Fig. 2A-C).

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

331 By 19 weeks, 100% of the fish from each treatment group exhibited fused ovarian cavities.
332 However, differences in ovary size among treatment groups were striking (Fig. 1), with
333 significantly smaller ovaries observed in the moderate and high groups compared to control (Fig.
334 2A). Average PN oocyte size was significantly different between the control and high treatment
335 groups, while the moderate group showed high variance and was not different from the other
336 groups (Fig. 2B). In agreement with data for ovary size, PN oocyte density was significantly
337 lower in the moderate and high treatment groups compared to the control (Fig. 2C). Over the
338 entire treatment period, the density/number of PN oocytes showed little increase in fish from the
339 high treatment group relative to fish from the moderate and control groups (Fig. 1, 2C).

340 *3.1.2. Somatic and germ cell-specific gonadal genes*

341 Targeted somatic cell genes associated with ovarian development included *cyp19a1a* and *foxl2a*,
342 which exhibited similar patterns of expression across treatment groups at 12 and 19 weeks (Fig.
343 3). Levels of both *cyp19a1a* and *foxl2a* were significantly elevated in the moderate and high
344 treatment group compared to the control group at 12 weeks. At 19 weeks, this pattern was

345 maintained for *cyp19a1a* and *foxl2a* in the high treatment group, whereas the moderate treatment
346 group had intermediate levels not significantly different from those of the control or high
347 treatment group (Fig. 3).

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

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

363 *3.1.3. Apoptosis-associated genes*

364 Caspase 8 and 3 represent cysteine proteases associated with the extrinsic apoptotic pathway. At
365 12 and 19 weeks, transcript levels for *casp8* in the moderate treatment group were significantly
366 higher than control, while levels in the high treatment group were higher than control at 12
367 weeks and comparable to both the control and moderate treatment group at 19 weeks (Fig. 4).
368 Transcript levels for *casp3* exhibited a declining pattern with exposure to elevated temperatures
369 at both 12 and 19 weeks (Fig. 4); levels in the high treatment group were significantly lower than
370 the control group, while the moderate group was intermediate. For *casp9*, no significant
371 differences were observed between treatment groups at either time point, though an increasing
372 trend with temperature was noted at 19 weeks (Fig. 4). Lastly, transcript levels of the tumor
373 suppressor *p53* were significantly higher in the moderate and high treatment groups relative to
374 control at both 12 and 19 weeks (Fig. 4).

375 *3.1.4. Plasma 17β-estradiol levels*

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

381 *3.2. One year post-treatment*

382 *3.2.1. Gonadal morphology and metrics*

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

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

398 *3.2.2. Plasma 17β-estradiol*

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

402 *3.2.3. Instances of sex reversal*

403 Phenotypic sex reversal was not observed in any fish sampled from the control or moderate
404 treatment group. However, 6% of the fish (2/35) sampled from the high treatment group
405 appeared to be sex reversed and possessed putative non-meiotic testes with no ovarian features
406 (Fig. 7A). Gonads of the two high temperature-generated neomales had undetectable expression
407 of the ovarian marker *cyp19a1a* and elevated expression of the testis marker *cyp11b* (Fig. 7B).
408 This corresponded well with results for MT-generated neomale and control male sablefish from a
409 previous study (Luckenbach et al., 2017) analyzed in parallel with samples from this study (Fig.
410 7B). Control females on the other hand exhibited the opposite pattern, having discernable
411 expression of *cyp19a1a* and no detection of *cyp11b* (Fig. 7B). The germ cell marker *vasa* was
412 detected in gonads of all individuals, indicating that the fish possessed germ cells and thus were
413 not reproductively sterile. Designation of the high temperature-treated fish as neomales was
414 further verified by confirming that they were indeed genotypic females using the *gsdf* genetic sex
415 marker (Fig. 7C).

416 **4. Discussion**

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

432 Despite the significant developmental impairment observed during the treatment period, these
433 effects were not permanent. After all fish were transferred to ambient seawater for one year,
434 oocyte development resumed and fish from the moderate and high treatment groups, with some
435 exceptions, developed ovaries indistinguishable from those of controls. Treated females therefore
436 demonstrated full compensatory recovery of ovarian development one year post-treatment.
437 Although reproductively sterile (germ-cell free) fish were not observed, 6% of the fish from the
438 high treatment group were sex reversed by temperature and possessed testes that expressed the
439 testis marker *cyp11b* and had no discernable expression of the ovarian marker *cyp19a1a*. These
440 represent the first documented instances of temperature-induced masculinization in this species.

441 High temperature exposure near the time of gonadal sex differentiation has been shown to induce
442 masculinization or reproductive sterilization in several fish species (Guiguen et al., 2010), and
443 the switch between masculinization and sterilization may be attributable to the interactive effects
444 of selected temperature and duration of exposure. In tilapias (genus *Oreochromis*), female-to-
445 male sex reversal has been documented at temperatures >32°C with relatively shorter durations
446 of exposure (Baroiller et al., 2009a), whereas irreversible sterilization was recently documented
447 in Nile and Mozambique tilapia (*O. niloticus* and *O. mossambicus*) exposed to ~37°C for longer
448 durations (Nakamura et al., 2015; Pandit et al., 2015). Because we observed a 6% rate of sex
449 reversal of sablefish in the high treatment group alone and no instances of sterility, a prolonged
450 treatment duration would be worth testing in the future to potentially achieve higher rates of sex
451 reversal or sterilization. This is supported by other work in our lab which indicated that dietary
452 MT treatment needed to extend well beyond the period of morphological sex differentiation (70-
453 150 mm FL) to induce masculinization of XX-genotype sablefish and that further
454 masculinization may occur post-treatment (Luckenbach et al., 2017; Luckenbach and Fairgrieve,
455 2016). Importantly, since the high temperature treatment approached the lethal level for juvenile
456 sablefish (24ºC; Sogard and Olla, 2001), it is unlikely that higher temperatures could be tested
457 without detrimental effects to the fish.

458 During the process of sexual differentiation, female- and male-determining factors expressed in
459 the gonads have antagonistic roles, with their interplay ultimately tipping the balance toward one
460 sex versus the other (e.g., Kim et al., 2006; Li et al., 2013; Siegfried, 2010). Two of the most
461 important genes to E2 biosynthesis and hence ovarian differentiation and maintenance in fishes
462 are the steroidogenic enzyme *cyp19a1a* and the female predominant transcription factor *foxl2a*

463 (Baroiller et al., 2009b; Guiguen et al., 2010; Shen and Wang, 2014; Siegfried, 2010). Foxl2a
464 regulates the expression of *cyp19a1a* and upregulation of *cyp19a1a* is necessary to trigger and
465 maintain ovarian differentiation, while suppression of *cyp19a1a* can lead to masculinization. In
466 several studies, high temperature has been shown to suppress gonadal *foxl2a* and *cyp19a1a*
467 expression, and thermal application particularly during the sexually labile period can override
468 sex differentiation of genotypic females, resulting in female-to-male sex reversal (Baroiller et al.,
469 2009b; D'Cotta et al., 2001; Kitano et al., 1999; Penman and Piferrer, 2008; Shen et al., 2018).
470 Previous work in sablefish has demonstrated that *foxl2a* and *cyp19a1a* are both highly expressed
471 during ovarian differentiation and low during testicular differentiation (Smith et al., 2013).
472 Interestingly, in the present study, levels of these mRNAs increased in response to exposure to
473 elevated temperatures. Upregulation of these genes was not anticipated based on studies in other
474 species and may indicate resilience of the endogenous female program in sablefish. This is also
475 supported by the relatively low rate of sex reversal observed.

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

483 For sex reversal to occur, it may have been essential that E2 levels were reduced at 12 weeks
484 (and possibly earlier in development). Lower levels of E2 have been shown to upregulate
485 gonadal expression of the transforming growth factor gene *amh* and thus drive sex reversal in
486 genotypic female fish (Fernandino et al., 2008). A number of studies have also documented
487 upregulation of *amh* and the male-predominant transcription factor *dmrt1* during high
488 temperature treatment (Baroiller et al., 2009b; Piferrer and Guiguen, 2008; Poonlaphdecha et al.,
489 2013; Wang et al., 2010). We found that sablefish *dmrt1* mRNA levels were not significantly
490 different among groups at any time point, but consistently trended higher at more elevated
491 temperatures. Gonadal levels of *amh* on the other hand were significantly upregulated at elevated
492 temperatures relative to controls. These results together suggest that male-determining factors
493 were generally activated in gonads of the monosex female sablefish exposed to elevated

494 temperatures and likely played a role in 'tipping the balance' toward testicular differentiation in
495 some individuals.

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

515 In addition to caspases, we investigated *p53*, a tumor suppressor responsible for preventing
516 unwanted cell growth, typically expressed at low levels under normal conditions (Fridman and
517 Lowe, 2003). Under different types of cellular stress, the *p53* apoptotic pathway may be
518 activated to suppress proliferation and development of damaged cells (Fridman and Lowe, 2003).
519 We found that the expression pattern for *p53* resembled *casp8* with significantly higher levels in
520 the moderate and high treatment groups at both 12 and 19 weeks, suggesting these genes together
521 could have played a role in the observed ovarian degeneration and/or sex reversal. Previous
522 studies in zebrafish (*Danio rerio*) and rice eel (*Monopterus albus*) have suggested that *p53*-
523 mediated gonadal apoptosis may be involved in the process of sex reversal (He et al., 2010;
524 Rodríguez-Marí et al., 2010). Furthermore, in coho salmon (*Oncorhynchus kisutch*), *casp8*,

525 *casp9*, and *p53* were some of the strongest markers of fasting-induced ovarian atresia
526 (Yamamoto et al., 2011).

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

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

544 In conclusion, continuous exposure to extreme elevated temperatures markedly inhibited early
545 ovarian development in sablefish, reducing ovarian growth, PN oocyte density and size, plasma
546 E2 levels, and germ-cell marker gene expression. Meanwhile, gonadal expression of key female-
547 and male-determining factors and some cell death-associated genes were stimulated. Following
548 transfer to cooler, ambient temperatures, sablefish exhibited striking compensatory recovery of
549 ovarian development. The exception to this was sex reversal of 6% of the individuals exposed to
550 the highest temperature. We posit that the reduction in E2 production and upregulation of
551 expression of *amh* and apoptotic factors led to the observed instances of sex reversal. To some
552 degree, the low percentage of sex reversal and lack of sterile individuals lends further support to
553 prior research suggesting sablefish are a strongly GSD species with relatively low sexual
554 plasticity. Sex-reversed sablefish have only previously been produced by dietary exposure to sex

555 steroids, therefore this is the first demonstration of sex reversal by rearing conditions alone.
556 These results may have positive implications for neomale broodstock production for aquaculture.

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755

756 **Figure 1**

757 Comparative ovarian development in monosex female sablefish after 4, 12, and 19 weeks of
758 continuous exposure to control (15.6°C ± 0.8°C), moderate (20.4°C ± 0.5°C), or high (21.7°C ±
759 0.5°C) water temperatures. All photomicrographs represent transverse histological sections of the
760 gonads. Arrowheads ( ) denote the dual ovary lobes. Perinucleolar oocytes, PN. Scale bars =
761 100 µm.

762

763 **Figure 2**

764 Quantitative analysis of ovarian development in monosex female sablefish after 4, 12, and 19
765 weeks of continuous exposure to control (15.6°C ± 0.8°C), moderate (20.4°C ± 0.5°C), or high
766 (21.7°C ± 0.5°C) water temperatures. (A) Average cross-sectional area of the ovaries, (B)
767 Average perinucleolar (PN) oocyte area, and (C) PN oocyte density. Data shown represent
768 means ± SEM of n = 10 fish per treated group at each time point. Shared lettering within a time
769 point indicates no significant difference ($p>0.05$) between treatment groups.

770

771 **Figure 3**

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

779

780 **Figure 4**

781 Relative mRNA levels for apoptosis-associated genes in gonads of monosex female sablefish
782 after 12 and 19 weeks of continuous exposure as weaned post larvae to control (15.6°C ± 0.8°C),
783 moderate (20.4°C ± 0.5°C), or high (21.7°C ± 0.5°C) water temperatures. Genes represent
784 cysteine proteases in the extrinsic and intrinsic apoptotic pathway, along with a tumor

785 suppressor. Box plots represent the median and interquartile range (IQR); whiskers extend to the
786 minimum and maximum observed values with n ≥ 8 per treated group at each time point. Shared
787 lettering within a time point indicates no significant difference (p>0.05) between treatment
788 groups.
789

790 **Figure 5**

791 Plasma 17β-estradiol (E2) levels of monosex female sablefish sampled after 12 and 19 weeks of
792 continuous exposure to control (15.6°C ± 0.8°C), moderate (20.4°C ± 0.5°C), or high (21.7°C ±
793 0.5°C) water temperatures. Box plots represent the median and interquartile range (IQR);
794 whiskers extend to the minimum and maximum observed values with n ≥ 8 per treated group at
795 each time point. Shared lettering within a time point indicates no significant difference (p>0.05)
796 between treatment groups.
797

798 **Figure 6**

799 Comparative ovarian development in yearling sablefish continuously exposed to control (15.6°C
800 ± 0.8°C), moderate (20.4°C ± 0.5°C), or high (21.7°C ± 0.5°C) water temperatures for 19 weeks
801 followed by one year at ambient temperatures (11.2°C ± 2.3°C). (A) Representative
802 photomicrographs of ovarian histological sections (Scale bars = 100 µm), (B) Average
803 perinucleolar (PN) oocyte area, (C) PN oocytes density, and (D) plasma 17β-estradiol (E2)
804 levels. Data shown represent means ± SEM of n = 10 per treatment. Shared lettering indicates no
805 significant difference (p>0.05) between treatment groups.
806

807 **Figure 7**

808 Gonadal analysis of sex-reversed, genotypic-female (XX) sablefish continuously exposed to high
809 (21.7°C ± 0.5°C) water temperatures for 19 weeks followed by one year at ambient temperatures
810 (11.2°C ± 2.3°C). (A) Representative histological section of a putative non-meiotic testis
811 composed of type-A spermatogonia (SG, Scale bar = 25 mm); (B) RT-PCR results for ovarian
812 (*cyp19a1a*), testicular (*cyp11b*) and germ cell (*vasa*) markers assessed in a control female (XX
813 genotype) and two high temperature-induced neomales (XX) from the present study, as well as a
814 methyltestosterone (MT)-induced neomale (XX) and control male (XY) from Luckenbach et al.

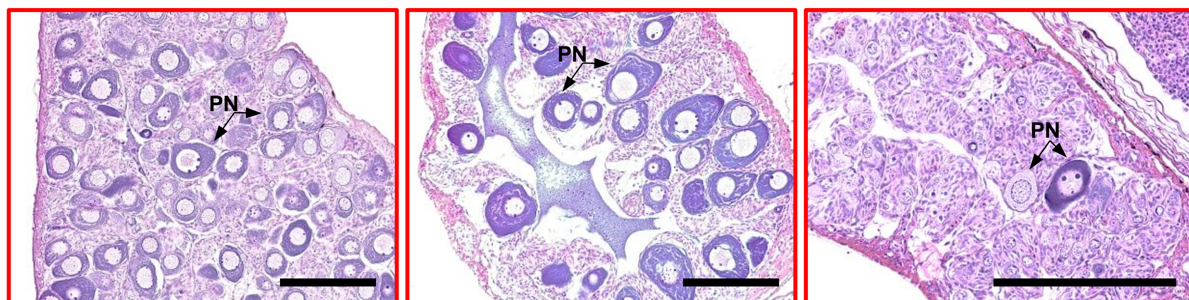
815 (2017); (C) PCR results for the sablefish genetic sex marker, *gsdf*, using genomic DNA isolated
816 from the same individuals. A positive control RT-PCR targeting *eef1a* and no template controls
817 (NTC) were included where appropriate. All PCRs were 32 cycles and electrophoresed on 1.5%
818 agarose gels. Ovaries, Ov; Testes, T.

Control

Moderate

High

4 weeks 12 weeks 19 weeks



Control Moderate High

Figure 1 (color)

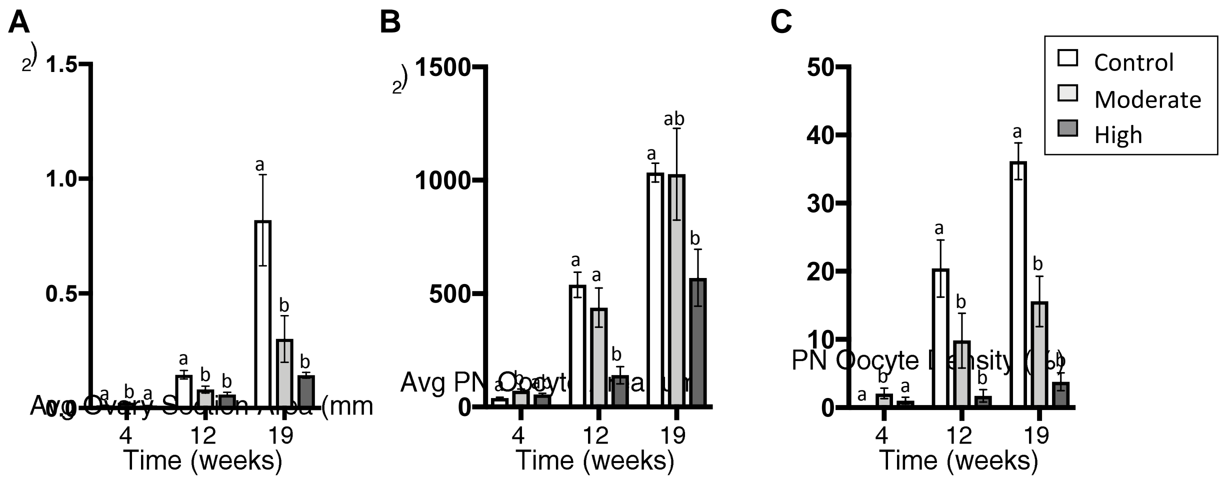
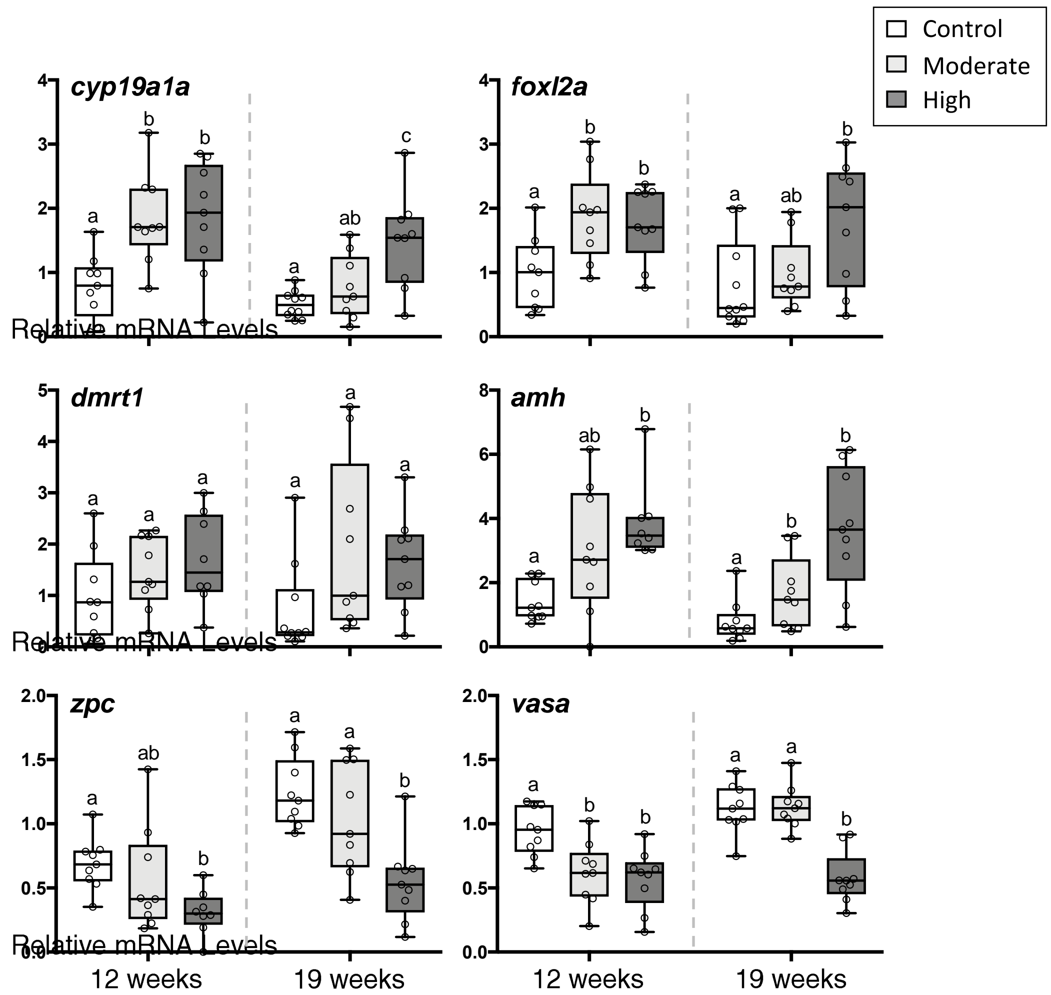


Figure 2



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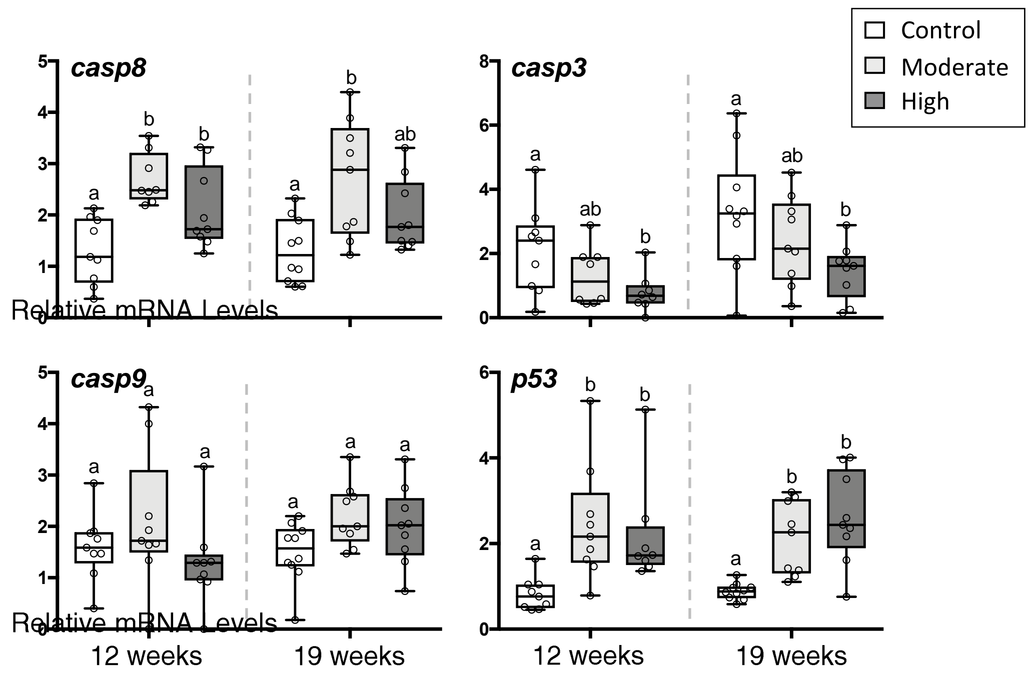

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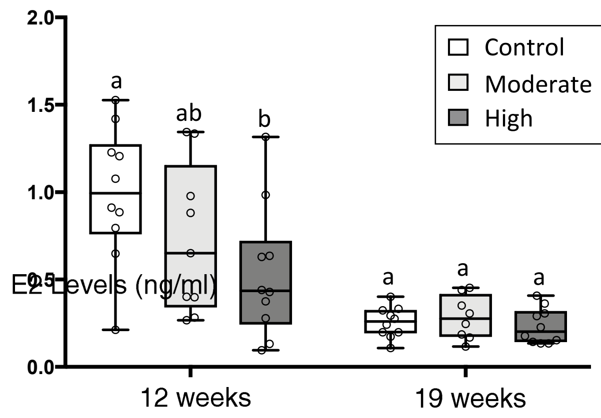
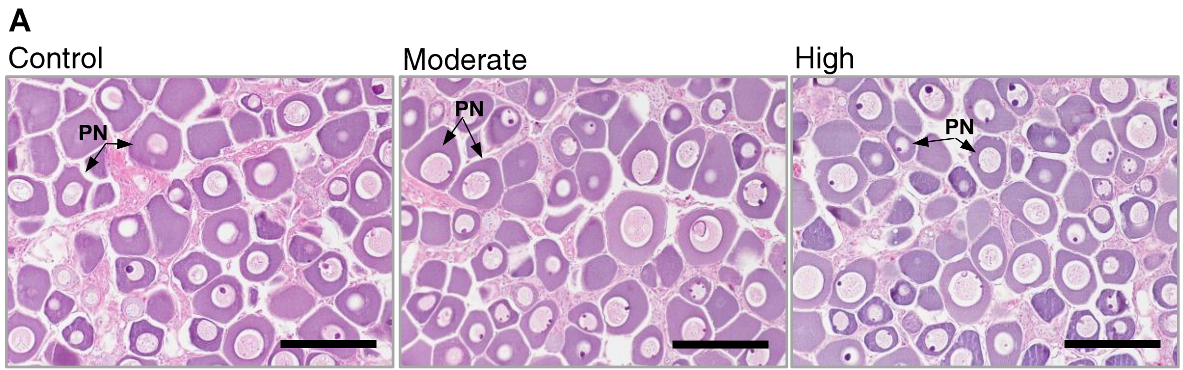
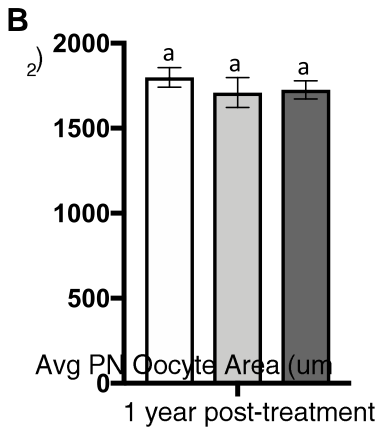
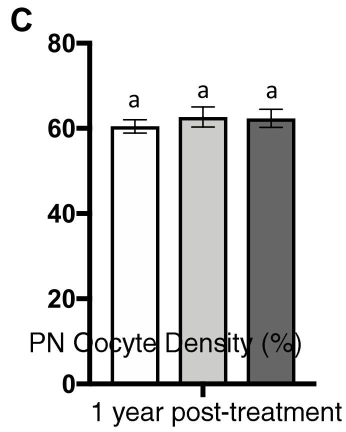


Figure 5







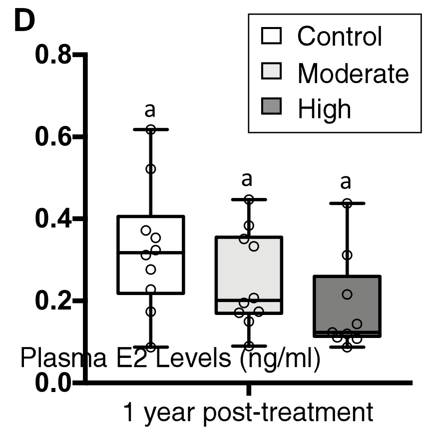
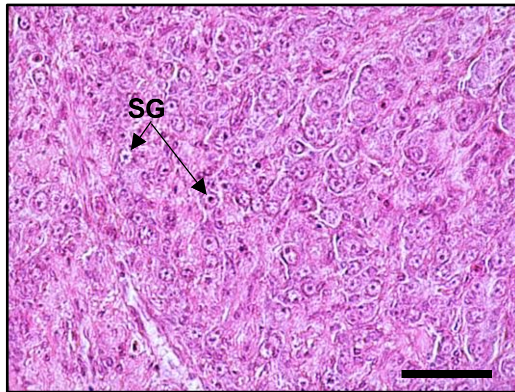
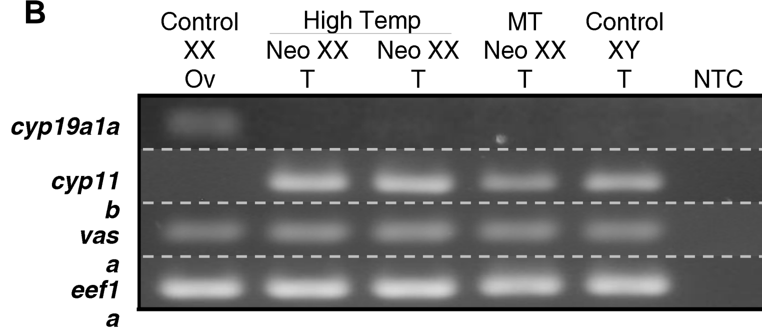


Figure 6 (color)

A



B



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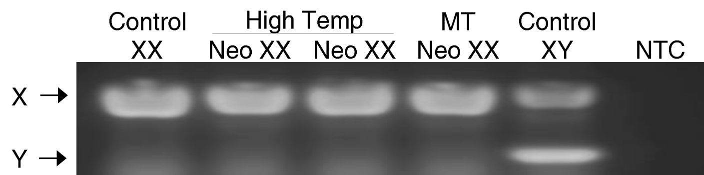

Figure 7 (color)

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