1 May 10, 2013
2 Manuscript prepared for the Transactions of the American Fisheries Society
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10 The effects of semi-chronic thermal stress on physiological indicators in steelhead
11 *Oncorhynchus mykiss*
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32 Abstract
33 The physiological response of juvenile steelhead *(Oncorhynchus mykiss)* to prolonged heat stress
34 was examined by exposing replicated groups of fish to 25 consecutive days at 15°C, 23°C, and
35 25°C followed by a 55 day recovery period at 15°C. We found that at temperatures ≥25°C,
36 steelhead consumed significantly less food per day and had elevated feed conversion rates but
37 experienced slower growth, reduced body size, lower body fat, and elevated heat shock protein
38 70 (hsp 70) levels relative to fish 2°C and 10°C cooler. Growth decreased 24.4% and 27.1% for
39 length and mass, respectively, between 15°C and 23°C, and an additional 60% and 56.5%
40 between 23°C and 25°C during exposures. While growth increments and lipid levels recovered
41 to control levels after water temperature was reduced, body size of the 25°C exposed fish lagged
42 throughout the experiment. Our results indicate a temperature threshold after which steelhead
43 exposed to semi-chronic thermal stress incur a physiological debt. Heat shock protein 70 levels
44 were detectable up to 25 days post-stress in fin and liver tissues, providing evidence that this is a
45 useful metric for thermal stress that can be assessed non-lethally, an important technique relevant
46 for monitoring thermal-habitat restoration efforts for threatened and endangered salmonids.
47
48 Introduction
49 Temperature influences multiple levels of biological organization, and dictates physiological
50 processes such as metabolism (Brett 1979; Moyle and Cech 2004), protein synthesis (Kültz
51 2005), and growth in fish (Geist et al. 2011). Salmonids, including steelhead *Oncorhynchus*
52 *mykiss* (anadromous rainbow trout) can be exposed to temperatures during summer months that
53 exceed 25°C when residing in streams (Matthews and Berg 1997; Spina 2007; Kammerer and
54 Heppell 2012). This temperature exceeds United States Environmental Protection Agency
55 standards for salmon-bearing streams (USEPA 2003), as it has been identified as a thermal limit
56 for rainbow trout (Jobling 1981) and other salmonids (Dent and Walsh 1997; Geist et al. 2009).
57 Average and maximum stream temperatures are expected to increase with climate change
58 (Mantua et al. 2009) high-lighting the importance of better understanding the physiological
59 effects of elevated temperatures on fish.
60

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61 To withstand conditions of extreme environmental stress, fish adapt by decreasing growth and
62 using fat reserves (Hurst et al. 2005; Geist et al. 2009). In addition, in response to elevated
63 temperatures, salmonids undergo a cellular stress response that includes the synthesis of heat
64 shock proteins 30 (hsp 30) and 70 (hsp 70) (Lund et al. 2002), the latter of which increases in
65 particular during acute thermal exposures and may last up to 2 weeks in Chinook salmon
66 *Oncorhynchus tshawytscha* (Mesa et al. 2002). Heat shock proteins act as molecular chaperones
67 to preserve vital protein function by maintaining appropriate protein folding (Kültz 2005).
68 Previous studies revealed that rainbow trout in the John Day River system in Oregon show a
69 physiological threshold at 23°C, above which hsp 70 increases (Feldhaus et al. 2010) and whole
70 body lipids drop (Feldhaus 2006). Whole body lipids act as an sink for energy storage and can
71 also be used as a physiological metric to assess thermal stress (Kammerer and Heppell 2012) as
72 elevated temperatures may affect their storage (Miller et al. 2006).
73
74 Short-term (1-2 h) heat stress experiments to evaluate hsp production have been performed in
75 redband rainbow trout *Oncorhynchus mykiss gairdneri* (Feldhaus et al. 2010), Atlantic salmon
76 *Salmo salar* (Lund *et al.* 2002), Chinook salmon (Mesa et al. 2002), and steelhead (Werner et al.
77 2005), but few studies have assessed the longer-term effects of elevated temperature on hsp's
78 and other performance metrics associated with thermal stress in rainbow trout. Our study seeks
79 to understand the effects of semi-chronic heat stress, which varies among the salmonids
80 (Hokason et al. 1977; Thomas et al. 1986) but has largely not been defined for steelhead. While
81 large daily fluctuations in temperature may be an ecologically-relevant parameter (Hokason et al.
82 1977; Myrick and Cech 2000), fish upper thermal tolerance and growth patterns may not differ
83 when fish are held under constant temperature regimes, as demonstrated in Lahontan cutthroat
84 trout *O. clarki henshawi* (Dickerson and Vinyard 1999; Meeuwig et al. 2004) and rainbow trout
85 with different thermal histories (Threader and Houston 1983). Testing the effects of large
86 cyclical temperature fluctuations were beyond the scope of the current study which evaluates
87 semi-chronic continuous exposures to elevated temperatures.
88
89 We conducted a laboratory study to elucidate the long-term impacts of elevated summer stream
90 temperatures on growth, the cellular stress response, and whole body lipids in steelhead. We also

91 wanted to test the hypothesis that there is a threshold at 23°C, above which physiological
92 performance declines, as previous studies indicate (Feldhaus 2006). Because many steelhead
93 populations in Oregon and California are listed as threatened or endangered under the U.S.
94 Endangered Species Act (USNMFS 2006), there are concerns about the impacts of lethal
95 sampling on any population being studied in the wild. To this end, in addition to measuring hsp
96 70 in liver, we also measured hsp 70 in fin tissue. This method may be applicable to non-lethal
97 measurements of thermal status and has potential for application to monitoring thermal habitat in
98 streams steelhead may inhabit.
99

100 Methods

101 *Animals and Experimental Design.-*
102 Juvenile summer steelhead (Skamania Columbia Basin stock) were obtained from the Oak
103 Spring Hatchery, a distribution hatchery located in Maupin, OR. During initial rearing at the
104 hatchery, fish were fed to satiation twice per day on Oregon MicroVita starter fry
105 119 feed (0.9 mm: 52% protein, 20% oil, 8.5% moisture, 1% fiber) and held in large, flow-
106 through freshwater holding ponds (319 m^3; temperature: 9-13°C, dissolved oxygen levels ≥95%
107 air saturation, density of 5.6 kg/m^3).
108

109 Fish were transferred to the Oregon State University (OSU) Fish Performance and Genetics
110 Laboratory and held in 2-m diameter, continuous flow-through holding tanks (dissolved oxygen
111 levels: ≥95% air-saturation, density 560 fish/tank) and fed *ad libitum* initially with BioVita 0.9
112 mm (Bio-Oregon, Longview, WA, USA) but transitioned to a larger, standard Bio-Diet Oregon
113 1.5 mm (Bio-Oregon, Longview, WA, USA) feed as they grew. Feed was withheld for 24 h prior
114 to any PIT-tagging, formalin treatments, the onset of temperature exposures, or sampling events.
115 Prior to experiments, fish were netted and anesthetized (50 mg/L tricaine methane sulfonate
116 (MS-222) buffered with 125 mg/L NaHCO3) until equilibrium was lost, and PIT tagged with
117 BioMark 8.4 mm 134.2 kHz tags (HPT8, BioMark, Boise, ID, USA) using a 6 G trocar injection
118 needle. Fish were treated prophylactically with oxolinic acid (2 mg/g food) for 10 days to treat
119 for the possibility of bacterial infection (Austin et al. 1983) and with formalin immersion
120 (1:10,000, 1:8000 then 1:6000 for 1.5 h each) to treat for the possibility of external parasites to

121 alleviate any hsp 70 stress response that could be induced by disease (Iwama et al. 1999; Forsyth
122 et al. 1997; Eyckmans et al. 2012) though hsp 70 does not appear to be affected by formalin
123 (Zarate and Bradley 2003).
124
125 After the completion of prophylactic treatments, steelhead were randomly allocated to one of
126 nine 1-m diameter circular tanks (n = 57 ± 2 per tank, 210 ± 20 L volume, standardized to one
127 complete turnover every 120 ± 5 min; flow rate velocities did not exceed 2L/min). Experimental
128 tanks were supplied with continuous flow-through water and were located in an indoor facility
129 that received both natural and artificial light on a natural photoperiod during June- September
130 (latitude 44.3° N). Following a 3-day tank-acclimation period, initial lengths and weights were
131 taken for all fish, and 10 fish per tank were sampled for growth, protein, and lipid analysis
132 (described below) as pre-treatment controls. Tanks were randomly assigned to a temperature
133 treatment and three tanks each were then heated, using gravity-fed well water heated in an
134 insulated tank with a spa heater (Hayward Electric Spa HeaterModel CSPA XI11, Hayward
135 Industries, Inc., N.J., USA), to create temperature treatments of 23 or 25°C for 25 days. These
136 exposures were designed to mimic the duration of elevated summer stream temperatures such as
137 in the John Day River watershed (Tattam 2006; Weber 2009; Feldhaus et al. 2010); three tanks
138 were held at ambient temperatures (maximum 15°C) to serve as experimental controls.
139 Temperature in elevated tanks was raised at a rate of 1.3°C/hr and maintained at maximum target
140 temperatures (23°C, 25°C). At the end of the temperature exposure all tanks were then rapidly
141 returned to ambient (maximum 15°C) and remained at that temperature for 55 days as a
142 temperature exposure recovery period. Temperature was monitored over the course of the
143 experiment at 10-minute intervals using Hobo Pendant temperature loggers (Onset Corp., Cape
144 Cod, MA, USA). During the experiment, fish were fed Bio-Diet Oregon 1.5 mm feed (Bio-
145 Oregon, Longview, WA, USA) at a ration of 2% of tank biomass per day (i.e. sum of fish
146 biomass x 0.02), during two separate feedings, and ration was recalculated every 10 days (data
147 not shown). The amount of uneaten food in each tank was recorded as a percent of uneaten food
148 after a 15 minute feeding period. Feed consumed per fish per day and conversion rate per
149 temperature treatment (Table 1) were calculated according to Cassinelli and Moffitt (2007, 2010)
150 where feed efficiency was calculated from the percent change in mass of feed consumed and

151 calculated per fish (per day) by tank. A feed conversion ratio was calculated using the mass of
152 feed consumed divided by the change in body weight per tank, averaged per treatment over time
153 period between sampling time points.
154
155 *Fish Sampling for Growth, Protein, and Lipid Analysis.-*
156 At each sampling event 10 fish per tank were placed in a lethal dose of MS-222 (200 mg/L
157 buffered with 500 mg/L $NaHCO_3$). Fish were then weighed to the nearest 0.01 g and measured to
158 the nearest mm. Lethal sampling occurred at day 0 (pre-treatment control), day 25 (end of heated
159 temperature exposures), day 40 (after 15 days recovery at 15°C), day 50 (after 25 days recovery
160 at 15°C), and day 80 (end of the experiment and 55 days of recovery); non-lethal weight and
161 length sampling occurred for all fish in each tank every 10 days, at which time food ration was
162 adjusted. Mortalities throughout the experiment were examined and tested for bacterial infection.
163 We calculated growth in length and mass over the first 30 days of the experiment [(size on day
164 30 - size on day 0)/30] to calculate growth increments among treatments during the temperature
165 treatment period. In addition to treatment-period growth increments, we calculated growth
166 increments over each 10-day interval for the duration of the experiment to evaluate the overall
167 pattern of growth both during and after the temperature treatment. Growth increments for each
168 temperature were calculated from only those fish that were not lethally sampled until day 80; we
169 calculated the change in length (l) or mass (m) between 10 day sampling periods, dividing by the
170 time interval in days (t), as: $(l^2-l^1)/(t^2-t^1)$ or $(m^2-m^1)/(t^2-t^1)$. In the interest of avoiding pseudo-
171 replication, the tank was treated as the sampling unit. Therefore, to compare growth increments
172 among treatments and the control we calculated an average growth increment for each tank, and
173 statistical analyses were based on n=3 for each treatment or control at each sampling interval.
174
175 Livers and fin tissue clips were collected from lethally sampled fish. Liver tissue was wrapped in
176 aluminum foil, while fin tissue was placed in a labeled 1.5 ml centrifuge tube, according to
177 Feldhaus et al. (2010). All tissue and carcasses were quickly frozen by immediately placing them
178 on dry ice. Following sampling, tissue samples were stored at ⁻80°C and carcasses stored at ⁻
179 20°C for later analysis.
180

181 *Tissue Sample Preparation and Western Blot Analysis of Heat Shock Protein 70.-*
182 Liver samples were homogenized on ice as described by Feldhaus et al. (2008, 2010). Briefly,
183 liver tissue was weighed on an analytical balance (± 0.1 mg) and homogenized in lysis buffer
184 and protease inhibitors in a ratio of 1:10 (mass: volume). Fin tissue was homogenized using 0.2
185 mL- 2 mL glass homogenizers and corresponding pestles (Wheaton #357848 & 357421,
186 Wheaton New Jersey, USA or Pyrex Tenbroeck, Corning, MA, USA). Liver and fin tissue
187 homogenates were spun at 3200 x g at 4°C for 15 minutes, and supernatants aliquoted and snap-
188 frozen on liquid nitrogen before storage at ¯80°C. Protein concentrations in homogenates were
189 assayed with the bicinchoninic acid (BCA) protein assay method (Pierce, Thermofisher
190 Scientific, IL, USA).
191
192 Heat shock protein 70 was measured using Western blot analysis according to Feldhaus et al.
193 (2010), modified as follows: 100 ng of protein were loaded per lane for liver hsp 70 analysis and
194 50 ng loaded per lane for fin tissue homogenates in 7.5% Tris-HCl gels (#161-1154 and#161-
195 1100, Bio-Rad, Hercules, CA USA) in a Mini Protean system (model 3 and Tetra Cell, Bio-Rad,
196 Hercules, CA USA). All gels were run with a calibrated molecular marker (Kaleidoscope
197 Precision Plus #161-0375, Bio-Rad, Hercules, CA USA) and 50 ng of recombinant Chinook
198 salmon hsp70 protein (SPP-763, StressGen Biotechnologies/Enzo Life Sciences, NY, USA) at
199 200 V for ~40 min. Staining also differed from Feldhaus et al. (2010) in that overnight block was
200 performed at 4°C and membranes for both sample types were probed with primary polyclonal
201 hsp 70 antibodies (SPA 758, StressGen/Enzo Life Sciences, NY, USA) diluted to a concentration
202 of 1:7500, while secondary goat anti-rabbit alkaline-phosphatase conjugated antibodies (SAB-
203 301, StressGen Biotechnologies/Enzo Life Sciences, NY, USA) were used in the concentration
204 of 1:5000. Proteins were analyzed colorimetrically using an alkaline phosphatase conjugate
205 substrate kit (170-6432, Bio-Rad, Hercules, CA USA) and relative hsp 70 band density was
206 calculated after quantification using densitometry software, ImageQuant TL (GE Healthcare Life
207 Sciences, Pittsburgh, PA USA) (Feldhaus et al. 2010).
208
209 *Whole-body Lipid Analysis.-*

210 Whole-body lipid content for each fish was determined following the methods of Anthony et al.
211 (2000) and Reynolds and Kunz (2001), though with livers removed. Fish were thawed, weighed,
212 and dried to a constant mass (i.e. ± 0.01 g of previous 24 h mass) in a convection oven set at
213 65°C. Fish were thoroughly homogenized with mortar and pestle and lipids were extracted from
214 dried samples using a Soxhlet apparatus and a 7:2 (v/v) hexane/isopropyl alcohol solvent system.
215 Fat mass was determined by subtracting lean dry fish mass from dried homogenized fish mass.
216 Whole body lipid fraction was calculated by dividing the fat mass by dry mass.
217
218 *Statistical Analysis.-*
219 The effect of temperature treatments on fish length, mass, growth increments of length and mass,
220 average feed consumed per day, and feed efficiencies were analyzed by analysis of variance
221 (ANOVA) by tank (n=3), taking into account repeated measures over time, by 10-day interval.
222 The interactive effects of temperature and time on relative hsp 70 expression and whole body
223 lipids were similarly analyzed, though over 10 to 25 day intervals. Because the experimental
224 unit was each tank of which temperature treatments were applied using a balanced design, a
225 repeated measures model was used for ANOVAs after the assumptions for normality (Shapiro-
226 Wilks) and equality of variance were tested. Tukey multiple comparison tests were performed
227 when means were significantly different. Paired t-tests were also used to test differences
228 between hsp 70 tissue types at each timepoint and temperature. All statistical tests were
229 performed in Sigmaplot (v.11 Systat Software, Inc. San Jose, CA USA) with significance set at P
230 < 0.05. Data are presented as the mean ± standard error.
231
232 Results
233 Temperatures were maintained at 13.43 ± 0.03°C (mean ± standard error), with a range from
234 12.6 - 15.1°C for the 15°C treatment, 22.2 ± 0.01°C, with a range from 21.3-23.1°C for the 23°C
235 treatment, and 24.54 ± 0.004°C, with a range from 23.8 - 25.2°C for the 25°C treatment.
236 Recovery period temperatures for both high temperature treatments averaged 13.5 ± 0.006°C,
237 with a range from 12.8-15.1°C. Mortalities only occurred in the 25°C tanks during this
238 experiment, starting 10 days after the start of the experiment; six fish, distributed evenly between
239 the 3 tanks, died by day 25 but subsequent bacterial pathogen tests were negative. The overall

240 health of the remaining fish was good, though we observed 10-20% of the fish in all tanks and
241 treatments to have frayed or split caudal fins by Day 60. Fish at Day 0 averaged 89.87 ± 0.03
242 mm (mean ± standard error) in length and 7.25 ± 0.01 g (mean ± standard error) in weight, with
243 no differences among treatments (ANOVA: F = 6.752; df = 16, 48; $P > 0.988$); ANOVA: F =
244 15.966; df = 16, 48; $P > 0.878$, respectively). There was a significant effect of temperature
245 treatment on length and mass (ANOVA: F = 21.110; df = 2, 48; $P = 0.002$; ANOVA: F =
246 37.476; df = 2, 48; $P < 0.001$, respectively; Figure 1 A&B). With the 8°C increase in temperature
247 from 15°C to 23°C, growth increments of length decreased by 24.4% while growth increments
248 based on mass decreased by 27.1% during the first 30 days of the experiment (Table 1). With the
249 2°C increase in temperature from 23°C to 25°C, growth increments based on length dropped an
250 additional 60% and growth rates based on mass an additional 56% during the initial 30 day
251 period (Table 1). Growth increments based on length were significantly different at 25°C than at
252 23°C and 15°C during this time (ANOVA: F = 45.086; df = 16, 48; $P < 0.007$), as were growth
253 increments based on mass (ANOVA: F = 5.374; df = 16, 48; $P < 0.013$). Following removal of
254 the temperature treatments, growth increments of fish in the 23°C and 25°C treatments achieved
255 that of the 15°C fish (Figure 1C&D). Fish in warm water treatments ate less per day (ANOVA:
256 F = 6.771; df = 2, 16; $P = 0.001$), though fish at 25°C had higher feed conversion efficiencies
257 than fish at 15°C and 23°C (ANOVA: F = 6.384; df = 16, 48; $P < 0.001$; Table 1).
258

259 Heat shock protein 70 levels in liver and fin tissue from fish at 25°C were significantly higher
260 than in fish held at 15°C at all time points (ANOVA: F = 4.098; df = 8, 16; $P = 0.008$; ANOVA:
261 F = 6.799; df = 8, 16; P < 0.001) except at Day 0 (pre-treatment) and after Day 50, when levels
262 returned to near pre-treatment control levels (Figure 2). At Day 25, liver hsp 70 levels in fish
263 exposed to 25°C had increased ~2-fold relative to controls and were not statistically different
264 than those fish exposed to 23°C (ANOVA: F = 4.098; df = 8, 16; P = 0.06). Hsp 70 levels in 23°
265 C treatments also differed from 15°C controls in both liver or fin tissue, at this time (ANOVA: F
266 = 4.098; df = 8, 16; $P = 0.018$; ANOVA: F = 6.799; df = 8, 16; $P < 0.001$, respectively). Hsp 70
267 expression in fin tissue was not different than that seen for liver ($P > 0.05$).
268

Time and the interaction of time and temperature treatments affected whole body lipids (ANOVA: F = 92.021; df = 4, 16; $P < 0.001$; ANOVA: F = 3.531; df = 8, 16; $P = 0.015$, respectively). Temporally, for fish at ambient temperature and at 23°C, whole body lipids increased significantly through Day 40 (ANOVA: F = 3.531; df = 8, 16; $P < 0.05$), and then did not change throughout the remainder of the experiment (ANOVA: F = 3.531; df = 8, 16; $P < 0.103$). Fish treated at 25°C saw a decrease in lipids over the initial part of the experiment, but had whole body lipid levels similar to that of the other treatments by Day 80 (Figure 3). As with growth increments, whole body lipids by Day 40 were significantly lower in fish held at 25°C compared to fish held at the other temperatures (ANOVA: F = 3.531; df = 8, 16; $P < 0.05$). By Day 80, however, whole body lipids were the same across treatments (ANOVA: F = 2.718; df = 8, 16; $P > 0.255$).

Discussion

Our data show that exposure to sustained high temperature results in decreased growth increments, decreased ability to store lipids, increased stress protein production, and elevated mortalities, with fish consuming less food but having higher conversion efficiencies at 25°C. These results suggest that the metabolic demand for these fish is not being met by their food intake at high temperatures, resulting in weight loss (Dickerson and Vinyard 1999; Meeuwig et al. 2004), and a re-partitioning of energy away from growth (Fang et al. 2010; Lohmus et al. 2010; Myrick and Cech 2000) until more optimal conditions allow the fish to recover.

The Q_{10} principle predicts a coefficient of increase in metabolism of ~2 for young rainbow trout (Railsback and Rose 1999; Kamler 2008), indicating that the steelhead in our experiment should approximately double their metabolic rate between 15°C to 23°C and potentially also increase in size. However, growth increments dropped substantially when temperatures increased from 15°C to 23°C (Table 1). More importantly, when the temperature was raised just 2°C from 23°C to 25°C, an even greater decrease in growth occurred (Table 1). Food conversion efficiency may increase at elevated temperatures (Huang et al. 2008; Fang et al. 2010), something we observed at 25°C, although consumption rate decreased. The influence of food consumption on differences in lipids, growth increments, and hsp production was expected to be minimal because feeding

299 was maintained at 2% body mass/day and food was consistently eaten except immediately
300 following weighing and measuring (Deng et al. 2009; Cassinelli and Moffitt 2010). Higher
301 temperatures may cause decreased absorption of food during digestion (Bogevik et al. 2011)
302 though there is also evidence of minimal impacts on feeding and foraging behaviors in strains of
303 thermally-adjusted steelhead (Spina 2007). For the stock of steelhead in these experiments, it is
304 apparent that the level and duration of the thermal stress had an impact on their metabolism with
305 resulting physiological consequences.
306
307 Our results suggest a physiological threshold at about 23°C, also observed by Feldhaus (2006)
308 for rainbow trout in a natural system and supported by earlier work on juvenile rainbow trout
309 (Hokason et al. 1977). A similar physiological threshold at constant thermal exposure was
310 observed for Lahontan cutthroat trout (Dickerson and Vinyard 1999). It is likely that part of the
311 decrease in growth above that threshold is due to energy reallocation to the process of hsp 70
312 production (Myrick and Cech 2000; Iwama et al. 1999).
313
314 Heat shock protein 70 is a molecule that provides protection from stress and indicates activation
315 of the cellular stress response (Feder and Hoffman 1999; Kültz 2005). In our elevated
316 temperature treatment, hsp 70 increased by Day 25 (Figure 2), consistent with other studies
317 (Feldhaus et al. 2010; Smith et al. 1999). Heat shock protein 70 has been shown to peak one day
318 after an acute (1 hr) exposure in Atlantic salmon (Lund et al. 2002), and following an initial sub-
319 lethal thermal shock fish can withstand subsequent greater heat shock (Kiang and Tsokos 1998;
320 Basu et al. 2002). Our experiment followed a substantially longer time course for hsp 70
321 synthesis than previous salmonid-based studies (Lund et al. 2002; Mesa et al. 2002; Feldhaus et
322 al. 2010), though hsp 70 remained elevated for at least 15 days after the stress was removed,
323 consistent with results in Chinook salmon after acute stress (Mesa et al. 2002). These
324 observations suggest that these fish retain hsp 70 cytoprotective mechanisms for an extended
325 period (Viant et al. 2003; Kültz 2005) even as they return to pre-stress homeostatic processes
326 during recovery.
327
328 Body fat provides a measure of overall physiological condition in thermally stressed steelhead
329 (Kammerer and Heppell 2012). At ambient temperature and at 23°C, whole body lipids increased

330 significantly for an extended period, after which they remained constant. Growth increments
331 based on length showed a similar pattern although growth increments based on mass continued
332 to increase (Figure 1). These results suggest that at ambient temperatures fish were storing
333 energy as fat instead of using it to grow longer. At 25°C fish had an initial elevation in lipids,
334 followed by a substantial decline and slow recovery; by the end of the experiment lipid levels
335 were similar across treatments (Figure 3). The decrease in whole body lipids 15 days post-
336 thermal exposure was also consistent with elevated hsp 70 levels (Figure 2), decreased lipids
337 during warm summer months in rainbow trout exposed to temperatures above 22-23°C (Feldhaus
338 et al. 2010) and Atlantic salmon during seasonally extreme temperatures (Naesje et al. 2006).
339 Starvation resulting from decreased lipid storage may affect fish reproductive status (Chatzifotis
340 et al. 2011), life history strategy (McMillan et al. 2011), and ocean survival (Triebenbach 2009).
341 Compensatory growth may occur after a stressor is no longer present in salmonids (Triebenbach
342 et al. 2009) and other species (Huang et al. 2008), characterized by elevated growth rates and
343 rapid restoration of lost energy reserves following a period of slow growth (Ali et al. 2003).
344
345 Temperatures in natural desert streams undergo a pronounced diel shift (Kammerer and Heppell
346 2012), so the effects observed here may not be as extreme in the wild, where a fish's thermal
347 history is more variable. However, Meeuwig et al. (2004) demonstrate that constant thermal
348 stress does not evoke physiologically dissimilar growth rates to fish exposed to stressful daily
349 thermal cycles. Variable thermal history does affect thermal tolerances of rainbow trout
350 (Threader and Houston 1983) lowering tolerance potentially by 1.5°C (Hokason et al. 1977),
351 although limited recovery of steelhead from thermal stress may occur each night as the
352 temperature drops (Werner et al. 2005). In natural systems fish also may retreat to thermal
353 refugia as temperatures in streams climb (Ebersole et al. 2003), depending on these areas to
354 survive (Matthews and Berg 1997; Ebersole et al. 2001). While our experimental design did not
355 mimic the natural desert environment, our work demonstrates three important principles in this
356 stock of steelhead: (1) thermal stress impacts performance, including induction of stress proteins,
357 decreased ability to store body fat, and decreased growth increments, (2) there is evidence of a
358 threshold thermal limit above which the biological impacts of semi-chronic elevated
359 temperatures become critical, and (3) evaluations of thermal stress can be conducted using
360 external tissue samples in a non-lethal manner.

361
362 We used fin tissue as our source tissue for measuring hsp 70 because of non-invasive manner
363 with which it is collected. There are other candidate tissues that could be used for measuring hsp
364 70. For example, heat shock protein 70 mRNA increases in brook trout *Salvelinus fontinalis*
365 (Lund et al. 2003) and rainbow trout (Lund et al. 2002) red blood cells after acute heat stress.
366 While red blood cell sampling represents another non-lethal method, carefully collecting and
367 processing blood in the field may not be feasible especially on the large scale, as it requires
368 catheterization or venipuncture (Cech et al. 1979). Certain compounds, such as oxolinic acid,
369 may impact blood chemistry (Lunden et al. 1998). However, all fish were treated equally and
370 well in advance of temperature treatments, as they were with formalin. While there were also
371 some potential complicating effects evident through caudal fin erosion, this result was observed
372 across all treatments and may be more illustrative of and aggressive feeding behavior (Noble et
373 al. 2006) and social tendencies in rainbow trout (North et al. 2006). Regardless of source tissue
374 or fish pre-treatments and behaviors, the non-lethal technique we outline can be particularly
375 useful in systems where restoration and monitoring efforts are being conducted on threatened
376 and endangered species (Kammerer and Heppell 2012), especially since physical stressors such
377 as PIT-tagging (Feldhaus et al. 2008) or handling (Vijayan et al. 1997; Iwama et al. 1999) do not
378 appear to affect hsp 70. Ultimately, while there are officially-mandated upper temperature
379 maxima for salmon bearing streams (USEPA 2003), our results demonstrate a physiologically-
380 relevant critical thermal threshold above which evokes a substantial physiological cost to this
381 stock of steelhead and of which we also present a non-lethal manner in which to assess it.
382
383 Acknowledgements
384
385 This project was support by the Oregon Watershed Enhancement Board (grant #208-8008) and
386 was conducted in compliance with Oregon State University (OSU) Institutional Animal Care and
387 Use Committee (ACUP #3658). This work was carried out with the help of Rob Chitwood in the
388 laboratory of Professor Carl Schreck, to both of whom we are grateful for technical assistance
389 and guidance. We would also like to thank Stephanie Archer, Ryan Lande, Robin Jenkins, and
390 Brian Whisenhunt for their help with fish sampling, PIT-tagging, and/or fish care, and Kelsey

391 Josi, Will Krett, and Ajand Shamloo for help with laboratory analysis of hsp or proximate lipid
392 composition. We would also like to thank John VanSickle and Raymond Wong for their
393 statistical counsel, Allison Evans for her editorial comments, and especially Joseph Feldhaus for
394 his kind advice throughout these experiments and analyses.

395

396 Figure and table captions

397

398 Figure 1.- The effects of water temperature on steelhead growth. Growth was measured by **(A)**
399 length (mm) and **(B)** mass (g) over time and by growth increment calculated from **(C)** length
400 (mm/day) and **(D)** mass (g/day) following exposures, where each treatment differed (see text).
401 Vertical lines indicate Day 25, the day when temperature treatments ended, in each panel.

402

403 Figure 2.- The response of relative heat shock protein 70 (hsp 70) content over time in steelhead
404 **(A)** liver and **(B)** fin tissue following exposure to 25°C, 23°C, or non-heated ambient (15°C)
405 temperature treatments, followed by a 55 day recovery period. Letters that differ indicate
406 statistical differences at a common time, while numbers that differ indicate differences with time
407 at a given temperature treatment.

408

409 Figure 3.- Whole body lipid changes in steelhead trout over time, following different
410 temperature treatments (25°C, 23°C, and 15°C) for 25 days, followed by a recovery period of 55
411 days at15°C. Letters that differ indicate differences at a common time, while numbers that differ
412 indicate differences with time at a given temperature treatment. The vertical line indicates Day
413 25, the day when temperature treatments ended.

414

415 Table 1.- Average growth increments (mean ± SE) in length (mm/day) and mass (g/day) from
416 three temperature treatments during the first 30 days of the experiment, encompassing the entire
417 temperature treatment period. Relative change in growth and percent change per degree Celsius
418 indicates the percentage decrease in average growth relative to the next coolest temperature.
419 Average feed consumed per day and average feed conversion was calculated per time period and
420 averaged (see text).

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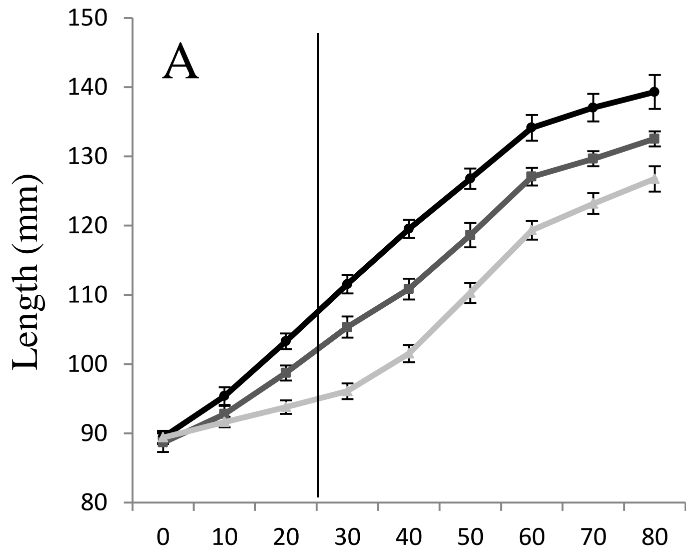
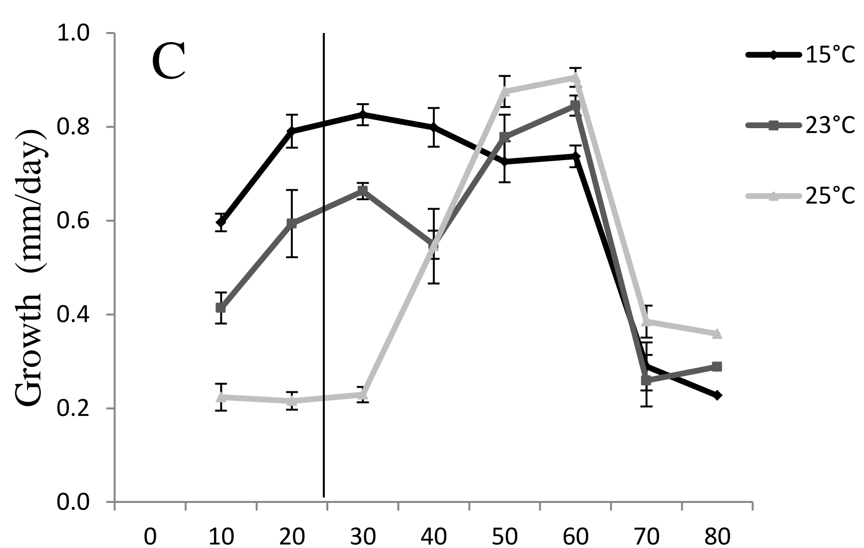
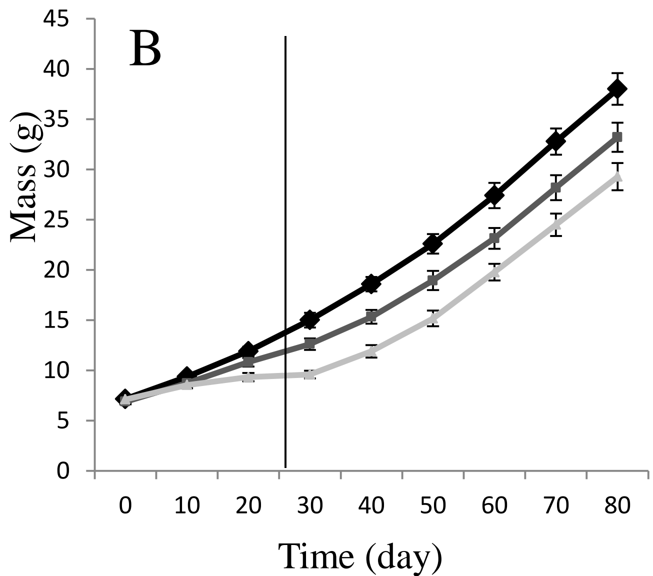
Figure 1







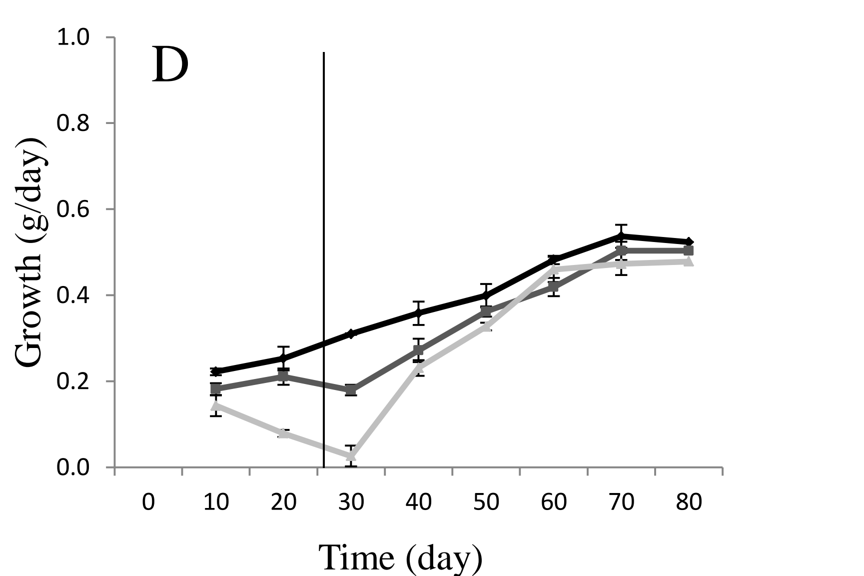


Figure 2

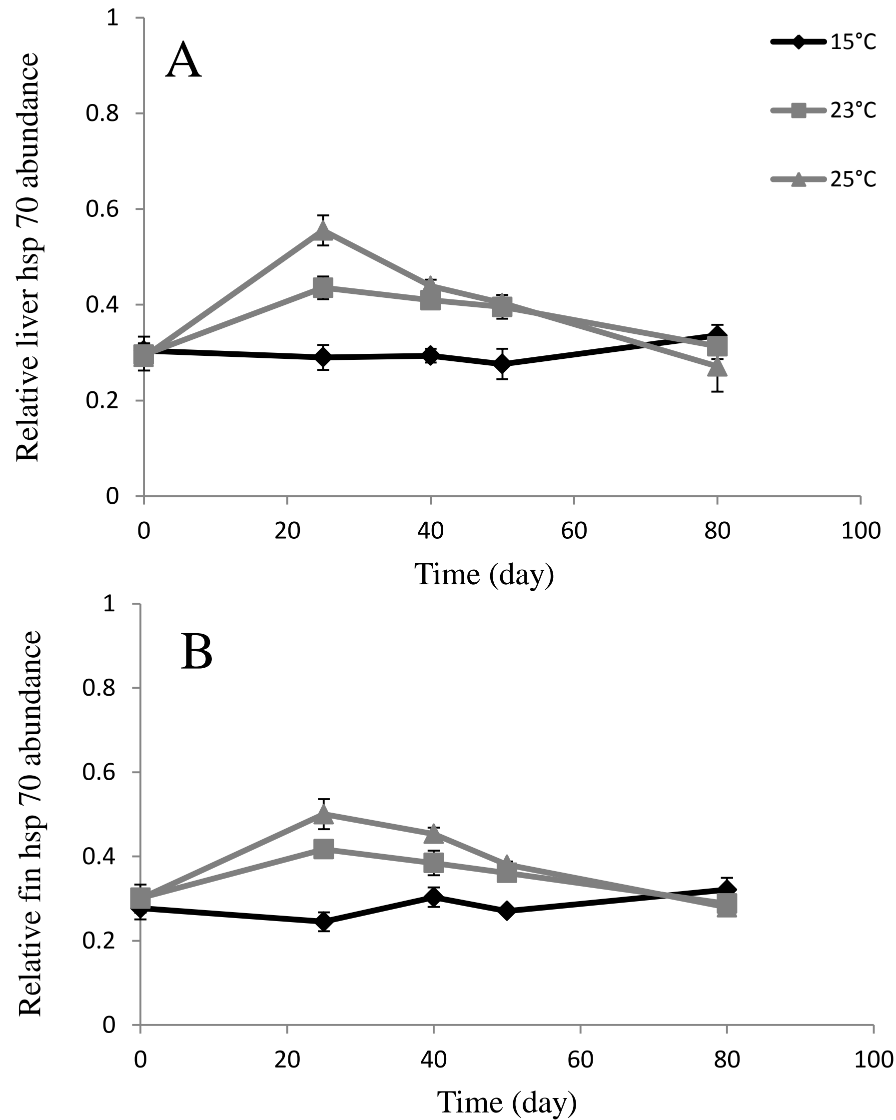


Figure 3

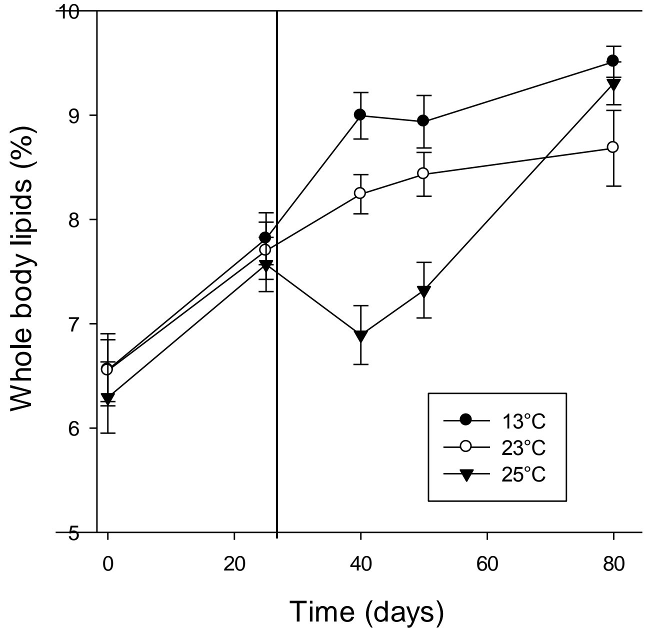


Table 1

Treatment	mm/day	Relative change growth (%)	%Change / degree C	g/day	Relative change growth (%)	%Change/ degree C	Average feed consumed (g) per fish per day	Average feed conversion
15	0.737 ±0.015			0.262 ±0.019			.32	0.70
23	0.557 ±0.011	-24.4	-2.4	0.191 ±0.010	-27.1	-2.7	.28	0.81
25	0.223 ±0.020	-60.0	-30.0	0.083 ±0.007	-56.5	-28.3	.25	1.26