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A physiological approach to quantifying thermal habitat quality for Redband Rainbow Trout (*Oncorhynchus mykiss gairdneri)* in the south Fork John Day River, Oregon

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Abstract We examined tissue-specific levels of heat shock protein 70 (hsp70) and whole body lipid levels in juvenile redband trout (*Oncorhynchus mykiss gairdneri*) from the South Fork of the John Day River (SFJD), Oregon, with the goal of determining if these measures could be used as physiological indicators of thermal habitat quality for juvenile redband trout. Our objectives were to determine the hsp70 induction temperature in liver, fin, and white muscle tissue and characterize the relation between whole body lipids and hsp70 for fish in the SFJD. We found significant increases in hsp70 levels between 19 and 22°C in fin, liver, and white muscle tissue. Maximum hsp70 levels in liver, fin, and white muscle tissue occurred when mean weekly maximum temperatures (MWMT) exceeded 20–22°C. In general, the estimated hsp70 induction temperature for fin and white muscle tissue was higher than liver tissue. Whole body lipid levels began to decrease when MWMT exceeded 20.4°C. There was a significant interaction between temperature and hsp70 in fin and white muscle tissue, but not liver tissue. Collectively, these results suggest that increased hsp70 levels in juvenile redband trout are symptomatic of thermal stress, and that energy storage capacity decreases with this stress. The possible decrease in growth potential and fitness for thermally stressed individuals emphasizes the physiological justification for thermal management criteria in salmon-bearing streams.

Keywords Lipids · Temperature · Heat shock proteins · hsp70

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Introduction

Habitat restoration projects designed to increase riparian zone integrity and lower stream temperatures are a widely used management strategy for protecting threatened salmonid populations in the Pacific Northwest. It is

generally accepted that temperature influences all levels of biological organization (Tait et al. 1994; Beitinger et al. 2000), and therefore managing for stream temperature is an important component of restoration. At the cellular level, temperature affects virtually all components of cellular processes, including protein stability and enzymatic rates (Hochachka and Somero 2002). Water temperature influences behaviours such as individual fish microhabitat choice (Baltz et al. 1987), behavior (Cunjak and Green 1986), and suppresses appetite and growth (Linton and Morgan 1998; Railsback and Rose 1999). At the population level, the distribution (Torgersen et al. 1999, Hughes and Grand 2000) and biomasss (Li et al. 1994, Zoellick 2004) of salmonids correlates with water temperature. There is also evidence that various strains of rainbow trout (*Oncorhynchus mykiss)* exhibit differences in growth above 22°C (Myrick and Cech 2000). Furthermore, population-specific differences in physiological function of redband trout (*O. mykiss spp.)* from southeastern Oregon were attributed to phenotypic differences (Gamperl et al. 2002). This means that while temperature is an important indicator of overall habitat quality, individual populations may respond differently to similar temperatures; a one-size fits all thermal regulatory rule may not be appropriate. Although specific water temperature standards have been developed to protect aquatic communities (e.g., Oregon Department of Environmental Quality (DEQ) 303(d) listed streams, http://www.deq.state.or.us/WQ/assessment/rpt02.htm), rapid techniques for examining and testing for population-specific responses and physiological adaptations to temperature are poorly developed.

Heat shock proteins (hsp) are a group of highly conserved cellular chaperone proteins (Feder and Hofmann 1999) that can be synthesized constitutively or in response to a stressor (Hochachka and Somero 2002). Quantifying hsp is a technique that has been used to measure thermal stress in salmonids (Lund et al. 2002; Lund et al. 2003; Werner et al. 2005). An increase in hsp in response to heat stress is called the heat shock response (Parsell and Lindquist 1994; Hochachka and Somero 2002), and this response is designed to protect vital cellular functions by binding denatured proteins and preventing inappropriate associations between proteins (Parsell and Lindquist 1994).

Given the impact that thermal stress has on productivity, it is plausible that physiological indicators of thermal stress could be used to define suitable habitat for threatened and endangered salmonids during recovery efforts. The goal of this study was to determine if juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri)* in the SFJD experience thermal stress during the summer, and whether heat shock proteins can be used as an indicator of stream thermal habitat quality. Redband rainbow trout (*O. mykiss gairdneri)* in the South Fork of the John Day River, Oregon (SFJD) are part of the middle Columbia River evolutionarily significant unit of summer run steelhead and are listed as threatened under the federal Endangered Species Act (U.S. National Marine Fisheries Service 2007). The objectives of this study were to determine the hsp70 induction temperature ranges in liver, fin, and white muscle tissue, and characterize the relation between whole body lipids and hsp70 for fish in the SFJD. This information can be used to develop physiological metrics to evaluate thermal habitat quality in salmon-bearing streams.

Study area

The John Day River and its tributaries, the North, Middle, and South forks comprise >800 km of free-flowing river (Torgersen et al. 1999); it is the second longest unimpounded river in the contiguous United States. The John Day basin is also one of the few drainage systems in the Columbia River basin that still supports wild runs of juvenile spring Chinook salmon (*O. tshawytscha*) and an anadromous summer run of redband rainbow trout (*O. mykiss gairdneri)* which are minimally influenced by hatchery fish (Li et al. 1994). Despite this free-flowing status, the Department of Environmental Quality (DEQ) has placed this river on the Oregon 303(d) list for water quality limited streams (http://www.deq.state.or.us/WQ/assessment/rpt02.htm) for salmon and trout rearing and migration streams because summer stream temperatures exceed the mandated 18°C seven-day-average maximum temperature threshold (DEQ, OAR 340-041-0028).

The South Fork of the John Day River (SFJD) drains an area of approximately 1,637 km^2 as it flows northward from the Ochoco and Aldrich mountains, dropping in elevation from 1,646 m to 710 m above sea level. Four main tributaries provide spawning habitat for adult summer steelhead below the anadromous fish barrier at Izee Falls (IZ) at river kilometer 46.7 (Fig. 1). This work focused on the mainstem SFJD, Black Canyon Creek (BC), Deer Creek (DC),

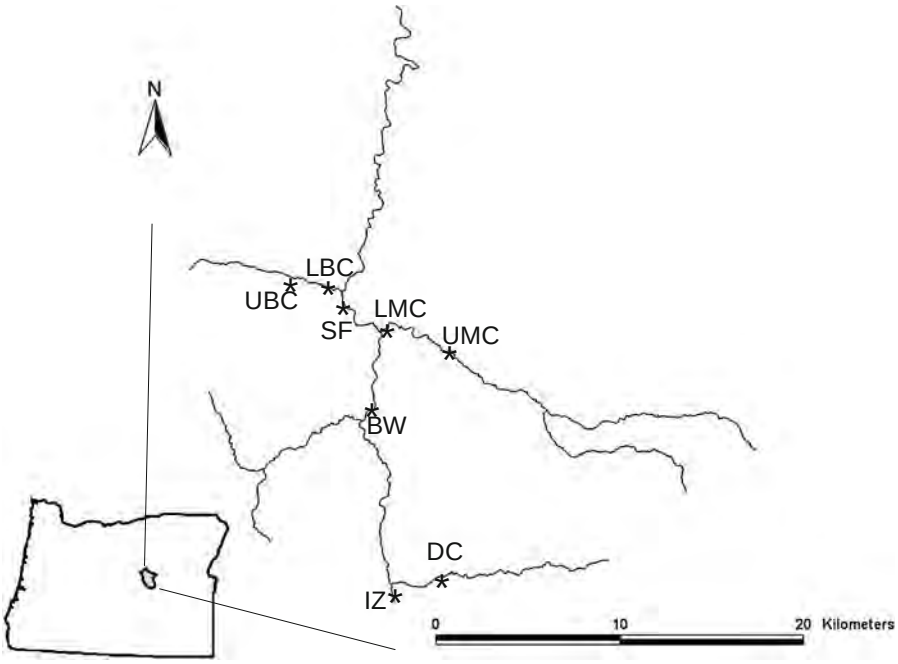

Fig. 1 Map of the juvenile redband steelhead trout collection sites in the South Fork John Day River (*SFJD*) catchment, Grant County, Oregon. Collections occurred at lower and upper Black Canyon creek (*LBC*, *UBC*), lower and upper Murderers creek (*LMC*, *UMC*), Deer creek (*DC*), in the SFJD at river kilometer 24 (*SF*), below Wind creek (BW), and below Izee falls (IZ)

and Murderers Creek (MC). Flowing from west to east, BC drains the Black Canyon Wilderness. Both DC and MC flow east to west. The lower section of MC flows through the Murderers Creek wildlife preserve.

Materials and methods

Laboratory experiment

To establish a context for our field sampling, a laboratory thermal manipulation experiment was conducted between 25 October and 20 November 2004 at Oregon State University's Fish Performance and Genetics Laboratory (FPGL) in Corvallis, OR. The experimental fish were raised at the FPGL in 1-m-diameter circular fiberglass tanks supplied by well water, and were first generation offspring of adult steelhead captured from the Umatilla River, Oregon and spawned at the FPGL. A total of 144 yearling steelhead trout were evenly and randomly divided among 9 indoor, 1-m-diameter circular fiberglass tanks (16 fish·tank^{-1}), with ambient flow-through well water entering the tanks at 13.0°C and maintained at a depth of 55 cm. Fish were acclimated to experimental tanks for 10 d. During acclimation, fish were kept on the same feeding regime they experienced prior to transfer to the experimental tanks, being fed once per day to apparent satiation with BioDiet Grower™.

Each experimental tank was randomly assigned a thermal stressor of 19, 22, or 25°C. Each stressor was applied to three different tanks. To better control the application of the stressor, only one tank of fish per day was subjected to a stressor. The order in which each tank was thermally manipulated was randomly assigned. Tank temperatures were monitored using Onset Optic Stowaway™ (Onset Corp) temperature loggers.

Prior to subjecting a tank of fish to a thermal treatment, fish in that tank were fasted for 24 h. Each day, before introducing heated water to the experimental tank, six fish were randomly netted from the tank to serve as pre-treatment controls and killed with a lethal dose of tricaine methanesulfonate (MS-222; 200 mg L^{-1}) buffered with 500 mg/L $NaHCO_3$. For each fish, length (mm FL) and weight (0.1 g) were recorded. Liver tissue was quickly excised and wrapped in aluminum foil. Fin tissue was removed from the lower caudal fin and placed in a labeled centrifuge tube. All tissue was flash frozen in liquid

nitrogen immediately after collection, then transported to Oregon State University and stored at -80°C for later analysis.

Flow-through well water was heated in a 387 L insulated tank with a Hayward Electric Spa Heater (Model CSPA XI11), and gravity fed into experimental tanks at 2 L·min^{-1} until the target temperature was reached. After 90 min of elevated temperature exposure, ambient water (13°C) was reintroduced to each experimental tank. To allow time for hsp synthesis, fish remained in the experimental tank for 12 h following the end of the temperature stress before six treatment fish were lethally sampled for liver hsp70 analysis as described for the control fish.

Field sampling

Fish were collected on 23-24 July, 2005 from both riffle and pool habitat by beach seine and electrofishing (Smith-Root Model 12B) from eight study locations in the SFJD catchment basin (Fig. 1, Table 1). Sampling occurred before daily water temperatures reached 18°C. All collections were completed within 2-3 h at each study location. Fish were individually anesthetized with MS-222, measured and weighed. Average fork length of all fish obtained from the SFJD was 126.3±11.9 mm and average mass was 23.9±11.9 g. Liver, lower caudal fin, and muscle tissue from the caudal peduncle region, hereafter referred to as white muscle tissue, were collected from each fish. White muscle and liver tissue were wrapped in aluminum foil, and caudal fin tissue was placed in a 1.5 ml eppendorf vial. All tissues were immediately frozen on dry ice. Fish carcasses were individually packaged, kept on ice, and transported to Oregon State University (Corvallis, OR). Tissue samples were stored at -80°C for later analysis of heat shock proteins. Fish carcasses were stored at -20°C.

Temperature data

Optic Stowaway™ and I-button™ (Company) temperature loggers set to record temperature (°C) each hour were placed at study locations in May and June 2005. The temperature logger at the IZ study location

Table 1 Sampling locations, coordinates (UTM; NAD 83), and temperature data (°C) for fish collected in the South Fork John Day River 23-24 July 2005. Weekly temperature averages are for the 7 days preceding the sampling date. Monthly temperature ranges are from 1 July to the sampling date

Sampling location	UTM Coordinates[a]	Sampling date	24 h Avg	24 h Min	24 h Max	Pre-visit Max Temperature; Date	MWAT	MWMT	MMAT	MMT_{max}	MDTR
Upper Black Canyon (UBC)	11 T 291492, 4913200	24 July	14.3	11.3	17.8	18.4; 19 July	14.4	17.9	13.8	17.2	6.3
Lower Black Canyon (LBC)	11 T 294907, 4912196	24 July	16.3	13.4	20.4	21.1; 19 July	16.7	20.6	15.9	19.7	6.9
SF at RKM 24 (SF)	11 T 296081, 4910855	24 July	21.6	17.9	25.4	25.4; 23 July	21.3	25.0	20.0	23.4	6.7
Upper Murderers Creek (UMC)	11 T 305099, 4906826	23 July	18.8	16.3	22.9	22.9; 22 July	17.3	21.8	16.5	19.5	6.1
Lower Murderers Creek (LMC)	11 T 297932, 4910336	23 July	22.4	19.9	25.8	25.8; 22 July	21.0	25.3	19.6	23.7	8.5
Below Wind Creek (BW)	11 T 297168, 4906262	24 July	20.2	16.4	24.0	24.6; 22 July	19.9	23.7	18.9	22.4	7.1
Izee Falls (IZ)	11 T 298082, 4896082	23 July	21.3	19.3	23.7	23.7; 22 July	20.0	23.0	19.1	22.2	6.2
Deer Creek (DC)	11 T 300210, 4896411	23 July	16.8	15.0	19.4	19.4; 22 July	14.9	18.0	14.0	16.8	5.4

SF = South Fork John Day River; the 24 h average, 24 h minimum, and 24 h maximum are for water temperatures during the 24 h preceding fish collection, pre-visit maximum is the maximum temperature recorded prior to fish collection

MWAT mean weekly average temperature, *MWMT* mean weekly maximum temperature, *MMAT* mean monthly average temperature, *MMTmax* mean monthly maximum temperature, *MDTR* mean daily temperature range, *NA* Not Applicable

[a] The first coordinate is meters east longitude; the second value is meters north latitude

failed, and for or this reason, temperature records from the U.S. Bureau of Reclamation Izee Falls gauging station, which is 10 km upstream of the IZ study location, were used for establishing the thermal profile for that location. Temperature data (average and maximum temperatures for 24 h, 7 d, and 1 month prior to fish collection) were summarized by sample date at each study location for use in regression analyses (Table 1).

Tissue sample preparation for protein and hsp70 determination

Liver and white muscle tissue was prepared following methods described in Feldhaus et al. (2008). Fin tissue was frozen with liquid nitrogen in a 1.5 mL eppendorf tube, and crushed into small pieces with a Teflon pestle. Lysis buffer and protease inhibitors were added and the tissue further homogenized by hand. Following homogenization, fin tissue was centrifuged (4500 × g) at 1°C. The resulting supernatant was aliquoted and replicate samples stored at -80°C. Protein concentrations in lysates were measured in duplicate using the bicinchoninic acid assay (BCA) method (Smith et al. 1985).

Heat shock proteins were measured using Western blots analysis. Briefly, for Western blot analysis, samples were diluted to 25 $\mu g \cdot protein^{-1}$ per lane and run on 8% tris–glycine gels (Invitrogen Corporation) for 2 h at 125 V. A calibrated molecular weight marker (Biorad) and 52 ng of recombinant Chinook salmon hsp70 protein (SPP- 763; StressGen Biotechnologies, Victoria, British Columbia) were applied to each gel to serve as internal standards for determining molecular weight and blotting efficiency. Proteins were transferred to a polyvinylidene difluoride membrane at 100 V for 1 h, then blocked overnight at 48°C in blocking solution (5% nonfat dry milk, 20 mM tris buffer, and 0.1% Tween-20).

The membrane was probed with a commercially available polyclonal hsp70 antibody (StressGen, SPA-758) followed by an alkaline phosphatase conjugated goat-anti rabbit IgG (StressGen, SAB-301) antibody (diluted 1:5000 in blocking solution) to detect the presence of hsp70. According to the manufacturer, the primary antibody detects proteins of the molecular mass of 70 and 73 kDa, corresponding to the apparent molecular mass of constitutive hsc70 (hsp73) and inducible hsp70 (hsp72) isoforms. All incubations were performed at room temperature for 1 h. Proteins were visualized colorimetrically using an alkaline phosphatase conjugate substrate kit (Biorad, 170-6432). Blots were developed for 15 min, after which they were rinsed with distilled water for 10 min to stop the reaction. Relative hsp70 band density was measured using ImageQuant 6.1 (Amersham Biosciences) densitometry software. Protein band density is expressed by subtracting background and dividing band density of the unknown sample by the hsp70 protein standard band density.

Only one hsp70 band resolved from protein extracted from both liver and fin tissue samples. For this reason, we do not attempt to distinguish between constitutive and inducible hsp70 isoforms in liver tissue. In white muscle tissue, either one or two bands resolved. Based on the specificity of the antibody supplied by the manufacturer, we determined the single band that was always present in white muscle tissue blots to be the constitutive form and the second, lower molecular weight band that was not always present to be the inducible hsp70. Hereafter, we refer to these two bands as constitutive and inducible hsp70.

Lipid determination

Total lipid content for each fish collected from the SFJD was determined following the methods described in Anthony et al (2000) and Reynolds and Kunz (2001). Fish were thawed and weighed, then dried to a constant mass (i.e., ± 0.01 g of previous 24 hr mass) in a convection oven set at 60°C. Fish were thoroughly homogenized with mortar and pestle. Lipids were extracted from dried samples with a Soxhlet apparatus and a 7:2 (v/v) hexane/isopropyl alcohol solvent system. Fat mass was determined by subtracting the mass of dried homogenized fish before Soxhlet extraction from the mass of lean dry fish mass after fat extraction. Whole body lipid (WBL) content was calculated by dividing the fat mass by lean dry mass.

Data analysis

Statistical analysis was completed with GraphPad Prism for Windows V5.01 (San Diego, CA, USA). Significance was determined at $P \leq 0.05$. Data were analyzed using regression techniques, general linear models, and one and two-way ANOVA. Paired t-tests were used to

compare liver and fin band density for samples from the same field location. Post-hoc comparisons were conducted with the Tukey-Kramer multiple comparison test. The induction temperature (i.e., increased protein synthesis over basal levels) for each temperature metric was estimated by fitting a sigmoid dose-response curve with parameters from the dose-response equation (i.e., three-parameter logistic equation; $y = bottom + (top - bottom)/\ (1 + 10\hat{}(logEC_{50} - X))$. The induction temperature was determined to be $LogEC_{50}$ where EC_{50} represents the midpoint (value=0.5) between the bottom and top of the curve.

Results

Laboratory experiment

The average fork length of fish in the laboratory experiment was 144.5±10.1 mm (mean±S.D) and average mass was 32.7±6.1 g. Peak temperatures recorded for thermal treatments were 19.5, 22.7, and 25.7°C. For the 19°C stressor, temperatures were between 19.0 and 19.5°C for an average of 119 min. For the 22°C stressor, temperatures were maintained between 22.0 and 22.7°C for an average of 105 min, and for the 25°C stressor, between 25.0 and 25.7°C for an average of 91 min.

Hsp70 levels measured in trout livers did not vary among similar treatments through time ($P > 0.05$), allowing us to pool samples among tanks for the same treatment. Liver hsp70 levels in treatment fish subjected to the 19°C temperature stressor were similar to controls (Fig. 2, P=0.88). Both the 22 and 25°C stressors significantly increased liver hsp70 band density over controls (P<0.0001). Treatment fish exposed to 25°C had significantly greater liver hsp70 band density measurements than treatment fish exposed to 22°C, and fish exposed to 22°C had significantly higher hsp70 levels than fish exposed to 19°C and control fish. A sigmoid dose-response curve fit the data better than a straight line (P<0.0001, r^2= 0.85). The induction temperature based on the dose-response equation $(y = 0303 + (0873 - 0303)/(1 + 10\hat{}(22.9 - \times)))$ was 22.9°C with a 95% confidence interval around $logEC_{50}$ between 22.8 and 23.1°C. After accounting for treatment effects, neither fish length nor mass were a significant predictor of liver hsp70 (P>0.50).

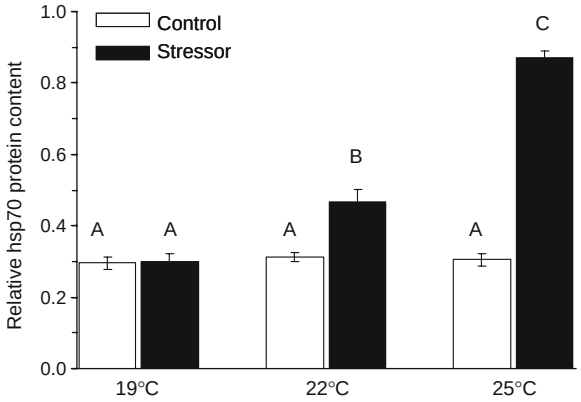

Fig. 2 Relative heat shock protein 70 (*hsp70*) content in liver tissue from rainbow trout (*Oncorhynchus mykiss*) acclimated to ambient water temperature (13°C) and exposed to an acute temperature stress of 19, 22, or 25°C followed by a 12-hour recovery at 13°C. Values represent mean densitometry values of protein bands (± 1 standard error of the mean) detected by Western blotting; n=6 for each bar. Open bars represent control fish and dark bars represent treatment fish. Shared letters indicate a non-significant difference (P>0.05)

Temperature metrics and hsp70 levels in fish from the South Fork John Day River

Temperature metrics, summarized in Table 1, varied significantly among the eight study locations. For example, the 24 h maximum temperature prior to fish collection and the mean weekly maximum temperature (MWMT) ranged from 17.8–25.8 and 17.9–25.3°C, respectively

Significant differences in the fork length ($F_{7,\ 72}$=3.43, P=0.003) and mass ($F_{7,72}$=3.21, P=0.005) of fish collected from the eight study locations precluded analysis of hsp70 expression based on fish size. The smallest fish were captured at DC (average fork length= 109 mm, average mass=15.0 g) and the largest fish were captured at Upper Murderers Creek (UMC; average fork length=139 mm, average mass=33.2 g; Table 2). Based on average fork length, fish collected from DC were significantly smaller than fish collected from the SFJD at RKM 24 (SF) and UMC (P<0.05), and upper Black Canyon creek (UBC) were smaller than fish from UMC and UBC (P<0.05). The average mass of fish collected from DC and was significantly less than fish collected from UMC.

There were significant differences in liver hsp70 levels for fish collected from each of the eight study locations ($F_{7,\ 68}$=35.04, P<0.0001). The lowest liver hsp70 levels were measured in fish collected from the

Table 2 Average mass (g) and fork length (mm±standard deviation) of fish sampled from the SFJD on 23-24 July 2005. For each sample location, the sample size (*N*) is 10 fish

Sampling location	Mass (g)	Homogeneous groups	Fork length (mm)	Homogeneous groups
Upper Black Canyon (UBC)	15.5 (± 3.8)	X	113 (± 9.7)	X X
Lower Black Canyon (LBC)	25.4 (± 4.0)	X X	129 (± 6.3)	X X X
SF at RKM 24 (SF)	28.6 (± 8.4)	X X	136 (± 12.9)	X X
Upper Murderers Creek (UMC)	33.2 (± 16.4)	X	139 (± 26.0)	X
Lower Murderers Creek (LMC)	24.9 (± 14.3)	X X	125 (± 22.3)	X X X
Below Wind Creek	25.4 (± 12.8)	X X	132 (± 22.1)	X X X
Izee Falls	23.0 (± 7.3)	X X	129 (± 9.5)	X X X
Deer Creek	15.0 (± 3.6)	X	109 (± 9.8)	X

Within each column of homogenous groups, shared X's form groups that are not significantly different ($P>0.05$)

two locations with the lowest water temperatures, UBC and DC. Liver hsp70 levels in fish from UMC and lower Murderers Creek (LMC), IZ, below Wind Creek (BW), and the SF were similar to each other, and significantly greater than liver hsp70 levels measured in fish from lower Black Canyon Creek (LBC; $P<0.0001$). In general, tissue samples with the highest hsp70 levels were collected from fish sampled at locations with the highest water temperatures (e.g., BW, SF, LMC). The calculated hsp70 induction temperatures for liver, fin, and white muscle tissue using different temperature metrics are shown in Table 3.

Fin tissue hsp70 measurements showed a similar pattern to liver hsp70 band density (Fig. 3). A significant relation between fin and liver tissue hsp70 was also found in a sub-sample of fin tissues analyzed in the laboratory experiment (data not shown). Excluding samples that did not have values for both fin and liver tissues, there was a significant linear correlation between liver and fin hsp70 (r^2= 70.5%). With the exception of UMC and IZ, where in both locations liver hsp70 values were significantly higher than fin hsp70 values (paired t-test, $P=0.038$ and 0.027, respectively), there were no significant differences between fin and liver tissue hsp70 density values.

In white muscle tissue, only the constitutive hsp70 band resolved from fish collected from the three coldest stream segments (UBC, DC, and LBC). At the other five sample locations, both the constitutive and inducible hsp70 bands resolved for each sample collected. There were significant differences in hsp70 band density measurements among locations for both the constitutive ($F_{7,78}=21.68$, $P<0.0001$) and inducible bands ($F_{7,78}=78.89$, $P<0.0001$; Fig. 4).

In general, liver, fin, and white muscle hsp70 levels reached maximal synthesis levels when MWMT reached 20–22°C. Based on sigmoid curve fits, the estimated induction temperature for all temperature metrics is an average of 0.2 to 2.3°C higher for white muscle and fin tissue than liver tissue (Table 3). The difference in the estimated induction temperature ranges for fin and white muscle tissue is ≤ 0.2°C.

Whole body lipids

There was a significant difference in WBL among fish in all eight-study locations ($F_{7,72}=5.27$, $P<$ 0.0001; Fig. 5). Mean WBL levels ranged from 13.9% for fish collected at LMC to 21.7% for fish collected at UMC. A second order polynomial regression of MWMT on WBL accounted for 19.9% of the variation between WBL and MWMT ($P=$ 0.0002). The maximum MWMT before lipid levels began to decrease with increasing water temperature was 20.4°C. The pre-visit maximum (i.e., maximum daily temperature prior to fish collection) temperature accounted for the largest amount of variation in the data (r^2=20.7%) and the MMAT accounted for the least amount of variation (r^2=9.6%). Polynomial equations and maximum temperature values for each temperature metric are summarized in Table 4.

To determine if temperature and hsp70 levels affected lipid levels, we used a general linear model to examine the interaction between each temperature metric and cellular hsp70 in liver, fin, and

Table 3 The relation between water temperature measurements preceding fish collection and estimated threshold temperatures for heat shock protein 70 (hsp70)

Temperature measurement	Dose response/polynomial equation	Estimated threshold temperature (°C)	correlation with hsp70
Liver hsp70			
24 h average	$y = 0.304 + (0.793 - 0.304)/(1 + 10^{\wedge}(17.8 - \times))$	16.9 to 18.7	0.717
24 h maximum	$y = 0.237 + (0.788 - 0.237)/(1 + 10^{\wedge}(20.6 - \times))$	20.3 to 20.9	0.775
pre-visit max	$y = 0.249 + (0.790 - 0.249)/(1 + 10^{\wedge}(21.3 - \times))$	21.0 to 21.6	0.774
MWAT	$y = 0.241 + (0.795 - 0.241)/(1 + 10^{\wedge}(16.7 - \times))$	16.4 to 17.0	0.745
MWMT	$y = 0.251 + (0.794 - 0.251)/(1 + 10^{\wedge}(20.8 - \times))$	20.5 to 21.1	0.769
MMAT	$y = 0.242 + (0.796 - 0.242)/(1 + 10^{\wedge}(15.9 - \times))$	15.6 to 16.2	0.744
MMTmax	$y = 0.253 + (0.772 - 0.253)/(1 + 10^{\wedge}(19.2 - \times))$	18.8 to 19.6	0.640
Fin hsp70			
24 h average	$y = 0.249 + (0.744 - 0.249)/(1 + 10^{\wedge}(18.6 - \times))$	18.2 to 19.0	0.742
24 h maximum	$y = 0.252 + (0.771 - 0.252)/(1 + 10^{\wedge}(22.0 - \times))$	22.5 to 23.2	0.756
pre-visit max	$y = 0.248 + (0.763 - 0.248)/(1 + 10^{\wedge}(22.8 - \times))$	22.5 to 23.2	0.758
MWAT	$y = 0.200 + (0.742 - 0.200)/(1 + 10^{\wedge}(17.1 - \times))$	16.8 to 17.4	0.765
MWMT	$y = 0.230 + (0.747 - 0.230)/(1 + 10^{\wedge}(21.6 - \times))$	21.2 to 21.9	0.760
MMAT	$y = 0.200 + (0.743 - 0.200)/(1 + 10^{\wedge}(16.3 - \times))$	16.0 to 16.6	0.764
MMTmax	$y = 0.214 + (0.740 - 0.214)/(1 + 10^{\wedge}(19.8 - \times))$	19.5 to 20.1	0.709
White Muscle Tissue hsp73 (hsc70)			
24 h average	$y = 0.075 + (0.824 - 0.075)/(1 + 10^{\wedge}(18.6 - \times))$	18.4 to 18.9	0.848
24 h maximum	$y = 0.080 + (0.857 - 0.080)/(1 + 10^{\wedge}(22.8 - \times))$	22.6 to 23.0	0.862
pre-visit max	$y = 0.073 + (0.845 - 0.073)/(1 + 10^{\wedge}(22.8 - \times))$	22.5 to 23.0	0.863
MWAT	$y = 0.008 + (0.821 - 0.008)/(1 + 10^{\wedge}(17.1 - \times))$	16.9 to 17.3	0.867
MWMT	$y = 0.047 + (0.826 - 0.047)/(1 + 10^{\wedge}(21.6 - \times))$	21.3 to 21.8	0.867
MMAT	$y = 0.008 + (0.821 - 0.008)/(1 + 10^{\wedge}(16.3 - \times))$	16.1 to 16.5	0.867
MMTmax	$y = 0.004 + (0.813 - 0.004)/(1 + 10^{\wedge}(19.8 - \times))$	19.5 to 20.0	0.787
White muscle tissue hsp72 (hsp70)			
24 h average	$y = 0.362 + (1.554 - 0.362)/(1 + 10^{\wedge}(18.7 - \times))$	18.3 to 19.0	0.780
24 h maximum	$y = 0.374 + (1.630 - 0.374)/(1 + 10^{\wedge}(22.9 - \times))$	22.7 to 23.2	0.801
pre-visit max	$y = 0.365 + (1.604 - 0.365)/(1 + 10^{\wedge}(22.9 - \times))$	22.6 to 23.1	0.800
MWAT	$y = 0.272 + (1.549 - 0.272)/(1 + 10^{\wedge}(17.2 - \times))$	16.9 to 17.4	0.787
MWMT	$y = 0.331 + (1.566 - 0.331)/(1 + 10^{\wedge}(21.7 - \times))$	21.4 to 21.9	0.795
MMAT	$y = 0.272 + (1.550 - 0.272)/(1 + 10^{\wedge}(16.4 - \times))$	16.1 to 16.6	0.787
MMTmax	$y = 0.328 + (1.542 - 0.328)/(1 + 10^{\wedge}(19.9 - \times))$	19.6 to 20.1	0.712

Estimated threshold temperatures were determined by constructing 95% confidence interval for the estimated threshold temperature at $LogEC_{50}$ where EC_{50} represents 50% of the hsp70 values greater than 0.5. Juvenile redband trout (*O. mykiss gairdneri*) were collected from 23-24 July 2005. The 24 h average, 24 h minimum, and 24 h maximum are for water temperatures during the 24 h preceding fish collection, pre-visit maximum is the maximum temperature recorded prior to fish collection

MWAT mean weekly average temperature, *MWMT* mean weekly maximum temperature, *MMAT* mean monthly average temperature, MMT_{max} mean monthly maximum temperature

white muscle tissue. The interaction between each temperature metric and hsp70 was significant for fin and white muscle tissue ($P<0.05$), but not liver tissue ($P>0.20$).

Discussion

Most studies of hsp70 expression in fish have occurred in laboratory settings. Fewer studies exam-

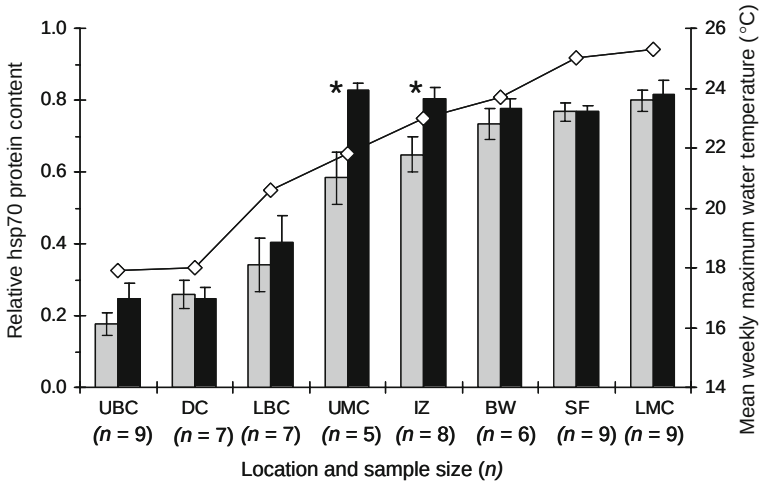

Fig. 3 Relative heat shock protein 70 (*hsp70*) band density (mean densitometry value±1 standard error of the mean) in fin (*grey bars*) and liver (*black bars*) from redband trout (*Oncorhynchus mykiss gairdneri*). Fish were collected from the South Fork John Day River (*SFJD*) on 23-24 July 2005 at upper and lower Black Canyon (*UBC*, *LBC*), Deer Creek (*DC*), upper and lower Murderers Creek (*UMC*, *LMC*), Izee falls (*IZ*), below Wind Creek (*BW*), and the SFJD at river kilometer 24 (*SF*). Temperature values (*diamonds*) represent the mean weekly maximum temperature (°C). The asterisk represents a significant difference (paired t-test, $P<0.05$) in hsp70 band density between liver and fin tissue

ine wild fish populations, and for this reason, changes in cellular hsp70 and subsequent impacts on wild fish populations are poorly understood. A complete understanding of the physiological temperature limits of wild fish populations helps guide discussions directed at establishing temperature standards for endangered fish populations and provides context for valuing stream restoration actions. The results presented in this paper suggest that measurements of hsp have potential to further the understanding of physiological temperature requirements for naturally producing salmonid populations.

We found a relationship between temperature and hsp70 induction, both in the laboratory and field data. Because the duration of the maximum temperature exposure in the laboratory was less than 2 h, the two most appropriate temperature metric comparisons between the field and laboratory studies were the pre-visit and 24 h maxima. Using pre-visit and 24 h maxima to compare our laboratory and field results, we found the estimated liver hsp70 induction temperature determined in the laboratory setting (22.8–23.1°C) were slightly higher than values from liver tissue collected from fish in the SFJD (20.3–21.6°C). These results are similar, but slightly higher, than the values reported by Werner et al. (2005), who examined hsp70 expression in white muscle tissue from steelhead parr collected from the Navarro River, CA.

The difference in the estimated liver hsp70 induction temperature range between the laboratory fish and fish from the SFJD represents one potential problem with trying to compare results from laboratory experiments and field observations. One explanation for these differences is a population specific hsp70 response to temperature stress. The laboratory fish were first generation progeny steelhead trout (*O. mykiss)* from the Umatilla River and represent a different subpopulation than fish from the SFJD. Significant differences in the hsp response, hepatic glycogen, and cellular energy metabolites between hatchery and wild populations of *O. mykiss* have been reported (Werner et al. 2006). A second explanation is seasonal differences (Fader et al. 1994). Fish from the SFJD were sampled in the late July while fish in the laboratory experiment were sampled in November.

As indexed by increases in hsp70, juvenile redband rainbow trout in certain sections of the SFJD River seem to be experiencing thermal stress during summer conditions. Signs of physiological dysfunction are indicated by the induction of hsp70 in liver, white muscle, and fin tissue, plus the decrease in WBL associated with this induction, thus confirming the utility of hsp70 as a potential biomarker of thermal stress. Our finding that hsp70 can be measured in fin tissue, and that these results are comparable to measurements in liver and muscle tissue is important

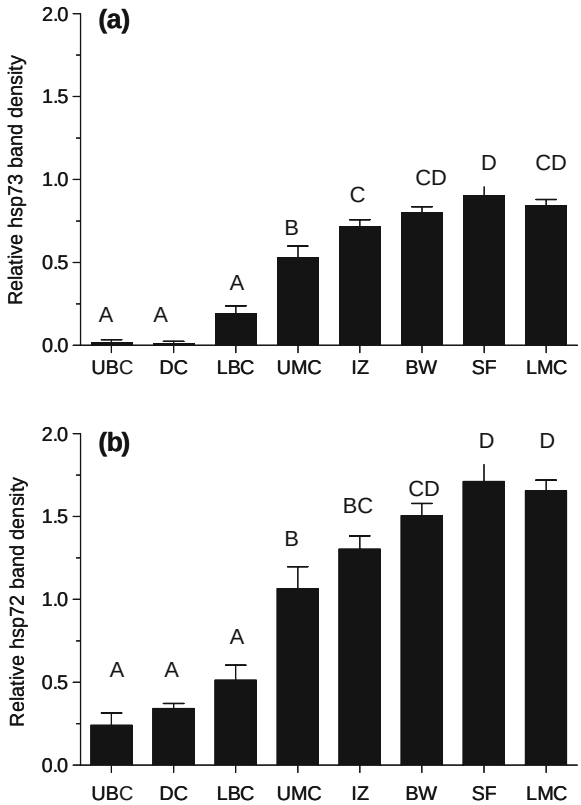

Fig. 4 Relative expression of **a** hsp72 (*hsp70*) and **b** hsp73 (*hsc70*) in white muscle tissue from redband trout (*Oncorhynchus mykiss gairdneri*). Hsp70 values represent mean densitometry values of protein bands (± 1 standard error of the mean). Shared capital letters above the vertical bars at different sampling locations indicate a non-significant difference in hsp70 ($P>0.05$) between locations. Each bar represents $N=10$, except UBC ($N=9$). Fish were collected on 23-24 July 2005 from upper and lower Black Canyon (*UBC*, *LBC*), Deer Creek (*DC*), upper and lower Murderers Creek (*UMC*, *LMC*), Izee falls (*IZ*), below Wind Creek (*BW*), and the South Fork John Day River at river kilometer 24 (*SF*)

because it provides a non-lethal procedure for measuring hsp70 levels, although this is not the first use of fin tissue to measure hsps. Pelvic fin tissue was used to measure hsps in green sturgeon (*Acipenser medirostris*; Allen et al. 2006).

For fish from the SFJD, the estimated induction temperature for hsp70 varied depending on tissue type and the temperature metric chosen for predicting the induction temperature. In general, estimates for white muscle hsp70 induction were higher than for liver tissue. This difference between tissue types is important because it highlights both the utility and danger in using hsp70 as an indicator of thermal habitat quality. Whereas the cellular stress response can be used to provide information about cellular temperature tolerances that help define an animal's physiological temperature tolerance, the different induction thresholds found among the different tissue types, and differences in the magnitude of the hsp response between juvenile and adult fish (Fowler et al. 2009), could lead to establishment of improper thermal criteria. For example, based on findings reported here, thermal criteria based on the cellular response in white muscle and fin tissue for fish from the SFJD could potentially result in approximately a 2°C higher thermal maximum criteria than would be established if liver tissue were used to set thermal maximum criteria.

One confounding factor in collecting fish from the SFJD and comparing hsp70 levels with longitudinal stream temperature profiles was fish movement (e.g., behavioral thermoregulation). In this study, fish collected from LBC had greater variability in hsp70 expression measured in fin and liver tissue than fish collected from any other location. The LBC study location is within 300 m of the mainstem SFJD. On average, summertime water temperatures are 3–5°C higher in the SJFD than in the lowest reach of BC. Therefore, it is possible that several fish collected at LBC on 24 July 2005 experienced high temperatures in the SFJD prior to seeking thermal refuge in LBC. This conclusion is supported by a concurrent fish movement study in LBC that detected trout moving upstream between 17 June and 20 July 2005 (Tattam 2006).

In the field component of this study, we found that the interaction between temperature and hsp70 measurements in fin and white muscle tissue, but not liver tissue, were significant predictors of WBL. In part, this finding is supported by the trend in the data for lipids levels to increase with temperature to a maximum and then decrease with increasing temperature. The lack of an interaction between hsp70 in liver tissue and temperature in predicting whole body lipids may represent a difference in function between tissues. Whereas white muscle tissue acts as a storage compartment for WBL (i.e., energy), two primary functions of the liver are energy regulation and blood filtering. Within these different tissues, it is reasonable to expect a different hsp response to temperature because both tissues perform different tasks at the same temperature. The lack of a significant interaction between hsp70 and temperature in liver tissue

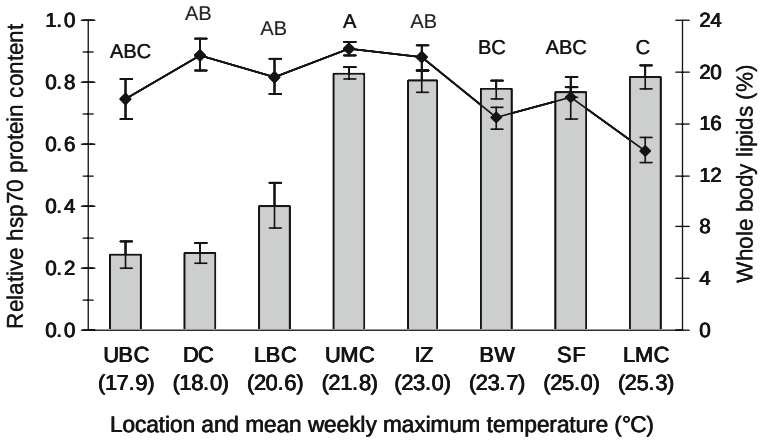

Fig. 5 The mean weekly maximum temperature (MWMT) compared to the percent (%) whole body lipids (black diamonds) and relative hsp70 band density (vertical grey bars) in liver tissue of juvenile redband trout (*Oncorhynchus mykiss gairdneri*). For hsp70 band density, values represent the mean (± 1 standard error of the mean), and each bar represents $n=10$ except BW and UBC ($N=9$) and UMC ($N=8$). Whole body lipid levels represent the mean (± 1 standard error of the mean), and each diamond represents $N=10$. Shared capital letters above the diamonds at different sampling locations indicate a non-significant difference ($P>0.05$) in whole body lipids between locations. Fish were collected from the South Fork John Day River on 23-24 July 2005 at upper and lower Black Canyon (UBC, LBC), upper and lower Murderers Creek (UMC, LMC), Deer Creek (DC), Izee Falls (IZ), below Wind Creek (BW), and the South Fork of the John Day River at river kilometer 24 (SF)

reconfirms the potential danger in comparing the hsp70 response among animals using different tissues.

In our study, there was variation in WBL within and between study locations. This variation in WBL might be related to either differences in food availability among study locations (Tait et al. 1994) or appetite suppression (Linton and Morgan 1998; Myrick and Cech. 2000), both due to variation in water temperature. The effects of temperature on food consumption, growth, and thermal tolerance presented in Myrick and Cech (2000) support our finding that temperature is an important variable effecting WBL levels. Alternatively, variation in WBL levels may be related to energetic demands of elevated hsp70 synthesis. The synthesis of proteins is energetically costly (Hochachka and Somero 2002), and there is some evidence of a relation between increased hsp72 and decreased metabolic condition (Viant et al. 2003). However, a study with steelhead parr (*O. mykiss*) concluded energy reserves did not decrease following

Table 4 Polynomial equations describing the relation between whole body lipid levels and the estimated maximum water temperature before lipid levels began to decrease with increasing water temperature

Temperature metric	Polynomial equation	Maximum temperature (°C)	p-value	r-squared
24 h avg	$y = -64.56 + 9.60\times - 0.269\times^2$	17.8	0.0006	17.4
24 h max	$y = -112.64 + 12.63\times - 0.298\times^2$	21.2	0.0001	19.5
pre-visit max	$y = -123.11 + 13.50\times - 0.315\times^2$	21.4	0.0001	20.7
MWAT	$y = -56.24 + 9.07\times - 0.267\times^2$	17.0	0.002	14.7
MWMT	$y = -85.84 + 10.42\times - 0.255\times^2$	20.4	0.0002	19.9
MMAT	$y = -49.90 + 8.83\times - 0.276\times^2$	15.8	0.008	9.6
MMTmax	$y = -74.33 + 9.93\times - 0.259\times^2$	19.2	0.0006	17.5

The 24 h average, 24 h minimum, and 24 h maximum are for water temperatures during the 24 h preceding fish collection, pre-visit maximum is the maximum temperature recorded prior to fish collection

MWAT mean weekly average temperature, *MWMT* mean weekly maximum temperature, *MMAT* mean monthly average temperature, MMT_{max} mean monthly maximum temperature

increased expression of hsp after heat shock (Werner et al. 2006). Changes in WBL in trout from the SFJD are probably best explained by a combination of the factors described above.

The cellular stress response is species, organ, and stressor specific, and factors other than temperature can alter hsp levels (Iwama et al. 2004). For example, bacterial kidney disease infection increased liver hsp70 levels in coho salmon (O. kisutch; Forsyth et al. 1997), and industrial effluents (Vijayan et al. 1998), heavy metals (Sanders 1993), and pesticides (Hassanein et al. 1999), have all been reported to alter hsp70 levels in fish. In contrast, handling stress (Vijayan et al. 1997), and electrofishing (Lund et al. 2002; Werner et al. 2005) have no apparent effect on hsp70 levels in salmonids, nor do anaesthesia, hypoxia, hyperoxia, capture stress, crowding, feed deprivation, and cold stress (Zarate and Bradley 2003). While we cannot completely dismiss other factors, the correlation between hsp70 and temperature in this study suggests that temperature is a variable that contributes to alterations in hsp70 levels of fish in the SFJD.

Future research on the physiological thermal tolerance of fish would benefit from simultaneous measurements of both hsp and ubiquitin. Ubiquitin is a highly conserved 76 amino acid protein that covalently binds to denatured protein for subsequent proteolysis by non-lysosomal proteases (Rechsteiner 1987; Ciechanover and Schwartz 1998). Thus, ubiquitin conjugates provide information about the amount of irreversibly damaged protein in cells. There are few examples of ubiquitin measurements in an ecologically and environmentally relevant context (see Hofmann and Somero 1995, 1996 for an example of ubiquitin measurements in intertidal mussels). Another way data on the hsp response could be applied is to use information on hsp70 induction and whole body lipid levels to model physiological limits under different habitat conditions. The hsp response provides a metric that gauges the cellular stress response, and lipid levels serve as an index of the animal's energy reserves.

Collections of fish in our field study, where maximum temperatures exceeded 25°C, support observations that redband trout can tolerate stream temperatures near 26°C (Behnke and American Fisheries Society 1992; Zoellick 1999). However, increased hsp70 levels suggest these fish are experiencing thermal stress, and this stress may compromise long-term fitness. With respect to local land use and climate change, the definition, interpretation, and understanding of physiological temperature limits of all life stages is important for helping guide management decisions and restoration efforts targeted at preserving not only threatened and endangered fish, but aquatic communities.

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