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Joseph W. Feldhaus for the degree of Master of Science in Fisheries Science presented on April 27, 2006.
Title: Heat Shock Proteins and Physiological Stress in Redband Rainbow Trout (*Oncorhynchus mykiss gairdneri*) in the South Fork John Day River, Oregon.

Abstract approved:

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The goal of this research was to characterize thermal habitat requirements for juvenile redband steelhead trout (*Oncorhynchus mykiss gairdneri*) in the South Fork John Day River (SFJD), Oregon using physiological indicators of fish condition. Physiological indices of fish condition measured were whole body lipid content and heat shock proteins, specifically hsp70. Hsp70 is a family of highly conserved molecular chaperones proteins that protect cellular function by preventing irreversible protein damage. Hsp70 levels were measured in liver, white muscle, and fin tissue.

This thesis includes a study of passive integrated transponder (PIT) tag handling stress in juvenile rainbow trout. PIT tags are used to identify individual fish. The objectives of the PIT tag study were to determine if there is a stress response, measured as a difference in plasma cortisol levels, between PIT tagged and non-PIT tagged rainbow trout (*O. mykiss*), to determine if PIT tag-related stress induces hepatic hsp70 synthesis in juvenile rainbow trout, and to examine the relation between cortisol and hsp70 levels. There was a difference in plasma cortisol six hours after tagging, with both treatment and tank effects. There were no differences detected after the 24h sampling period. Plasma cortisol levels were highly variable, but changes in plasma cortisol did not alter hepatic hsp70 levels.

A laboratory experiment demonstrated that hsp70 levels increase significantly between 19 and 22ºC in both fin and liver tissue. The finding that hsp70 can be quantified in fin tissue is significant because it provides a non-lethal technique for assessing thermal stress in rare or endangered fish. The response of hsp70 in relation to temperature was sigmoid.

During the summers of 2004 and 2005, a field study in the SFJD was conducted. The objectives were to: (1) determine if SFJD redband rainbow trout experience thermal stress, (2) develop a non-lethal technique for measuring cellular hsp70 levels, (3) and characterize the relation between whole body lipids and hsp70 for fish in the SFJD. Maximum hsp70 levels in liver and white muscle tissue in field collected fish occurred when mean weekly maximum temperatures (MWMT) exceeded 22-23ºC. Short and long term stream temperature averages of 15.6-18.2ºC and temperature maximums of 18.8-21.6ºC significantly increased hsp70 levels in liver tissue. Both the hsp72 and hsp73 isoforms were significantly elevated in white muscle tissue when long and short term average stream temperatures were 16-18.5ºC and 19.6-23ºC for temperature maximums. Lipid levels began to decrease when MWMT exceeded 23ºC.

Results of this research suggest increased cellular hsp70 levels in juvenile redband rainbow trout in the SFJD River is symptomatic of cellular stress related to thermal conditions, as are decreasing lipid levels. Measurement of cellular hsp70 levels provides another tool that can be used to characterize physiological suitability of thermal habitat and potentially to define thermal limits, as differences of 1 or 2°C are sufficient to change expression of thermal stress proteins detected using these techniques. When using hsp70 as an index of thermal stress in different *O. mykiss* subpopulations, differences in hsp70 expression between tissues should be considered.

Heat Shock Proteins and Physiological Stress in Redband Rainbow Trout (*Oncorhynchus mykiss gairdneri*) in the South Fork John Day River, Oregon

by
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I understand that my thesis will become part of the permanent collection of the Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Joseph W. Feldhaus, Author

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Chapter 1: General Introduction

Introduction

A Biological Opinion (BiOp) was issued by the US National Oceanic and Atmospheric Administration Fisheries (NOAA-Fisheries) in 2000 that described operations of the Federal Columbia River Power System. One result of the BiOp was a mandated federal research, monitoring, and evaluation program (RME) for the Columbia River Basin. The goal of the tributary Federal tributary RME program is to describe the "health" of fish population processes and their habitat so it can be determined if mitigation or changes to current management are required to ensure survival of Columbia River Basin salmon and steelhead listed under the Endangered Species Act (Bouwes 2005). Prior to wholesale application of the RME approach, it is first being tested through pilot projects in the Wenatchee, John Day, and Salmon river subbasins. These pilot projects will help identify and prioritize restoration actions that will increase freshwater salmonid production (Bouwes 2005).

Monitoring of longitudinal summer stream temperatures is one approach being tested as a method for rapidly assessing the carrying capacity of threatened and endangered salmonids. It has been hypothesized that fish will select habitat offering the best opportunities for growth and survival as outlined by the Ideal Free Distribution (IFD) concept (Li and Bowen, 2003). The ideal free distribution (IFD) theory proposed by Fretwell and Lucas (1970) predicts animals will distribute themselves in habitat of different availability so that animals will receive an equal proportion of available resources. Hughes and Grand (2000) modified the IFD concept and incorporated a physiological model of fish growth that predicts input-matching of fish to resource renewal rates is an exception and that temperature-based size-segregation is more common in nature. By linking summer temperatures with physiological performance indices, it should be possible define summer water temperatures as physiologically suitable, marginal, and unsuitable.

The South Fork John Day subbasin in Northeastern Oregon is on the Oregon 303d list for Water Quality Limited Streams[1]. Anthropogenic disturbances within catchment basins have increased summer stream temperatures, and are believed to be a contributing factor that limits juvenile steelhead trout production. The SFJD is an important rearing and spawning ground for a population of Middle Columbia River steelhead trout (*O. mykiss)* that were listed in 1999 by NOAA-Fisheries as a threatened Evolutionarily Significant Unit (ESU). Steelhead trout east of the Cascade Mountains are considered to be part of the redband trout complex (Behnke 1992) and are sometimes referred to as redband steelhead trout (*O. mykiss gairdneri*). Redband trout can tolerate stream temperatures in excess of 26ºC (Behnke 1992; Zoellick 1999). One explanation for the persistence of inland populations of redband rainbow (resident population of *O. mykiss gairdneri*) trout in warm stream reaches is behavioral thermoregulation and not stock adaptation (Li et al. 1994). Another explanation is physiological adaptation to thermal history leading to temperature-dependent swimming performance and aerobic metabolism that allow for short term (< 2h) exposure to temperatures greater than 24ºC (Gamperl et al. 2002).

One technique that can be used to examine and document thermal stress in salmonids is measurement of cellular heat shock proteins (Lund et al. 2002, Lund et al. 2003, Werner et al. 2005). Heat shock proteins (hsps), or stress proteins, are highly conserved cellular chaperone proteins (Feder and Hofmann 1999). The genes encoding hsps have been found in every species examined (Feder and Hofmann 1999). Molecular chaperones can be synthesized constitutively or in response to a stressor (Hochachka and Somero 2002). Heat shock proteins assist proper protein folding and are involved with the cellular immune response (Zugel and Kaufmann 1999). Heat shock proteins are also involved in cellular signalling that regulate growth and development and have been hypothesized to alter physiological signals in response to stress or disease states (Nollen and Morimoto 2002). The increase in molecular chaperones in response to heat stress is called the heat shock response (Parsell and Lidquist 1994, Hochachka and Somero 2002). This heat shock, or cellular stress

[1] A complete list of 303(d) listed streams can be found at http://www.deq.state.or.us/wq/303dlist/303dpage.htm,

response, protects vital cellular functions by binding denatured proteins and preventing inappropriate associations between proteins (Parsell and Lidquist 1994). The cellular stress response can be elicited from a wide range of stressors; include extreme temperature, heavy metals, ultraviolet light, gases, hypoxia, hyeroxia, and exposure to alcohols (Feder and Hofmann 1999; Hochachka and Somero 2002, Sanders 1993). Heat shock proteins have also been identified as potential biomarkers for environmental stress in fish (Iwama et al. 1998).

Before heat shock proteins can be used as an indicator of cellular stress, it is important to establish that the response is not induced by handling the organism during collection (Sanders, 1993). In rainbow trout (*O. mykiss*), handling stress did not alter levels of hepatic hsp70 (Vijayan et al., 1997), and hepatic hp60 levels were not altered in muscle, gill, or heart tissue (Washburn et al., 2002). Common forms of hatchery related stress, including anaesthesia, formalin exposure, hypoxia, hyperoxia, capture stress, crowding, feed deprivation, and cold stress do not alter levels of gill hsp30, hsp70, or hsp90 (Zarate and Bradley, 2003) in Atlantic salmon (*Salmo salar*). The effect on hepatic hsp70 of passive integrated transponder (PIT) tags, commonly used in the Columbia river system to track salmonid movement, levels has not been explicitly studied.

Before hepatic hsp70 levels can be reliably measured in PIT tagged fish, it must first be determined if PIT tags alter hepatic hsp70 levels. PIT technology has been used to estimate survival and travel time of juvenile salmonids between hydroelectric dams equipped with PIT tag readers (Prentice et al. 1990b, Muir et al. 2001, Peterson et al. 1994, Hockersmith et al. 2003), and is being used in the SFJD River to assess the effect of lay flat panel stanchion irrigation dams on seasonal movement, habitat use, growth, and migration timing of juvenile steelhead trout (*Oncorhynchus mykiss*). Using PIT tags to track movement of *O. mykiss* in relation to thermal patterns would be useful.

However, there is evidence that cortisol, the primary stress hormone, plays a role in mediating hsp70 levels in fish tissue following physiological stress (Basu et al. 2001). Chronic increases in plasma cortisol levels can increase disease susceptibility, decrease growth rates, and inhibit reproduction (Hazon and Balment 1998, Schreck

2000, Schreck et al. 2001). No study has directly measured physiological indicators of stress related to PIT tagging, including cortisol and heat shock proteins. If increases in cortisol associated with PIT tagging impair the heat shock response, it would indicate PIT tagging fish in temperature impaired streams (e.g., SFJD River) has unforeseen physiological consequences impacting thermal tolerance not detected in laboratory studies of growth and survival.

The goal of this research is to examine the utility of using heat shock proteins, specifically hsp70, to measure thermal stress in wild populations of redband steelhead trout in the South Fork John Day River. The specific objectives addressed in Chapter 2 are to: (1) determine if there is a stress response, measured as a difference in plasma cortisol levels between PIT tagged and non-PIT tagged rainbow trout (*O. mykiss*), (2) determine if PIT tagging induces hepatic hsp70 synthesis in juvenile rainbow trout, and (3) examine the relation between cortisol and hsp70 levels in juvenile rainbow trout. In Chapter 3, the specific objectives are to (1) determine if redband rainbow trout in the SFJD River are experiencing thermal stress, (2) determine the temperature that increases cellular hsp70 levels, (3) develop a nonlethal technique for measuring cellular hsp70 levels, and (4) characterize the relation between whole body lipids and hsp70 for fish in the SFJD.

Information from this research will increase knowledge about the physiological impacts of elevated stream temperatures on juvenile redband steelhead trout production in the SFJD River. This information will also broaden understanding of how summer water temperatures relate to early life history strategies, growth, survival, distribution patterns, and the carrying capacity and production of summer trout habitat. Physiological criteria for suitability of juvenile redband steelhead trout habitat will also provide a useful index of habitat quality. A physiological index of habitat quality can be used to meet the goals of the Federal tributary RME program.

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Chapter 2: Passive Integrated Transponders do not alter hepatic hsp70 or plasma cortisol levels

Introduction

Passive integrated transponder (PIT) tags provide a method to uniquely identify (Prentice et al. 1990a) and track individual fish movement in streams (Ombredane et al. 1998, Roussel et al. 2000 and 2004; Cucherousset et al. 2005). In the Columbia River Basin, USA, this technology has been used to estimate survival and travel time of juvenile salmonids between hydroelectric dams equipped with PIT tag readers (Prentice et al. 1990b, Muir et al. 2001, Peterson et al. 1994, Hockersmith et al. 2003). Studies examining survival and tag retention of PIT tagged salmonids (Prentice et al. 1990a, Peterson et al. 1994, Gries and Letcher 2002, Dare 2003, Hockersmith et al. 2003) have found high survival and tag retention rates in juvenile salmonids.

In the South Fork John Day (SFJD) River in Northeastern, Oregon, PIT tags are being used to assess the seasonal movement, habitat use, growth, and migration timing of juvenile redband steelhead trout (*Oncorhynchus mykiss gairdneri)* as it relates to longitudinal temperature patterns and barriers to movement, including lay flat panel stanchion irrigation dams. The SFJD is on the Oregon 303d list for Water Quality Limited Streams[2], and elevated stream temperatures are believed to be a limiting factor in juvenile steelhead trout production. The ability to uniquely identify juvenile trout with PIT tags provides an opportunity to monitor movement and habitat use of individual salmonids in thermally impaired streams such as the SFJD.

No study has directly measured physiological indicators of stress related to PIT tagging, including cortisol and heat shock proteins (hsps). Physiological stress (e.g., handling) results in an elevation in plasma cortisol, the primary stress hormone, that can increase disease susceptibility, decrease growth rates, and inhibit reproduction (Hazon and Balment 1998, Schreck 2000, Schreck et al. 2001). Plasma cortisol also is important for energy metabolism, ion regulation, and can interact with other hormonal

[2] A complete list of 303(d) listed streams can be found at http://www.deq.state.or.us/wq/303dlist/303dpage.htm,

systems (Hazon and Balment). Heat shock proteins assist proper protein folding, are involved with the cellular immune response (Zugel and Kaufmann 1999), and play important roles in cellular signalling that regulate growth and development and have been hypothesized to alter physiological signals in response to stress or disease states (Nollen and Morimoto 2002).

Heat shock proteins, also called stress proteins, are molecular chaperone proteins that are classified into families based on molecular mass (kilodaltons; kDa). Constituitive hsp isoforms are synthesized under normal cellular conditions and inducible isoforms can be synthesized in response to both biotic and abiotic stressors (Hochacka and Somero 2002). Heat shock proteins are potential biomarkers for environmental stress in fish (Iwama et al. 1998) and have been used to evaluate thermal stress in juvenile salmonids (Lund et al. 2002, Werner et al. 2005).

The hsp70 protein family is highly conserved, correlated with thermal tolerance (Basu et al. 2002, Feder and Hofmann 1999), and highly temperature responsive (Sanders 1993, Parsell and Lindquist 1993). Measuring heat shock protein levels has advantages over measuring stress hormones (e.g., plasma cortisol) because levels of hsp70 in rainbow trout are not altered by handling stress (Vijayan et al. 1997, Washburn et al. 2002). Electrofishing has been reported to have no effect on heat shock protein levels (Lund et al. 2002, Werner et al. 2005). Common forms of hatchery stress including anaesthesia, hypoxia, hyperoxia, capture stress, crowding, feed deprivation, and cold stress were reported to have no effect on gill hsp70 levels in Atlantic salmon (*Salmo salar*; Zarate and Bradley 2003).

There is evidence that cortisol plays a role in mediating hsp70 levels in fish tissue following physiological stress (Basu et al. 2001), and may attenuate gill hsp30 (Ackerman et al. 2000) and hsp90 mRNA expression (Sathiyaa et al. 2001). If PIT tags increase plasma cortisol levels, this increase could adversely affect the ability of trout cells to initiate a heat shock protein response to subsequent thermal stress and may alter normal cellular responses to physiological signals. Therefore, the role between cortisol and hsp's should be investigated prior to using hsp levels as an indicator of physiological stress in PIT tagged fish, and the direct effect of PIT tagging on changes in plasma cortisol should be determined. If increases in cortisol levels

associated with PIT tagging impair the heat shock response, it would indicate PIT tagging fish has unforeseen physiological consequences that compromise thermal tolerance.

Given this background, the objectives of this study were to: (1) determine if there is a difference in plasma cortisol levels between PIT tagged and non PIT tagged rainbow trout (*O. mykiss*), (2) determine if PIT tagging induces hepatic (liver) hsp70 synthesis in juvenile rainbow trout, and (3) examine the relation between cortisol and hsp70 levels in juvenile rainbow trout.

Materials and methods

Fish and rearing conditions

The experiment was carried out at the Fish Performance and Genetics Laboratory of the Oregon State University in Corvallis, OR, between August 12 and 14 September 2004. Fish were zero-age hatchery steelhead trout (*Oncorhynchus mykiss*) from the Roaring River Fish Hatchery on the McKenzie River, Oregon. On 12 August 2004 a total of 180 rainbow trout were divided between four outdoor, 1-meter diameter circular fiberglass tanks (45 fish/tank) and exposed to natural photoperiod. Tanks were supplied with 13-14ºC flow through well water adjusted to1 liter/minute flow. The fish were acclimated to experimental tanks for 21 days prior to beginning the experiment. During the acclimation period, fish were fed by hand to satiation twice per day during the week and once per day on weekends with BioDiet Grower, a semi-moist commercial pellet manufactured by Bio-Oregon. Fish were considered satiated when feeding slowed and uneaten pellets remained on the tank bottom. The fish were fasted for 48h before the start of the experiment. For three days after the start of the experiment, all fish were fed 9-10 g of food per tank each day, and then fed to satiation one time each day for the duration of the study. This study was approved by the Institutional Animal Care and Use Committee at Oregon State University under permit #3055.

Experimental design and sampling procedures

After random assignment, Tanks 1 and 3 were assigned to handling control treatments, and Tanks 2 and 4 were assigned as PIT tagging treatments. Before processing each tank of fish, a fresh solution of buffered tricaine methanesulfonate (MS-222) was prepared in 5 liters of water for both light anesthesia (50 mg/l MS-222, 125 mg/l $NaHCO_3$) and lethal dose solutions (200 mg/l MS-222, 500 mg/l $NaHCO_3$). The light anesthesia was used during tagging and handling. At 0h, six fish were rapidly netted and killed from each tank. These fish provided baseline values for cortisol and hsp70 values prior to handling. Fish were lethally sampled at 2, 6, 24, and 120h post handling. A total of six fish were lethally sampled from each tank during each time period.

Immediately following the initial lethal sampling of a tank, the remaining fish were carefully and rapidly netted from that tank and handled according to the treatment (i.e., PIT tagging or handling control) assigned to the tank. Fish were considered anaesthetized and ready for handling after equilibrium was lost and opercular movements became irregular. It took between eight and ten minutes to process each tank of fish (N = 39/tank).

PIT tagging each fish took approximately three to five seconds per fish. PIT tagging involved inserting a 124.2-kHz PIT tag (11.5 x 2.1 mm; Biomark, ID) into the body cavity using a modified hand held syringe following the methods described in Prentice et al. (1990b). The fish in the non-PIT tag tanks were anesthetized in a manner identical to fish from the PIT tagged tanks and each individual fish was held out of the water for three to five seconds to serve as handling controls. Following handling, all fish were carefully placed back in the experimental tank and allowed to recover.

Length (mm) and mass (g) were recorded for all fish, and blood was collected into ammonium-heparinized capillary tubes by severing the caudal peduncle with a razor blade. Blood was transferred into microcentrifuge tubes, centrifuged, and plasma was separated and stored on ice. Immediately following blood collection, liver samples were removed, wrapped in aluminum foil, and flash frozen in liquid nitrogen. Plasma and liver samples were stored at -80ºC for later analysis.

Tissue sample preparation for hsp70 Western blotting

Liver samples were lysed in ice-cold lysis buffer (50 mM, 20 mM NaCl, 5 mM EDTA, pH 7.5) containing protease inhibitors (0.5 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluroride (PMSF), 1mg/ml aprotinin, and 0.7 mg/mL pepstatin). Liver samples were homogenized with a tissue tearor (Model 985-370; Biospec Products, Inc.) and tissue lysates were centrifuged for 30 minutes at 2700 x g at 0ºC, and the resulting supernatant was aliquoted and stored at -80ºC. Protein concentrations in lysates were assayed with the bicinchoninic acid (BCA) protein assay method (Sigma-Aldrich, Saint Louis, MO). Plates were read on a Molecular Devices Vmax Kinetic Microplate reader at 570 nm.

Western blotting

Western blot analyses were performed based on methods described by Towbin et al (1979). Briefly, protein samples were mixed with an equal amount of SDS sample buffer (Laemmli 1970), and then heated at 95ºC for 3 minutes. Equivalent amounts of protein (25 μg) were separated by gel electrophoresis on 8% Tris-Glycine gels (Invitrogen corporation) for 2 hours at 125 volts. A calibrated molecular weight marker (Biorad) and 52-ng recombinant chinook salmon hsp70 protein (StressGen Biotechnologies Corp., Victoria, British Columbia; SPP-763) were applied to each gel to serve as internal standards for molecular weight determination and blotting efficiency. Proteins were transferred to PVDF membrane (Millipore Corp., Billerica, MA, USA) at 100 volts for 1 hr, then blocked overnight at 4ºC in blocking solution (5% non-fat dry milk, 20 mM tris buffer, and 0.01% Tween-20). Membranes were incubated at room temperature for 1h with a polyclonal primary antibody for hsp70 (StressGen, SPA-758) at a 1:5000 dilution; then washed three times (10 minutes per wash), in tris-buffered saline solution (TBS), TBS with 0.5% Tween-20 (TBS/Tween), and TBS. According to the manufacturer, this antibody detects proteins of the molecular mass of 70 kDa and 73 kDa, corresponding to the apparent molecular mass of constitutive hsc70 (hsp73) and inducible hsp70 (hsp72) isoforms. In liver tissue from this study, only one band was detected of approximately 70-75 kDa. With one-dimensional gel electrophoresis, it is possible that hsp bands assigned to a size-class

have more than one hsp homologue. Blots were incubated at room temperature for 1h with a 1:5000 dilution of alkaline phosphatase conjugated goat-anti rabbit IgG (StressGen, SAB-301). Blots were rinsed as previously described, and proteins were visualized colorimetrically using an alkaline phosphatase conjugate substrate kit (Biorad, 170-6432) according to manufacturer instructions. Blots were developed for 15 minutes and the reaction was stopped by rinsing with distilled water for 10 minutes. Relative hsp70 band density was quantified by densitometry. Each stained blot was scanned at 600 dpi and 256 shades of grey using a Hewlet Packard 3970 desktop scanner. Band density was measured using ImageQuant 6.1 (Amersham Biosciences). Relative protein band density for each sample is expressed by subtracting background and dividing by the hsp70 protein standard band density.

Cortisol Assay

Plasma cortisol levels were analyzed using a radioimmunoassay as described in Foster and Dunn (1974) and adapted by Redding et al. (1984). All values below the lowest standard (3.9 ng/ml) were designated to contain 1.95 ng/ml cortisol.

Statistics

All heat shock protein and plasma cortisol data were tested for normality and equality of variance. Data for cortisol were log transformed to increase homogeneity of variance. Significance was set at $P < 0.05$. Results are reported as the mean ± one standard error of the mean. Variation in plasma cortisol levels among tanks at Time 0h suggested tank effects were a potential confounding factor. To account for tank effects in analysis of plasma cortisol data, a nested factorial model was used and tank was nested within treatment. Across time, one-way ANOVA was used to examine changes in fish plasma cortisol levels within individual tanks. Analysis of liver hsp70 levels did not show significant difference in fish liver hsp70 band density among tanks at Time 0h. Therefore, tank effect was not included in statistical models. Hsp70 data was analyzed with one and two-way ANOVA. If significant differences were found, a

Tukey-Kramer multiple comparison test was used. Analyses were conducted with the statistical software packages S-Plus 6.2 (Insightful Corp., Seattle, WA) and SAS (SAS Institute 2003).

Results

PIT tag retention during this experiment was 100%. The average fork length (mm) of fish sampled in this experiment was 110 mm and ranged from 79 to 142 mm. The average mass (g) was 16.7 g with a range of 7.1-24.6 g.

Plasma cortisol

Within each time block, there was no difference in fish length among the four experimental tanks (ANOVA, $P > 0.05$). There was no evidence that log plasma cortisol levels were explained by fish length at Time 0h ($F_{1, 22} = 0.97$, $P = 0.336$), Time 2h ($F_{1, 22} = 3.79$, $P = 0.0645$), Time 6h ($F_{1, 22} = 0.18$, $P = 0.678$), Time 24h ($F_{1, 22} = 1.21$, $P = 0.283$), or Time 120h ($F_{1, 22} = 0.45$, $P = 0.510$). Since the length of fish sampled among tanks within a time block was not significantly different and because length was not a significant explanatory variable of log plasma cortisol levels, fish length was not included as an explanatory variable in subsequent analysis.

Prior to the handling stress (Time 0h), there was a significant difference in log plasma cortisol among fish sampled from each of the four experimental tanks ($F_{3, 20} = 4.11$, $P = 0.02$) suggesting a tank effect (Figure 1). For this reason, ANOVA models with log plasma cortisol as the response include treatment and tank in analysis for each of the four post treatment time blocks (Time 2, 6, 24, and 120h). At 0h, the only significant difference in log mean plasma cortisol level among tanks was measured in fish sampled from Tank 2 and 3 (log cortisol values = 0.002 to 1.320; Tukey-Kramer $P = 0.05$; Figure 1).

Between 0 and 2h, plasma cortisol levels more than doubled in all tanks (Figure 1). This increase is significant in Tank 3 and 4 (Tukey-Kramer, $P < 0.0001$) but not Tank 2 (Tukey-Kramer, $P = 0.126$). There is suggestive, but inconclusive evidence that plasma cortisol levels from fish measured in Tank 1 (Tukey-Kramer, *P*

= 0.047) increased between Time 0h and 2h. At Time 2h, treatment effect depends on tank ($F_{2, 20} = 3.84$, = 0.039). After accounting for the tank by treatment interaction, the effect of treatment at 2h was not significant ($F_{1, 20} = 1.81$, $P = 0.19$). A comparison of mean log plasma cortisol levels suggests no significant difference between Tank 1, 2, and 3 (Tukey-Kramer, $P > 0.12$). At 2h, there is suggestive, but inconclusive evidence of a difference in log mean plasma cortisol measured in fish sampled from Tank 2 and 4 (Tukey-Kramer, $P = 0.053$).

Between 2h and 6h, mean plasma cortisol levels measured in fish decreased in all tanks (Figure 1). This decrease is significant in the two control tanks (T1 and T3; Tukey-Kramer, $P < 0.05$) but not the PIT tag tanks (T2 and T4; Tukey-Kramer, $P > 0.10$).

At 6h, both the interaction (treatment*tank; $F_{2, 20} = 3.81$, = 0.040) and treatment effects were significant ($F_{1, 20} = 12.85$, $P = 0.002$; Figure 1). Within the 6h time block, there was no significant difference in plasma cortisol levels detected between the two control tanks (T1 and T3; Tukey-Kramer, $P = 0.095$) or the two treatment tanks (T2 and T4; Tukey-Kramer, $P = 0.617$). The log plasma cortisol levels from fish in Tank 1 were significantly different than values measured in Tank 2 (Tukey-Kramer; $P = 0.024$) and Tank 4 (Tukey-Kramer; $P = 0.0015$). Log plasma cortisol levels for fish sampled from control Tank 3 were similar to plasma cortisol levels for fish sampled from both treatment tanks (Tukey-Kramer, $P > 0.25$; Figure 1).

There was no treatment by tank effect at Time 24 or 120h (ANOVA, $P > 0.20$). There were no significant differences in log plasma cortisol levels between treatment and control fish at Time 24h ($F_{1, 22} = 0.65$, $P = 0.43$) or Time 120h ($F_{1, 22} = 1.76$, $P = 0.1985$).

Hepatic hsp70 levels

Hepatic hsp70 levels were measured at Time 0, 24, and 120h (Figure 2). Hepatic hsp70 levels were similar in all tanks at Time 0h ($F_{3, 20} = 0.68$, $P = 0.57$). Because hepatic hsp70 levels in fish livers were not significantly different at Time 0h among tanks, tank effect was not included in analysis at Time 24 or 120h. There is no evidence that PIT tagging significantly altered hsp70 levels between control and

treatment fish sampled at 24 ($F_{1, 22} = 0.148$, $P = 0.70$) or 120h ($F_{1, 22} = 0.028$, $P = 0.87$). Hepatic hsp70 levels were not correlated with fish length ($F_{1, 70} = 0.86$, $P = 0.356$, $r^2 = 0.046$) or mass ($F_{1, 70} = 0.837$, $P = 0.36$, $r^2 = 0.012$). Through time, hepatic hsp70 levels did not change ($F2,69 = 2.41$, $P = 0.0974$).

Discussion

Both handling and PIT tagging were stressful events, as indicated by increased plasma cortisol levels. An increase in plasma cortisol levels at 2h post treatment followed by a decrease at 6h indicates a quick response to handling stress followed by rapid recovery. This type of response to handling has been documented by others (Sharp et al. 1998). Jepsen and co-workers (2001) in a radio tagging study with juvenile chinook salmon (*O. tshawytscha*) found that 3h after tagging, radio tagged fish had increased plasma cortisol levels that remained elevated for 24-48 hours after tagging, returning to levels equal to control fish after 7 days. If radio tags, which are larger than PIT tags and involve a much more invasive implantation procedure than PIT tagging did not elicit a chronic increase in plasma cortisol, then it could be expected that PIT tags should not significantly alter cortisol levels relative to fish that were handled as controls.

The cortisol data was more variable than expected. There is evidence PIT tagged fish have increased plasma cortisol levels 6h after tagging compared to non-tagged fish, but these results are inconclusive and may be confounded by tank effects (Figure 1). The absence of a significant decrease in plasma cortisol levels from Time 2h to Time 6h among the two PIT tagging tanks and a significant decrease in plasma cortisol levels among the control tanks offers evidence that PIT tagged fish might have greater plasma cortisol levels than control fish for up to 6 hours post tagging. However, this conclusion is conservative because there is still substantial variation among tanks at Time 6h (Figure 1). If PIT tagging was more stressful on these fish than handling, it could be expected that plasma cortisol levels would be higher (e.g., more than doubled) as observed in this study. In this experiment, a sample size of six fish per tank with two replicates for each treatment may not be sufficient to conclude

PIT tagged fish have higher plasma cortisol levels 6h post tagging compared to a non-PIT tagged fish. Collectively, these data suggests PIT tagging does not cause chronic (> 24h) increases in plasma cortisol levels, but there might be short term effects (≤ 24h). Further investigation is needed to examine short term (≤ 24h) effects of PIT tagging on plasma cortisol levels. To account for possible tank effects, subsequent studies should increase the number of replicate treatment tanks and increase the sample size at each time period.

Unlike the cortisol data, there was less variability in the liver hsp70 data and neither tank nor treatment effects were found. There was no relation between fish length and hepatic hsp70 levels, and there was no correlation between hsp70 and plasma cortisol levels. The conclusion that PIT tag implantation does not alter hepatic hsp70 levels supports other research that shows no effect of handling on hsp70 levels (Vijayan et al. 1997, Washburn et al. 2002, Zarate and Bradley 2003).

Once cellular hsp70 protein production is induced, levels will remain elevated for extended periods of time (> 24h). In brook trout (*Salvelinus fontinalis*), peak hsp70 proteins in both red blood cells and white muscle tissue reached peak levels 12h following an acute temperature stress, and these levels remained elevated for over 48h (Lund et al. 2003). With juvenile chinook (*O. tshawytscha*), a single exposure to 26ºC for 10-15 minutes was sufficient to produce significantly elevated hepatic hsp70 levels for 14 days (Mesa et al. 2002). Therefore, we focused our analysis of hsp70 protein levels at 24 and 120h post-treatment. If PIT tagging caused an increase in hsp70 levels, this response would have been expected to be detected in both the 24h and 120h sampling periods.

There was no evidence in this study that plasma cortisol levels altered hepatic hsp70 levels. This is important because elevated cortisol levels can suppress hepatic hsp70 levels in juvenile rainbow trout subjected to an acute heat stress (Basu et al. 2001), and chronically stressed fish may have decreased ability to produce hepatic hsp70 (Basu et al. 2002). If PIT tags increased plasma cortisol levels in a chronic fashion, it could decrease a fish's ability to mount a heat shock response to an hsp70 inducing stressor (e.g., temperature, disease, toxins) which could compromise survival. Alternatively, the increase in plasma cortisol observed at 2h post-handling

may have suppressed synthesis of hsp70 proteins. Also, one-dimensional gel electrophoresis and the antibodies used in this study may not be sensitive enough to detect changes in different hsp70 isoforms.

In conclusion, PIT tagging and the associated handling does not alter liver hsp70 levels. Furthermore, PIT tags have little or no effect on short or long term plasma cortisol levels in juvenile rainbow trout (*O. mykiss*). Since PIT tagging does not alter hepatic hsp70 levels, it should be possible to use PIT tags in combination with lab or field manipulations to examine the effect of specific or multiple stressors on hepatic hsp70 levels. Therefore, PIT tagging fish as a method to study long term effects of stressors on survival, growth, and reproduction of fish looks promising. Together, these results support a growing body of literature documenting the suitability of PIT tags as a low impact tagging procedure for juvenile salmonids.

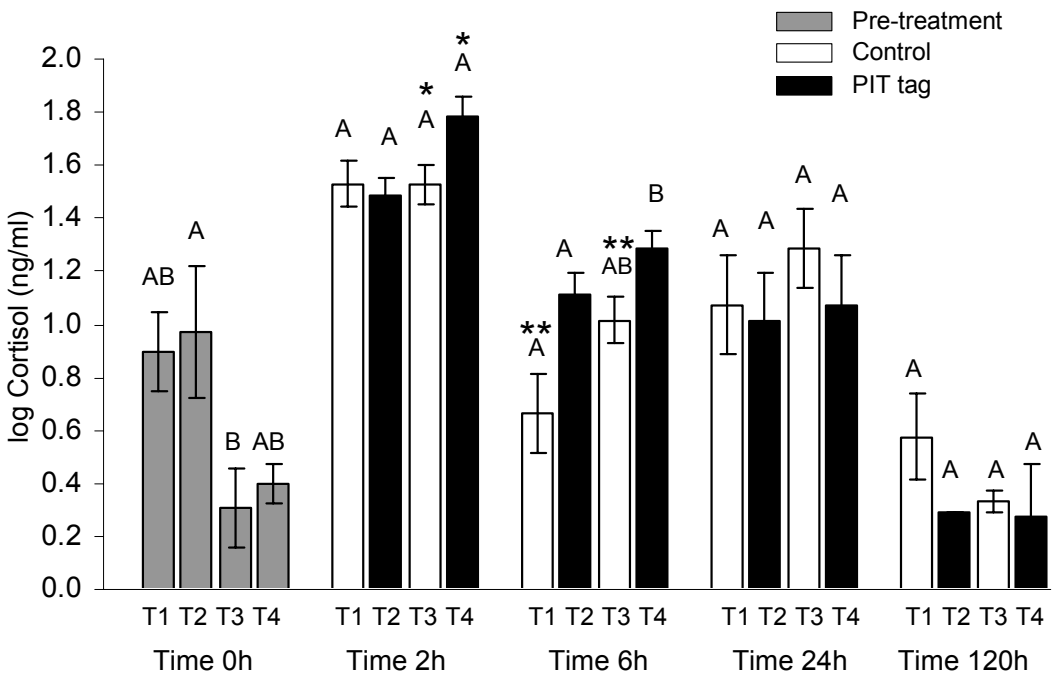

Figure 1- Log mean plasma cortisol levels (ng/ml) ± one SE prior to treatment (0 h), and at 2, 6, 24, and 120h after treatment. Open bars represent fish from control tanks (T1, T3) and dark bars represent PIT tag tanks (T2, T4). Shared letters within a time block represent non-significant differences ($P > 0.05$) at that time interval. A single asterisk indicates a significant difference ($P < 0.05$) in a tank from Time 0h to Time 2h. A double asterisk indicates a significant difference in a tank from Time 2h to Time 6h; $n = 6$ for each bar.

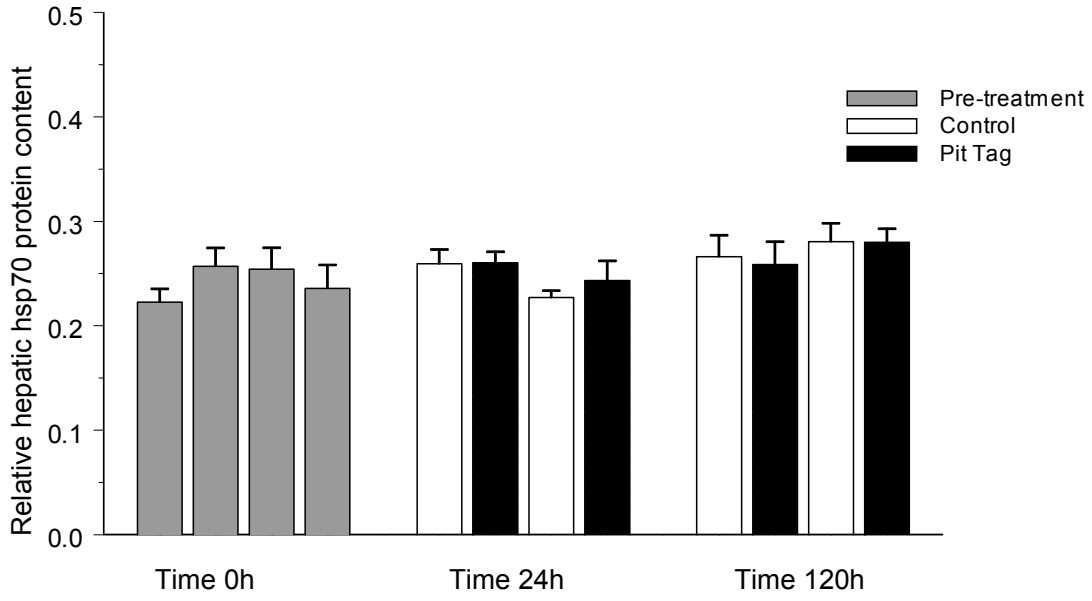

Figure 2- Relative heat shock protein 70 (hsp70) levels prior to experimental treatment (0h), and 24, and 120h after treatment. Open bars represent fish from handling control tanks, dark bars represent fish that were pit tagged. Values represent mean densitometry values of protein bands expressed as density relative to the positive control in the western blot (± 1 standard error of the mean) band density; $n = 6$ for each bar.

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Chapter 3: Heat Shock Proteins and Physiological Stress in Redband Steelhead Trout (*Oncorhynchus mykiss gairdneri*) in the South Fork John Day River, Oregon.

Introduction

A challenge for fisheries ecologists is choosing the correct scale to study aquatic habitats (Feist et al. 2003). Studying aquatic habitat at the wrong scale can lead to a lack of information and tools for managers to effectively conserve and protect stream fish populations (Fausch et al. 2002). A promising approach for bridging the gap between conservation and research is studying systems using a top-down approach that is capable of integrating information from the bottom up (i.e., riverscape, Fausch et al. 2002). A well studied top-down controller of aquatic ecosystems is temperature. Temperature influences all levels of biological organization, and for this reason, temperature has been called the "abiotic master" (Beitinger et al. 2000).

Water temperature is a criterion that can be used for different purposes. For example, as an indicator of habitat quality it can be manipulated to enhance, protect, and restore aquatic ecosystems. Water temperature can also have community level effects that alter food webs (Tait et al. 1994). At the population level, water temperature has also been found to correspond with distribution patterns of spawning adult chinook salmon (*Oncorhynchus tshawytscha*; Torgersen et al. 1999). Research suggests the biomass of redband rainbow trout (*O. mykiss gairdneri*) is negatively correlated with solar insulation and maximum stream temperatures in streams of the John Day River basin in eastern Oregon (Li et al. 1994) and southwestern Idaho (Zoellick 2004). Moreover, it is hypothesized that temperature is the dominant environmental force driving salmonid evolution and life history strategies (Brannon et al. 2004). Physiological performance and bioenergetics studies on various strains of *O. mykiss* have also pointed to minor differences in growth above 22ºC (Myrick and Cech 2000). Water temperature can also influence individual fish microhabitat choice (Baltz et al. 1987), behavior (Cunjak and Green 1986), and suppress appetite and growth (Linton et al. 1998, Railsback and Rose 1999). At the cellular level,

temperature affects virtually all components of cellular processes including protein stability and enzymatic rates (Hochochka and Somero 2002). Within this context, the uncertainties of global climate change effects on natural ecosystems demands a better understanding of how temperature affects the physiological limits of juvenile salmonids, especially as it is transmitted from the individual to the population.

Cellular thermal stress in wild salmonid populations can be measured using heat shock proteins (Lund et al. 2002, Lund et al. 2003, Werner et al. 2005). Heat shock proteins (hsps), or stress proteins, are highly conserved cellular chaperone proteins (Feder and Hofmann 1999). The genes encoding hsps have been found in every species examined (Feder and Hofmann 1999). Molecular chaperones can be synthesized constitutively or in response to a stressor (Hochachka and Somero 2002). Heat shock proteins assist proper protein folding and are involved with the cellular immune response (Zugel and Kaufmann 1999). Heat shock proteins are also involved in cellular signalling that regulate growth and development and have been hypothesized to alter physiological signals in response to stress or disease states (Nollen and Morimoto 2002). The increase in molecular chaperones in response to heat stress is called the heat shock response (Parsell and Lidquist 1994, Hochachka and Somero 2002). This heat shock, or cellular stress response, protects vital cellular functions by binding denatured proteins and preventing inappropriate associations between proteins (Parsell and Lidquist 1994). Because the cellular stress response can be elicited from a wide range of stressors, heat shock proteins have been identified as potential biomarkers for environmental stress in fish (Iwama et al. 1998). Examples of stressors that induce stress proteins include extreme temperature, heavy metals, ultraviolet light, gases, hypoxia, hyeroxia, and exposure to alcohols (Feder and Hofmann 1999; Hochachka and Somero 2002, Sanders 1993).

A particularly well studied heat shock protein is the highly conserved hsp70 family (Sanders 1993). The hsp70 family is also correlated with acquired thermal tolerance (Parsell and Lindquist 1994). However, synthesis of proteins is energetically costly, and the energy required to synthesize additional stress proteins may constitute a significant proportion of an organism's energy budget (Hochachka and Somero 2002). Hargis (1998) determined the induction temperatures for hsp30 and hsp70 to be

between 20 and 21ºC in juvenile chinook salmon (*O. tshawytscha*). The induction temperature correlated with nearly complete inhibition of growth and was found to be an indicator of poor fitness, reduced growth rates, and decreased survival. Basu and co-workers (2001) reported unpublished work that suggests the maximal hsp70 induction in trout occurs at 21ºC regardless of season, magnitude of heat stress, or acclimation temperature. In a laboratory experiment with juvenile steelhead parr (*O. mykiss*), chronic exposure to 20ºC lead to hsp72 synthesis and decreased liver and muscle levels of the high-energy compounds ATP, phosphocreatine, and glycogen (Viant et al. 2003). In brook trout (*Salvelinus fontinalis)*, the threshold for hsp synthesis was found to correspond to temperatures shown to influence the distribution of this species (Lund et al. 2003). Recently, Werner et al. (2005) measured expression of hsp72 in white muscle tissue of juvenile steelhead parr (*O. mykiss*) in the Navarro River watershed, California, and concluded these steelhead parr were experiencing thermal stress when maximum daily water temperatures reached 20-22.5ºC. Furthermore, they found higher basal levels of hsp72 in fish collected from inland sites than coastal sites but were unable to determine if this was due to acclimatization or genetic adaptation of the subpopulations to thermal conditions. Together, these findings demonstrate that hsps are involved with individual growth, survival, and thermal tolerance. These findings have implications for understanding distribution limits of salmonid subpopulations, and for guiding management actions relating to summer water temperatures.

The South Fork John Day subbasin in Northeastern Oregon is on the Oregon 303d list for Water Quality Limited Streams[3]. Anthropogenic disturbances within catchment basins have increased summer stream temperatures. Elevated stream temperatures are believed to be a contributing factor that limits juvenile steelhead trout production. The South Fork John Day River (SFJD) is an important rearing and spawning location for a population of Middle Columbia River Steelhead trout (*O. mykiss)* that were listed in 1999 by the National Marine Fisheries Service as a threatened Evolutionarily Significant Unit (ESU). Steelhead trout east of the Cascade

[3] A complete list of 303(d) listed streams can be found at http://www.deq.state.or.us/wq/303dlist/303dpage.htm,

Mountains are considered to be part of the redband trout complex (Behnke 1992) and are sometimes referred to as redband steelhead trout (*O. mykiss gairdneri*). Redband trout have been observed to tolerate stream temperatures in excess of 26ºC (Behnke 1992; Zoellick 1999). One explanation for the persistence of inland populations of redband rainbow (resident population of *O. mykiss gairdneri*) trout in warm stream reaches is behavioral thermoregulation and not stock adaptation (Li et al. 1994). Another explanation is physiological adaptation to thermal history leading to temperature-dependent swimming performance and aerobic metabolism that allow for short term (< 2h) exposure to temperatures greater than 24ºC (Gamperl et al. 2002).

Given this background, the objectives of this study were to (1) determine if redband rainbow trout in the SFJD River are experiencing thermal stress, (2) determine the temperature that increases cellular hsp70 levels, (3) develop a nonlethal technique for measuring cellular hsp70 levels, and (4) characterize the relation between whole body lipids and hsp70 for fish in the SFJD. Results from this study will increase knowledge about the physiological ecology of juvenile redband steelhead trout in the SFJD River. Information from this research will improve understanding about the effects of the physiological impacts of elevated stream temperatures on juvenile redband steelhead trout production. In turn, this knowledge will improve awareness of how temperature relates to early life history strategies, growth, survival, distribution patterns, and the carrying capacity and production of summer habitat. Physiological criteria for suitability of juvenile redband steelhead trout habitat will also provide a useful index of habitat quality.

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). The John Day River is one of only 42 streams in the contiguous United States exceeding 200 km that remains unimpounded (Benke 1990). It is also one of the few drainage systems in the Columbia River basin that still supports wild runs of juvenile fall and spring chinook

salmon (*O. tshawytscha*) and summer steelhead (anadromous redband rainbow trout *O. mykiss gairdneri*; Li et al. 1994).

The SFJD River is located in Grant County, Oregon, and flows northward from the Ochoco and Aldrich mountains. The SFJD River enters the mainstem John Day River at Dayville, Oregon, drains an area of approximately 1637 square kilometers (Leitzinger 1993), and ranges in elevation from 710 to 1646 meters above sea level. The SFJD River is also considered the driest and most sparsely populated area of the John Day subbasins (The Upper John Day River Local Advisory Committee 2002).

Within the SFJD catchment, four tributaries provide spawning habitat for adult summer steelhead. These tributaries, between Dayville and the upstream anadromous fish barrier, Izee falls at river kilometer (RKM) 46.7, are Black Canyon Creek (RKM 22.7), Murderers Creek (RKM 26.6), Wind Creek (RKM 33.8), and Deer Creek (RKM 45.1). Murderers Creek is a 4th order stream and Deer, Wind, and Black Canyon Creek are 3rd order streams. Both Black Canyon and Wind Creek flow west to east, and Black Canyon drains from the Black Canyon Wilderness. Deer and Murderers Creek flow east to west and the lower section of Murderers Creek flows through the Murderers Creek wildlife preserve. The primary commercial activity in the SJFD catchment is cattle ranching, and most of this occurs within the lower 22.5 kilometers and upstream of Izee falls. Limited cattle ranching activities occur upper and lower Murderers Creek and the lower section of Wind Creek. A dirt logging road follows almost the entire length of Deer Creek, but logging in this catchment is limited.

Materials and Methods

Fish Sampling

Sampling was completed during the summers of 2004 and 2005 in the South Fork John Day River (SFJD). In 2004, ten fish were collected from each of four different locations on 10-11 June, 30-31 July, and 11 September. In 2005, ten fish were collected from each of eight different study locations from 23-24 July (Table 1, Figure 3). Four of the eight locations visited in 2005 were identical to 2004, and fish

were collected from both riffle and pool habitat. During 2004, all fish were collected by electrofishing using a Smith-Root Model 12B backpack electrofisher. In 2005, fish were collected by beach seine and electrofishing. Collections occurred before water temperatures reached 18ºC and were completed within 2-3 h at each study location. Collected fish were held in 5 gallon buckets and water was refreshed with stream water every 10-15 minutes. Fish were removed from the collection bucket, individually anesthetized with a lethal solution of tricaine methanesulfonate (200 mg/l MS-222, 500 mg/l $NaHCO_3$) and measured for fork length (mm) and weight (g), and visually examined for signs of parasites or disease. In 2004, 21 of the sampled fish carcasses were donated to the Oregon Department of Fish and Wildlife (ODFW) for disease profiling and these carcasses were not analyzed for lipid content.

For each individual fish, all tissues were removed and frozen on dry ice within five minutes following death. In 2004, only liver and caudal fin tissue was collected. In 2005, liver, lower caudal fin, and white muscle tissue from the caudal peduncle region was collected. Liver and white muscle tissue was quickly excised and wrapped in aluminum foil. Caudal fin tissue was placed in a labeled 0.7 mL eppendorf tube. Fish carcasses were individually packaged, placed in labeled plastic bags, kept on ice, and transported to Oregon State University (Corvallis, OR). Tissue samples were stored at -80ºC for later analysis of heat shock proteins. Fish carcasses were stored at -20ºC. This study followed the Institutional Animal Care and Use Committee (IACUC) guidelines at Oregon State University under permit #3080.

Temperature Data

Optic Stowaway temperature loggers set to record temperature (°C) each hour were placed at all study locations in June and early July 2004. In 2005, Optic Stowaway and I-button temperature loggers were placed at study locations in May and early June. The logger at the Izee falls and Deer Creek study locations were not launched until the 2nd and 12th of July, respectively. Discharge and temperature data were also available from gauging stations operated by the US Bureau of Reclamation located in Deer Creek (UTM =11T 302735mE, 4896710mN; elevation = 1222 meters, stream kilometer 5.6), Murderers Creek (UTM = 11T 297905mE, 4910076mN;

elevation = 908 meters, stream kilometer 0.6), and in the SFJD 10 kilometers upstream of Izee Falls (UTM = 11T 300646mE, 4888621mN; elevation = 1198 meters).

In 2004 and 2005, some temperature records were lost because of logger failure or exposure of the logger to air because of drought conditions. For this reason, temperature records from gauging stations were compared with nearby temperature loggers and data from the gauging stations were used to supplement missing temperature records. Data from the Deer Creek and Izee falls gauging stations were substituted for missing temperature records in the Deer Creek and Izee falls study locations from 1 May through 11 July 2004. The Izee falls gauging station is 10 kilometers upstream of the IZ study location. The Deer Creek gauging station is three kilometers upstream of the Deer Creek study location. In July 2005, temperature data from the Izee falls gauging station were used for the Izee falls study location. The 2004 temperature records for lower Black Canyon were collected by the U.S. Bureau of Land Management. Unless otherwise noted, all other temperature records were collected under the auspices of this project.

Laboratory temperature exposure and experimental design

The temperature experiment was carried out at the Fish Performance and Genetics Laboratory of the Oregon State University in Corvallis, OR, between 25 October and 20 November 2004. Fish were first generation yearling steelhead trout (*Oncorhynchus mykiss*) spawned from wild parents captured from the Umatilla River, Oregon near the Umatilla Fish Hatchery. Adult steelhead were transported to the Fish Performance and Genetics Laboratory, and spawned in spring 2003. A total of 144 yearling fish were evenly and randomly divided between 9 indoor, 1-meter circular fiberglass tanks (16 fish/tank) in an indoor facility. Ambient water entering the tanks was 13.0°C flow through well water and water depth in all tanks was 55 centimeters. Fish were acclimated to experimental tanks for 10 days. During acclimation, fish were fed by hand once per day to satiation with BioDiet Grower, a semi-moist commercial pellet manufactured by Bio-Oregon. Fish in each individual tank were fasted for 24 h before the start of the experiment.

Flow through well water was heated in a 387 liter insulated tank by a Hayward Electric Spa Heater (Model CSPA XI11). Heated water was gravity fed into experimental tanks at 2.0 liters/minute. The experiment was a block design with three blocks. Each block represented three days. Within each block were three tanks, and each tank was randomly assigned a temperature stressor of 19 (stressor 1), 22 (stressor 2), or 25°C (stressor 3). The order for completing each temperature treatment within a block was randomly determined and one treatment was completed each day. In each tank, between six and nine Onset Optic Stowaway temperature loggers recorded temperature (°C) once every minute. These temperature records were used to calculate rate of heating in each tank and were used to determine the length of time fish were exposed to the respective temperature stressor. Temperature loggers were placed on the bottom of the tank, as well as suspended from the standpipe 18 and 36 centimeters below the water surface. After 24 h of food restriction, and immediately before introducing warm water to the tank, six control fish were netted and placed in a lethal solution of buffered tricaine methanesulfonate at a concentration of 200 mg/l with 500 mg/l $NaHCO_3$. Next, warm water was introduced to the experimental tank at a rate of 2 L/minute. During the experiment, water temperature was manually monitored with a thermometer. Temperatures in each tank were not allowed to exceed target temperatures (i.e., 19.0, 22.0, or 25.0 °C) by more than 0.5°C. The time at which the target temperature was first achieved for each experimental tank was recorded. Cold ambient water (13ºC) was reintroduced to each experimental tank at a rate greater than 5 L/min 90 minutes after the target temperature for the experimental treatment was first recorded. Fish remained in the experimental tank for 12 hours following the end of the temperature stress (i.e., reintroduction of 13ºC water) and were lethally sampled in buffered MS-222.

Length (mm) and weight (g) were recorded for all lethally sampled trout. For each fish, tissue was removed from the lower caudal fin and placed in a labeled 0.7 mL eppendorf tube, and flash frozen in liquid nitrogen. Fish livers were quickly excised, wrapped in aluminum foil, and flash frozen in liquid nitrogen. Tissue samples were transported to Oregon State University (Corvallis, OR) and stored at -80ºC for later analysis.

Liver hsp70 levels were determined for all fish sampled prior to the temperature stressor (pretreatment; control) from each tank. Following the temperature stressor, six fish from each tank were randomly selected for further analysis. The remaining samples were not analyzed. To compare fin and liver hsp70 levels, a total of eight fish were randomly selected from the entire collection of control samples, and eight fish were randomly chosen from each of the 19, 22, and 25ºC temperature stressors.

Tissue sample preparation for hsp70 determination

Liver samples were lysed in ice-cold lysis buffer (50 mM Tris base, 20 mM NaCl, 5 mM EDTA, pH 7.5) containing protease inhibitors (0.5 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluroride (PMSF), 1mg /ml aprotinin, and 0.7 mg/ml pepstatin). Liver samples were homogenized with a tissue tearor (Model 985-370; Biospec Products, Inc.) and lysates were centrifuged for 5-10 minutes at 13000 x g at 1ºC or 4500 x g for 30 minutes at 1ºC, and the resulting supernatant was aliquoted and stored at -80ºC. Fin tissue was weighed on an analytical balance (± 0.1 mg), placed in a 1.5 mL eppendorf tube, frozen with liquid nitrogen and crushed into small pieces with a Teflon pestle. Lysis buffer and protease inhibitors (volume adjusted for fin mass) were added and the tissue further homogenized by hand. Fin samples were spun at 4500 x g at 1ºC for 30 minutes, aliquoted, and frozen at -80ºC. Protein concentrations in lysates were assayed with the bicinchoninic acid (BCA) protein assay method (Sigma-Aldrich, Saint Louis, MO). Plates were read on a Molecular Devices Vmax Kinetic Microplate reader at 570 nm.

Western blotting

Western blot analyses were performed based on methods described by Towbin et al (1979). Briefly, protein samples were mixed with an equal amount of SDS sample buffer (Laemmli 1970), and then heated (95ºC) for 3 minutes. Equivalent amounts of protein for liver (25 μg) and fin (25 μg) were separated by gel electrophoresis on 8% Tris-Glycine gels (Invitrogen corporation) for 2 hours at 125

volts. A calibrated molecular weight marker (Biorad) and 52-ng recombinant chinook salmon hsp70 protein (StressGen Biotechnologies Corp., Victoria, British Columbia; SPP-763) were applied to each gel to serve as internal standards for molecular weight determination and blotting efficiency. Proteins were transferred to PVDF membrane (Millipore Corp., Billerica, MA, USA) at 100 volts for 1h, then blocked overnight at 4ºC in blocking solution (5% non-fat dry milk, 20 mM tris buffer, and 0.01% Tween-20). Membranes were incubated at room temperature for 1 hour with a polyclonal primary antibody for hsp70 (StressGen, SPA-758) at a 1:5000 dilution; then washed three times (10 minutes per wash) in tris-buffered saline solution (TBS), TBS with 0.5% Tween-20 (TBS/Tween), and TBS. According to the manufacturer, this 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. Two bands were detected in white muscle tissue that apparently correspond with hsp72 and hsp73, but one band of approximately 70-75 kDa was detected in liver and fin tissue. With one-dimensional gel electrophoresis, it is possible that hsp bands assigned to a size-class have more than one hsp homologue. Blots were then incubated at room temperature for 1 hour with a 1:5000 dilution of alkaline phosphatase conjugated goat-anti rabbit IgG (StressGen, SAB-301). Blots were rinsed as previously described, and proteins were visualized colorimetrically using an alkaline phosphatase conjugate substrate kit (Biorad, 170-6432) according to manufacturer instructions. Blots were developed for 15 minutes and the reaction was stopped by rinsing with distilled water for 10 minutes. Relative hsp70 band density was quantified by densitometry. Each stained blot was scanned at 600 dpi and 256 shades of grey using a Hewlet Packard 3970 desktop scanner. Band density was measured using ImageQuant 6.1 (Amersham Biosciences). Protein band density is expressed by subtracting background and dividing by the hsp70 protein standard band density.

It is unknown if the single band of hsp proteins detected in the liver and fin tissue are homologous to the apparent hsp72 and hsp73 protein bands detected in white muscle tissue. Fin and liver tissue samples run side by side on the same gel with the same quantity of protein (25 μg) had bands develop on the same horizontal plane

at approximately 70-75 kDa. Fin and liver hsp70 results are compared using paired t-tests, but it should be noted that these may be different isoforms.

Lipid determination

Total lipid content for each fish was determined following the methods described in Anthony et al. (2000) and Reynolds and Kunz (2001). Briefly, to determine laboratory wet mass, fish were thawed and weighed on an analytical balance (± 0.1 mg). Total body water (TBW, g) was determined by drying fish to a constant mass (i.e., ± 0.01 g of previous 24hr mass) in a convection oven set at 60ºC and TBW was calculated by subtracting the difference between the laboratory wet mass and the dry mass. Fish were then 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. Fish wet mass recorded at the time of capture was not used to calculate fat content because internal organs and tissue were removed from each fish and subsequent variation in WBL based on field wet mass would be increased. Percent water (PW) was calculated by dividing TBW by laboratory wet mass.

Statistics and Data Analysis

All data were tested for normality and equality of variance. Significance was determined at $P < 0.05$. Results are reported as ± one standard error of the mean. In the laboratory experiment, block (time) by treatment effects were tested using two-way ANOVA. There was no effect of time on control fish so data were subsequently pooled, and the three temperature stressors were compared using one-way ANOVA and multiple linear regression. Comparisons of fin and liver hsp70 data in the laboratory experiment were done with one-way ANOVA followed by a Tukey-Kramer multiple comparison test. For fish collected from the SFJD River in 2004, one-way

ANOVA was used to test for differences among locations within months and in a location between months. A two-way ANOVA was used to test for differences between year and location at lower Black Canyon, Below Wind Creek, Izee Falls, and Deer Creek. A Tukey-Kramer multiple comparison test was applied when significant differences were found (SAS Institute 2003). The estimated induction temperature (i.e., increased protein synthesis over basal levels), and associated 95% confidence intervals, were determined with sigmoid curves constructed with the statistical software package GraphPad Prism (San Diego, CA). All other analyses were completed with the statistical software package S-Plus 6.2 (Insightful Corp., Seattle, WA).

Results

South Fork John Day River temperature data

When using gauging station data for supplemental temperature records for 2004, temperature data from the Deer Creek (DC) and Izee gauging stations were compared with nearby instream temperature loggers. There were few differences between the maximum daily temperatures recorded at Izee falls gauging station and the instream temperatures recorded by temperature loggers at the Izee falls (IZ) study location (Figure 4). From 2 July-15 September 2004, the Izee gauging station recorded max daily water temperatures that were an average of 0.3ºC lower than the downstream temperature logger at the IZ study location. In 2004, the maximum water temperature recorded at IZ and the Izee falls gauging station were 23.5°C and 23.7°C, on 25 July. During 2004, the DC temperature logger recorded maximum daily water temperatures that were an average of 0.2ºC higher than temperatures recorded at the DC gauging station from 12 July-15 September 2004. In 2004, the maximum water temperature (19.4ºC) at the DC study location occurred on 25 July. On the same day, the DC gauging station recorded a temperature maximum of 19.2ºC. The coldest study location in 2004 was DC.

In 2005, the location with the lowest maximum daily water temperature recorded prior to fish capture was Upper Black Canyon Creek (18.4°C), while maximum daily water temperatures approached 26°C at Lower Murderers Creek (25.8°C) and in the South Fork John Day River at river kilometer 24 (25.4°C; Table 2). Additional temperature measurements are summarized in Table 2. From 1 July to 23 July 2005, a similar temperature profile is displayed at the Izee falls gauging station and the Below Wind Creeks study location (

Figure **5**). The similarity in temperature records between BW and the IZ gauge in 2005 suggests that in 2004, temperatures at the Below Wind study location were equal to or slightly higher than the temperatures recorded at the Izee Falls gauging station in 2004.

Laboratory experiment

The average fork length of fish in this experiment was 144 mm (range = 115-170 mm) and average mass was 32.1g (range = 15.9-56.5 g). The temperatures reported for this experiment are the average of 6 or 9 Optic Stowaway temperature loggers. The vertical thermal profile indicates that temperatures were uniform throughout the tanks without any obvious stratification.

Peak temperatures recorded for trials 1-3 were 19.5, 22.7, and 25.7ºC. For stressor 1, temperatures were maintained above 19ºC for an average of 119 minutes (range = 111-126 minutes). On average it took 246 minutes to change the water temperature from 13 to 19ºC (stressor 1) with an average heating rate of 1.5ºC/hr (range = 1.2-1.7ºC/hr). For stressor 2, temperatures were maintained above 22ºC for an average of 105 minutes (range = 102-111 minutes). It took an average of 286 minutes to change the water temperature from 13 to 22ºC, with an average heating rate of 1.9ºC/hr (range = 1.7-2.1ºC/hr). Water temperature was maintained above 25ºC (stressor 3) for an average of 91 minutes (range = 76-107 minutes). On average, it took 333 minutes to change the water temperature from 13 to 25ºC, and the average heating rate was 2.2ºC/hr (range = 1.9-2.3ºC/hr). Fish were lethally sampled 12 hours after reintroduction of 13ºC (ambient) well water. A representative temperature profile for each temperature stressor is shown in Figure 6.

There was no interaction between block and treatment ($F_{4, 99} = 0.385$, $P = 0.887$), but there was a significant treatment effect ($F_{2, 99} = 20.43$, $P < 0.0001$). After accounting for block, there was no effect of the 19ºC temperature stressor on liver hsp70 band density ($F_{1, 102} = 0.05$, $P = 0.815$). There was a significant difference in relative hsp70 band density between control and treatment fish following the 22ºC ($F_{1, 102} = 46.79$, $P = <0.0001$) and the 25ºC temperature stressors ($F_{1, 102} = 560.45$, $P = < 0.0001$). The mean relative hsp70 band density in fish exposed to 22ºC was 0.164 units greater than control fish, and this difference was significant ($t_{102} = 6.84$, $P < 0.0001$). Treatment fish exposed to 25ºC had significantly greater hsp70 band density measurements than treatment fish exposed to 22ºC ($t_{102} = 13.745$, $P < 0.0001$; Figure 7). Based on this analysis, the induction temperature for hsp70 protein in the livers of rainbow trout tested in this experiment is between 19 and 22ºC, with a significantly elevated response occurring at 25ºC (Figure 7).

The relative hsp70 band density measured in fin tissue was similar to liver tissue. Band density in both tissues increased with increasing temperature. The greatest band density measured in fin and liver tissue was from fish exposed to the 25ºC treatment (Figure 8). Temperature had a significant effect on relative hsp70 band density in fin tissue ($F_{3, 28} = 352.5$, $P < 0.0001$). There was no difference in fin hsp70 band density following 19ºC exposure when compared to controls ($F_{1, 28} = 0.67$, $P = 0.42$), but there was a significant effect of the 22ºC ($F_{1, 28} = 286.5$, $P < 0.0001$) and 25ºC ($F_{1, 28} = 751.4$, $P < 0.0001$) temperature stressors (Figure 8). Treatment fish exposed to 22ºC had significantly elevated levels of hsp70 in fin tissue compared to those exposed to 19ºC ($F_{1, 28} = 10.53$, $P < 0.0001$). Treatment fish exposed to 25ºC had significantly greater fin hsp70 levels than fish exposed to 22ºC ($F_{1,28} = 109.96$, $P < 0.0001$). There is evidence that mean hsp70 band density measurements in liver and fin tissue are not the same at each temperature treatment ($F_{3, 56} = 3.19$, $P = 0.031$). Hsp70 band density measurements are different between liver and fin tissue at 22ºC (see Figure 8; paired t-test, $P = 0.043$), but not at any other temperature ($P > 0.05$). Based on this analysis, the induction temperature for hsp70 protein in the fin tissue of rainbow trout tested in this experiment is between 19 and 22ºC. The difference in

hsp70 levels in fin and liver tissue at 22ºC suggests there is a difference in the cellular hsp70 response between these two tissues (Figure 8).

Fish size and whole body lipids

The fork length (mm) of fish handled in June 2004 was 125.4 ± 20.7 (average ± SD, range 95-125 mm), 145.6 ± 19.2 mm (114-181 mm) in July 2004, 145.5 ± 19.4 mm (113-198 mm) in September 2004, and 126.3 ± 19.5 (94-180 mm) in July 2005. Whole body lipids (WBL) were determined on 99 juvenile rainbow trout in 2004 and 80 in 2005.

In 2004, there were no differences in WBL between locations for fish sampled at lower Black Canyon (LBC), Below Wind (BW), Deer Creek (DC), and Izee Falls (IZ) on 10-11 June ($F_{3, 24} = 1.04$, $P = 0.394$) or 11 September 2004 ($F_{3, 32} = 1.98$, $P = 0.136$; Figure 9). There was a significant difference in WBL among locations for fish sampled at LBC, BW, DC, and IZ on 30-31 July 2004 ($F_{3, 31} = 7.36$, $P < 0.001$). In July 2004, fish collected at BW had significantly lower WBL than fish collected at DC (Tukey-Kramer, $P = 0.001$), LBC (Tukey-Kramer, $P = 0.0164$), and IZ (Tukey-Kramer, $P = 0.003$), (Figure 9). In general, WBL levels increased from June to July (Figure 9). In 2004, the interaction between month and location did not explain WBL levels ($F_{6, 87} = 0.893$, $P = 0.504$), but the effect of location ($F_{3, 87} = 8.25$, $P < 0.0001$) and month were highly significant ($F_{2, 87} = 13.49$, $P < 0.0001$). After accounting for location and month, the addition of mean weekly maximum temperature (MWMT) prior to fish capture at each location did not explain WBL ($F_{4, 89} = 0.66$, $P = 0.62$).

From June through September, there was a significant change in WBL from fish sampled in DC ($F_{2, 24} = 9.73$, $P = 0.0009$) and LBC ($F_{2, 22} = 3.966$, $P = 0.034$), but not IZ ($F_{2, 22} = 3.04$, $P = 0.068$) or BW ($F_{2, 23} = 1.62$, $P = 0.22$). In Deer Creek, there was a significant increase in mean WBL from June to July (Tukey-Kramer, $P = 0.003$) and a significant decrease from July to September (Tukey-Kramer, $P = 0.003$; Figure 9). At lower Black Canyon, there was a significant increase in WBL from June to July (Tukey-Kramer, $P = 0.029$) but not July to September (Tukey-Kramer, $P = 0.16$; Figure 9).

The same pattern for WBL determined for July 2004 was observed in July 2005 at DC, LBC, BW, and IZ (Figure 10). In 2005, there was a significant difference in WBL among all eight study location on 24-25 July ($F_{7, 72} = 5.27$, $P < 0.0001$, Figure 11) In 2005, mean WBL levels ranged from 13.9% for fish collected from lower Murderers Creek (LMC) to 21.7% for fish collected at upper Murderers Creek (UMC). Arranging the study locations from the lowest mean weekly average temperature recorded at upper Black Canyon (UBC) to the highest maximum daily temperature recorded at LMC suggests a pattern where mean WBL increase from UBC to IZ and then begins to decrease as temperature increases at BW, SF, and LMC (Figure 11). A second order polynomial regression of MWMT on WBL explains 19% of the variation between WBL and MWMT.

Liver hsp70 levels from June-September 2004

One protein band was detected on western blots of liver tissue and this band contains proteins of approximately 70-75 kDa (Figure 12). On 10-11 June 2004, there were no significant differences in liver hsp70 band density measured at DC, LBC, IZ, or BW ($F_{3, 36} = 2.56$, $P = 0.070$; Figure 13). Significant differences in hsp70 band density were detected among locations on 30-31 July ($F_{3,34} = 30.56$, $P < 0.0001$) and 11 September ($F_{3 ,35} = 21.51$, $P < 0.0001$).

In July 2004, liver hsp70 band density increased with increasing water temperature among locations ($r^2 = 0.729$). The liver hsp70 band density measured in fish collected from DC and LBC were similar (Tukey-Kramer, $P = 0.06$; Figure 13). There was a significant difference in liver hsp70 band density between LBC and IZ (Tukey-Kramer, p<0001). There was no difference in hsp70 band density between BW and IZ (Tukey-Kramer, $P = 0.09$; Figure 13).

In September, fish from DC had the lowest hsp70 band density measurement and this was similar to IZ (Tukey-Kramer, $P = 0.137$), but not LBC or BW (Tukey-Kramer, $P > 0.05$, Figure 13). There was no significant difference in hsp70 band density between LBC and IZ in September (Tukey-Kramer, $P = 0.208$) and all locations were significantly different than BW.

Across months, there was a significant difference in hsp70 band density in fish collected at LBC ($F_{2, 26} = 4.85$, $P = 0.016$), BW ($F_{2, 27} = 116.5$, $P < 0.0001$) and IZ ($F_{2,27} = 25.72$, $P < 0.001$) but not at DC ($F_{2, 27} = 2.26$, $P = 0.123$). From June to July, there was a significant increase in hsp70 band density measured in fish collected at LBC, BW, and IZ (Tukey-Kramer, $P < 0.05$). From July to September, a significant decrease in hsp70 band density was measured in fish collected from DC, IZ, and BW (Tukey-Kramer, $P < 0.05$) but not LBC (Tukey-Kramer, $P = 0.093$; Figure 13).

Liver hsp70 levels in field collected fish from 23-24 July 2005

In July 2005, there were significant differences in liver hsp70 levels between study locations ($F_{7, 68} = 35.04$, $P < 0.0001$). In 2005, the lowest hsp70 levels were measured in fish collected from UBC and DC. Liver hsp70 levels measured in fish collected from LBC were significantly different than liver hsp70 levels measured in fish collected from DC (Tukey-Kramer, $P = 0.0313$) and UBC (Tukey-Kramer, $P < 0.0001$). Liver hsp70 band density measured in fish collected from UMC, IZ, BW, and the SF at river kilometer 24 were similar, and these levels were significantly greater than band density measured in fish collected from LBC (Tukey-Kramer, $P < 0.0001$).

The July 2005 hsp70 levels in fish liver demonstrated a sigmoid (threshold) relation with the maximum pre-visit water temperature (Figure 14). In liver tissue, a sigmoid curve was fit using mean monthly maximum temperature (MMT_{max}), mean weekly maximum temperature (MWMT), mean monthly average temperature (MMAT), and maximum, minimum, and average temperature (24-h max, 24-h min, 24-h avg) twenty four hours prior to capture (Table 3). The relation between mean weekly average temperature (MWAT) and hsp70 band density was not sigmoid (Appendix E) and for this reason, the estimated threshold temperature was not calculated (Table 3). Based on the sigmoid curve fits and 95% confidence intervals around the inflection point, the estimated induction (increased protein synthesis over basal levels) temperature in liver tissue is 15.6-16.2ºC for MMAT and 16.9 to 18.2 ºC for the 24 h average (Table 3). The estimated induction temperature for temperature

maximum (24-h max, pre-visit max, MWMT, MMT_{max} , Table 3) ranges from a low of 18.8ºC (MMT_{max}) to a high of 21.6ºC for the pre-visit maximum temperature.

A comparison of liver hsp70 band density for fish collected in July 2004 and 2005 at DC, LBC, IZ, and BW shows the same general pattern (Figure 15). There was no difference in liver hsp70 band density explained by an interaction between year and location ($F_{3, 69} = 1.70$, $P = 0.176$), but there was a difference among locations ($F_{3, 69} = 53.05$, $P < 0.0001$). At these four study locations in 2005, there is a linear relation between liver hsp70 band density and location ($r^2 = 0.68$), and band density among locations increases with water temperature.

Caudal fin hsp70 levels

Fin tissue was collected from the lower caudal fin of 80 fish in 2005. Sufficient tissue existed to conduct analysis on 66 samples. Similar to liver tissue, one band of approximately 70-75 kDa was detected in fin tissue (Figure 12). Fin hsp70 band density showed a similar pattern to liver hsp70 band density (Figure 16). There was a significant difference in caudal fin hsp70 levels among locations ($F_{7, 65} = 31.93$, $P < 0.0001$). After excluding samples that did not have values for both fin and liver tissues, hsp70 band density was compared across locations between these tissues. Within a location, there was a significant difference in hsp70 band density measurements between fin and liver tissue at UMC (paired t-test, $P = 0.0384$) and IZ ($P = 0.0266$, Figure 16).

White muscle hsp70 levels

Two bands were detected in white muscle tissue and correspond to the apparent molecular mass of the constitutive hsc70 (hsp73) and inducible hsp70 (hsp72) isoforms (Figure 17). The overall pattern for hsp70 band density for both the constitutive and inducible isoforms is similar to the pattern observed in liver tissue where band density increases with increasing temperature. There were significant differences in band density measurements among locations in both the constitutive (F_7,

78 = 21.68, p< 0.0001) and inducible bands ($F_{7, 78}$ = 78.89, p<0.0001; Figure 18). The band density of both the constitutive and inducible hsp70 isoforms displayed a sigmoid (threshold) relation with the 24-hour average, 24-hour minimum, 24-hour maximum, maximum temperature within 5 days of capture, MWMT, MWAT, MMAT, and MMT_{max}. Based on sigmoid curve fits, the estimated induction temperature is on average, from 0.2 to 2.3 ºC higher in white muscle tissue than liver tissue (Table 3). Temperature threshold in white muscle tissue are 16-18.5ºC for temperature averages (24-h avg, MMAT, MWAT) and 19.6-23ºC temperature maximum (24-h max, pre-visit max, MWMT, MMT_{max} , Table 3).

Hsp70 levels and lipid content

In July, but not June or September 2005, there was a significant inverse linear relation between hsp70 band density and lipid levels ($F_{1,31}$= 7.94, p=0.008; r^2 = 0.204). In 2005, the relation between hsp70 and lipids was not linear. Liver and white muscle hsp70 levels reached maximal synthesis levels when mean weekly maximum temperatures (MWMT) reached 22-23ºC. Although hsp70 levels remained constant, lipid levels began to decrease when MWMT exceeded 23ºC (Figure 19).

Discussion

Juvenile redband steelhead trout in the South Fork John Day River (SFJD), Oregon experience summertime temperatures sufficient to increase cellular quantities of hsp70 measured in liver, white muscle, and fin tissue. The finding that hsp70 can be measured in fin tissue is important because it provides a non-lethal procedure for measuring hsp70 levels. There was also a general pattern of decreased WBL with increasing temperature. The decrease in whole body lipids (WBL) was associated with maximal hsp70 levels, but it is likely that other factors besides increased hsp70 levels contribute to this general pattern.

In the laboratory experiment, the estimated induction temperature for hsp70 protein synthesis in both fin and liver tissue was between 19 and 22ºC. The temperature range determined in the laboratory overlaps estimated induction, or

"threshold", temperature values for sublethal protein damage (i.e., increased hsp70 synthesis) determined for field collected fish. The estimated induction temperature for field collected fish was variable and changed with different temperature summaries. For example, in liver tissue, the average estimated induction temperature using the pre-visit maximum temperature was 21.3ºC (95% confidence interval = 20.99 to 21.62). In contrast, the estimated induction temperature in liver tissue using MMAT was 15.9ºC (95% confidence interval = 15.63 to 16.17). There was also a difference in estimated induction temperature between tissues. Among the various temperature summaries for the estimated induction temperature, estimates for hsp70 induction were between 0.2 to 2.3 ºC higher for white muscle tissue than liver tissue. In general, estimated threshold induction temperatures based on average temperatures were lower than estimates based on maximum temperatures. The variability in estimated induction temperature using different temperature summaries, and different tissues, is important because it demonstrates how different temperature summaries lead to different conclusions about sublethal temperatures causing protein damage.

There is a highly significant sigmoid "threshold" relation between water temperature and hsp70 in liver tissue (Figure 14), and in both the constitutive and inducible hsp70 bands from white muscle tissue (Figure 18) for fish collected in July 2005. Fish from Deer Creek and upper Black Canyon experienced the lowest maximum summertime water temperatures (< 19.4ºC), and these fish expressed the lowest hsp70 content measured in all three tissues. Trout from lower Black Canyon (LBC) Creek had the greatest variability in band density measurements; and hsp70 expression was intermediate between those tested from Deer Creek and upper Murderers Creek. Upper Murderers Creek had maximum water temperatures about 2ºC higher than LBC (Table 2). The LBC study location is within 300 meters of the mainstem SFJD river. Water temperatures in the SFJD River are on average, 3-5ºC higher than water temperatures in Black canyon during the summer. If trout collected at LBC had resided in SFJD and then swam into Black canyon seeking thermal refuge shortly prior to collection, then we would expect increased variability in hsp70 protein levels measured in LBC. PIT tag tracking data in lower Black Canyon Creek detected 11 rainbow trout moving at least one kilometer upstream between 26 June and 21 July

2005 (Ian Tattam, Oregon State University, unpublished data), and one of these 11 fish had been previously tagged at the end of December in the SFJD 12 kilometers downstream of Black Canyon Creek. Therefore, it is reasonable to assume that several of the fish collected at lower Black Canyon in July 2005 had experienced previous thermal stress in the SFJD.

Estimated "threshold" temperature values for hsp70 protein synthesis in liver tissue for fish collected from the SFJD subbasin in 2005 was 15.6-18.2ºC for temperature averages (based upon 24-h avg, MMAT) or 18.8-21.6ºC for temperature maximum (based upon 24-h max, pre-visit max, MWMT, MMT_{max}). In white muscle tissue, threshold temperatures were 16-18ºC for temperature averages and 20-23ºC for temperature maximum. Differences in estimated threshold values between liver and muscle tissue are slight and both maximum and average temperature based estimates of thresholds overlap. Werner and co-workers (2005) reported threshold values of 18-19ºC for short and long term temperature averages, and 20-22.5ºC for maximum temperature during the 24 hours preceding fish collection for fish collected from the Navarro River, CA. Fish from the SFJD had a slightly lower estimated threshold temperature for temperature averages (16-18ºC) compared to fish from the Navarro river (18-19ºC). However, estimated threshold temperatures between these two stocks of fish using temperature maximum were almost identical and are between 20 and 23ºC. These findings suggest that the induction temperature for hsp70 synthesis in rainbow trout is similar among at least two subpopulations.

Unpublished data discussed by Basu et al. (2001) suggests that maximal hsp70 induction in trout occurs at 21ºC regardless of season, magnitude of heat stress, or acclimation temperature. If the unpublished findings of Basu et al. (2001) are comparable to results for rainbow trout collected from the SFJD and the Navarro River, this would suggest there is no difference in the induction temperature for hsp70 expression across multiple rainbow trout subpopulations. Alternatively, techniques used to quantify hsp70 expression in response to temperature are not precise enough to detect differences. Differences in laboratory techniques and variability in temperature and hsp70 measurements may also increase variability and mask differences in induction temperature among different rainbow trout subpopulations. Another

possibility is that the induction temperature for rainbow trout populations is similar (20-23ºC) but warm-water adapted subpopulations of rainbow trout, such as those from interior populations of eastern Oregon, synthesize a greater quantity of hsps and are able to respond more efficiently to the repair of denatured protein. This hypothesis is not completely satisfactory because other research suggests too much or too little hsp70 expression can result in developmental malformations and cell death (Nollen and Morimoto 2002). The strict regulation of all cellular chaperone proteins, not just hsp70, suggests that the cells ability to handle thermal stress has more to do with isoform efficiency than quantity.

Norris and co-workers (1995) studied outbred tropical topminnows, *Poeciliopsis gracili*s, and could not demonstrate any variation in the constitutive hsp70 isoforms and reported a high degree of polymorphism in the inducible isoforms. Furthermore, Norris et al. (1995) showed that *Poeciliopsis* adapted to desert environments had lower levels of inducible hsp70 isoforms than tropical species and hypothesized that the inducible and constitutive isoforms had different cellular functions that were under different evolutionary constraints. Variability in the constitutive and inducible hsp70 isoforms has also been shown in Antarctic notothenioid fishes and is highlighted by 1) the inability of one species to upregulate the inducible transcript (Place et al. 2004), and 2) the loss of the heat shock response (Hoffman et al. 2005). Collectively, these findings suggest there are probably differences in type and function of hsp70 isoforms expressed among different rainbow trout populations. Additional study is needed to determine if there are differences in hsp70 isoforms and function before further inference can be made into how hsp70's are related to the apparent thermal resistance among various salmonid populations.

In the laboratory experiment, difference between liver and fin tissue hsp70 band density were detected following the 22ºC temperature stress. Differences in hsp70 band density between fin and liver tissues was also observed in tissues samples of fish collected from the SFJD River at upper Murderers Creek and Izee falls in July 2005, but in this instance, hsp70 levels were significantly higher in liver tissue than in fin tissue. Difference in hsp70 expression suggests cells within these tissues are responding differently to temperature stress. Also, hsp70 isoforms may be expressed

in different tissues so it is possible that when exposed to temperature maximums of 21-23ºC, the hsp70 response is different because different isoforms are being expressed. To my knowledge, this is the first study to document the use of fin tissue to measure hsp70 as it relates to temperature stress in wild populations of rainbow trout. The utility of fin tissue for this purpose was briefly discussed by Hargis (1998), but no data were shown.

In this study, whole body lipid (WBL) content was used as an index of energy reserves, and analysis of WBL shows that WBL begins to decrease after hsp70 band density reaches maximal levels (i.e., asymptote of sigmoid curve; Figure 19). However, the decrease in WBL does not occur until MWMT exceeds 23ºC. The estimated threshold level for hsp70 induction in liver and white muscle tissue was between 20 and 23ºC. Collectively, this suggests increased synthesis of hsp70 proteins might provide a benefit by allowing for maintenance of body condition. Since the physiological consequences of maintaining elevated hsp70 levels for extended periods of time are unknown (Werner et al. 2005) and the synthesis of proteins is energetically costly (Hochachka and Somero 2002), it is reasonable to assume that prolonged synthesis of heat shock proteins would eventually cause a negative impact on energy reserves. Some support for this hypothesis was presented by Viant et al. (2003) who report that juvenile steelhead parr (*O. mykiss*) exposed to a chronic 20ºC temperature stress had increased hsp72 synthesis in white muscle tissue and this was associated with decreased liver and muscle levels of ATP, phosphocreatine, and glycogen. The decrease in WBL observed in trout from the SFJD when MWMT exceeds 23ºC might be an indication of this cost. Alternatively, the changes in WBL might be more strongly related to either 1) differences in food availability among study locations related to community level impacts of water temperature (Tait et al. 1994), or 2) appetite suppression (Linton et al. 1998; Myrick and Cech. 2000). However, previous work by Myrick and Cech (2000) reports that rainbow trout consume less food at 22ºC and show a decline in growth as temperatures neared 25ºC. Therefore, changes in WBL shown in trout from the SFJD are probably best explained by a combination of physiological costs (e.g., increased metabolic

rates), behavioral modification (e.g., appetite suppression), and the effect of temperature on food web dynamics.

In 2004, there were significant increases in liver hsp70 levels from June to July for fish examined from the LBC, BW, and IZ study locations. There were also significant differences in hsp70 levels among the fish collected from the study locations in September 2004. The change from June to July is likely a function of increased water temperature. However, it is uncertain if the differences in September are related to continued thermal stress, or are the legacy of thermal stress experienced in July. For example, following a single acute temperature stress where water temperatures were raised in experimental tanks from 12 to 26ºC at a rate of 2ºC/hr and maintained at 26ºC for 10-15 minutes, hepatic hsp70 levels measured in juvenile chinook salmon (*Oncorhynchus tshawytscha*) were significantly elevated over controls for 14 days (Mesa et al. 2002). After a 15ºC heat shock (7.4-22.4ºC) for 2 hours, Mazur (1996) reported elevated hsp70 levels in the gills of cutthroat trout up to 3 weeks following the stressor. Fish at each of the four study locations experienced different thermal histories. Difference in thermal history and the uncertainty of how long it takes hsp70 levels to return to basal levels following thermal stress suggest differences in hsp70 levels for fish collected in September from LBC, BW, DC, and IZ are a function of past and not current thermal stress.

An alternative explanation for changes in hsp70 levels from June to July and from July to September is a seasonal difference. Fader and co-workers (1994) showed seasonal changes in heat shock proteins of approximately 70 kDa in four different fish species, with the lowest levels measured in the winter and the highest levels measured in the spring. In the current study, the highest hsp70 levels measured in 2004 were from fish collected at the end of July. With the exception of Deer Creek, the coldest location sampled in 2004, there was an increase in hsp70 levels across all locations from June to July. These findings are contrary to the pattern detected by Fader et al. (1994) which showed decreases in hsp70 expression from Spring to Summer and from Summer to Spring. In the SFJD study, there was a significant change in hsp70 expression through time at all study locations except Deer Creek. Contrary to what would be expected from results presented by Fader and co-workers, hsp70 levels in

fish sampled from Deer Creek did not change from June through September. One component that is missing from Fader et al. (1994) is a comparison of fish sampled on a single date from multiple locations. For example, for fish collected from SFJD River, there is greater variation in hsp70 levels among locations (Figure 13) and temperatures (Table 2) on 10-11 June 2004 (variation among locations was not statistically significant), than there is in Deer Creek from June to September. The variation among locations is even more dramatic in July and September. In contrast, Fader et al (1994) only collected *Salmo trutta* from one location in Valley Creek (Valley Forge, PA). Fader and co-workers are correct in cautioning against incorrectly interpreting changes in hsp70 that do not consider seasonal variation. However, researchers also need to consider 1) variation within a season and among different locations, and 2) the effects of past thermal history on hsp70 expression.

The cellular stress response is species, organ and stressor specific, and factors other than temperature can alter cellular hsp levels (Iwama et al. 2004). For example, hsp70 levels in liver and kidney tissue increased in coho salmon (*Oncorhynchus kisutch*) when infected with bacterial kidney disease (Forsyth et al. 1997). Other factors shown to increase hsp levels in fish include industrial effluents (Vijayan et al. 1998), heavy metals (Sanders 1993), pesticides (Hassanein et al. 1999), and certain chemicals (Bierkens 2000). There is evidence that the stress hormone cortisol is involved with mediating hsp70 levels in fish tissue following physiological stress (Basu et al. 2001), and may attenuate gill hsp30 (Ackerman et al. 2000) and hsp90 mRNA expression (Sathiyaa et al. 2001). In contrast, handling stress (Vijayan et al. 1997), insertion of passive integrated transponders (PIT tags) into the body cavity of rainbow trout (this study), and electrofishing (Lund et al. 2002, Werner et al. 2005) have no apparent effect on hsp70 levels in salmonids. Common forms of hatchery stress including anaesthesia, hypoxia, hyperoxia, capture stress, crowding, feed deprivation, and cold stress have been reported to have no effect on gill hsp70 levels in Atlantic salmon (*Salmo salar*; Zarate and Bradley 2003). Increased muscle activity induces hsp70 in muscle tissue and blood of mammals (Milne and Noble 2002), but a recent exhaustive exercise study in male and female rainbow trout found no upregulation of hsp70, hsp30, or hsp90 in heart or muscle tissue (Clarkson et al.

2005). Clarkson and co-workers (2005) concluded that the maintenance of core body temperature and a lack of exercise-induced protein damage explained why hsps were not upregulated. Collectively this suggests that handling and exercise stress do not explain changes in hsp70 levels observed in fish collected from the SFJD or in the laboratory experiment.

It is not entirely possible to dismiss other factors, including the effects of pesticides or pollutants, on hsp70 levels in fish collected from the SFJD. However, the strong correlation between hsp70 and temperature found in both July 2004 and 2005 suggests temperature is the primary factor explaining alterations in hsp70 levels. If factors besides temperature affect hsp70 expression, the three mainstem study locations (SF, BW, and IZ falls) have the greatest potential to be impacted by confounding factors because of increased risk for exposure to agricultural runoff. Water quality measurements are not available to test this conclusion, but given the remoteness of the study locations and the relatively minor agricultural use, altered hsp70 expression because of agricultural pollutants or industrial waste is not a major concern.

Thermal tolerance is an important physiological trait that helps define suitable habitat and indices used to define this habitat should be chosen carefully (Rodnick et al. 2004). Although results of this research suggest juvenile redband rainbow trout in the SFJD River can tolerate summer daily maximum temperatures in excess of 22ºC, it is important to recognize that (1) they are physiologically compromised as indicated by the induction of hsps, (2) little is known about how fish respond when exposed simultaneously to multiple or sequential stressors (Schreck 2000), and (3) there is a difference between "stress" and "distress." For example, this research has shown an increase in hsps consistent with the *cellular stress* response, but it does not show that cellular stress has led to erratic behavior or physical deterioration associated with distress (see Schreck et al. 1997 and Schreck 2000 for further discussion of stress in fish). Moreover, Feder and Hofmann (1999) discuss the use of hsps as biomarkers in environmental toxicology and how it can be complicated because multiple simultaneous stressors can yield significant hsp70 expression even if no single

toxicant is at harmful levels. Collectively, this has led to the warning that the simple use of hsps as an indicator of *stress* in fish is premature (Iwama et al. 2004).

Measurement of hsps show promise for better defining physiological temperature requirements for juvenile rainbow trout in the natural environment, but this research needs to proceed with caution. Subsequent research should carefully define the scope of inference, and methods for investigating hsps need to expand beyond the use of one-dimensional gel electrophoresis (e.g., proteomics). The changes in hsps at the cellular level are dynamic. Positive or negative impacts of altering hsp levels and the subsequent impacts on the physiological status of fish are poorly understood. In the future, research on the physiological tolerance of fish to temperature would benefit from measurements of both hsps and ubiquitin. Ubiquitin is a highly conserved protein of 76 amino acids that covalently binds to denatured protein, and proteins bound by ubiquitin are marked for proteolysis by nonlysosomal proteases (Rechsteiner 1987, Ciechanover 1998). Therefore, ubiquitin conjugates provide information about the amount of irreversibly damaged protein. There are few examples of ubiquitin measurements in an ecologically and environmentally relevant context for aquatic organisms (but see Hofmann and Somero 1995, Hofmann and Somero 1996 for work on intertidal mussels). Ubiquitin was not measured in the current study.

Similar to Behnke (1992) and Zoellick (1999), findings from this work support observations that redband trout can tolerate stream temperatures near 26ºC (Behnke 1992; Zoellick 1999). Measurements of heat shock proteins strongly suggest these fish are experiencing thermal stress in the summer, but this does not provide convincing evidence that the redband rainbow trout in the SFJD River have greater thermal tolerance than any other rainbow trout subpopulation. Therefore, it is likely that the ability of redband rainbow trout in the SFJD to tolerate summer temperatures in excess of 22ºC is best explained by physiological adaptation to thermal history leading to temperature-dependent swimming performance and aerobic metabolism that allow for short term (< 2h) exposure to temperatures greater than 24ºC (see Gamperl et al. 2002).

In summary, the management of thermal habitat requirements for juvenile salmonids requires an understanding of physiological temperature limits. For this reason, it is important for studies of physiological limits to incorporate both spatial and temporal factors. The next challenge is to integrate this information from "the top down" and "the bottom up" (e.g., riverscape; Fausch et al. 2002) and provide a biologically meaningful interpretation of the results. Ultimately, the definition of these physiological limits will be used to help guide management of local land and water use practices. In turn, an understanding of these limits will contribute to a better understanding of the impacts of global climate change on fish populations.

Table 1- Sampling locations and coordinates (UTM; NAD 83) for collection locations in the South Fork John Day Basin in 2004 and 2005.

Sample Location	2004 Sampling Dates[a]	2005 Sampling Dates	UTM Coordinates[b]	Elevation (M)
Black Canyon				
Lower Black Canyon	10 Jun, 31 Jul, 11 Sep	25 Jul	11T 294907, 4912196	894
Upper Black Canyon	NA	25 Jul	11T 291492, 4913200	1037
Murderers Creek				
Lower Murderers Creek	NA	24 Jul	11T 297932, 4910336	908
Upper Murderers Creek	NA	24 Jul	11T 305099, 4906826	1001
Deer Creek	10 Jun, 30 Jul, 11 Sep	24 Jul	11T 300210, 4896411	1158
South Fork John Day				
SF RKM 24	NA	25 Jul	11T 296081, 4910855	881
Below Wind Creek	11 Jun, 31 Jul, 11 Sep	25 Jul	11T 297168, 4906262	939
Izee Falls	11 Jun, 30 Jul, 11 Sep	24 Jul	11T 298082, 4896082	1050

[a.] NA = not sampled

[b.] The first coordinate is meters east longitude, the second value is meters north latitude.

Table 2- Temperature data (ºC) for sampling locations in the South Fork John Day River from 10-11 June 2004; 30-31 July 2004; 11 September 2004; and 23-24 July 2005. June 2004 temperature records for Deer Creek and Izee Falls are from respective gauging stations operated by the U.S. Bureau of Reclamation. Weekly temperature averages are for the 7 days preceding the sampling date In 2004, monthly temperature ranges are for the 25 days preceding fish collection in June and September and from 1 or 2 July to 30-31 July at Lower Black Canyon (LBC), and 12 July to 30 July at Deer Creek. Monthly temperature ranges for 2005 are from 1 July to the sampling date. SF = South Fork John Day River; MWAT = mean weekly average temperature; MWMT = mean weekly maximum temperature; MMAT = mean monthly average temperature; MMT_{max} = mean monthly maximum temperature; MDTR = mean daily temperature range; NA = Not Applicable.

Sampling location	Sampling date	24-h Average	24-h Minimum	24-h Maximum	Pre-visit Max Temperature; Date	MWAT	MWMT	MMAT	MMT_{max}	MDTR
Lower Black Canyon	10 June 04	10.2	9.4	11.7	17.6; 4 June 04	11.2	13.6	10.3[a]	12.7[a]	4.2[a]
Lower Black Canyon	31 July 04	17.4	14.2	21.0	22.2; 24 July 04	17.3	21.3	16.5	20.5	7.5
Lower Black Canyon	11 Sept. 04	13.3	10.4	16.7	20.9; 18 Aug 04	12.9	16.0	14.4	17.2	5.3
Below Wind Creek	11 June 04	NA	NA	NA	NA	NA	NA	NA	NA	NA
Below Wind Creek	30 July 04	NA	NA	NA	NA	NA	NA	NA	NA	NA
Below Wind Creek	11 Sept. 04	NA	NA	NA	NA	NA	NA	NA	NA	NA
Izee Falls	10 June 04	12.3	11.6	13.4	21.3; 4 June 04	15.2	17.8	13.4	15.6	4.2
Izee Falls	30 July 04	18.6	15.1	22.2	23.7; 25 July 04	18.8	22.7	18.2	21.9	7.2
Izee Falls	11 Sept. 04	14.0	11.2	16.5	21.5; 17 Aug 04	13.3	15.8	15.3	17.7	4.9
Deer Creek	10 June 04	7.7	6.9	8.7	14.5; 4 June 04	9.9	11.9	8.8	10.6	3.4
Deer Creek	30 July 04	16.0	13.3	17.9	19.2; 25 July 04	15.9	18.2	15.8	17.8	4.4
Deer Creek	11 Sept. 04	11.8	9.6	14.2	18.7; 20 Aug 04	11.2	13.5	13.1	15.0	3.8
Upper Black Canyon	24 July 05	14.3	11.3	17.8	18.4; 19 July 05	14.4	17.9	13.8	17.2	6.3
Lower Black Canyon	24 July 05	16.3	13.4	20.4	21.1; 19 July 05	16.7	20.6	15.9	19.7	6.9
SF at river kilometer 24	24 July 05	21.6	17.9	25.4	25.4; 23 July 05	21.3	25.0	20.0	23.4	6.7
Upper Murderers Creek	23 July 05	18.8	16.3	22.9	22.9; 22 July 05	17.3	21.8	16.5	19.5	6.1
Lower Murderers Creek	23 July 05	22.4	19.9	25.8	25.8; 22 July 05	21.0	25.3	19.6	23.7	8.5
Below Wind Creek	24 July 05	20.2	16.4	24.0	24.6; 22 July 05	19.9	23.7	18.9	22.4	7.1
Izee Falls	23 July 05	21.3	19.3	23.7	23.7; 22 July 05	20.0	23.0	19.1	22.2	6.2
Deer Creek	23 July 05	16.8	15.0	19.4	19.4; 22 July 05	14.9	18.0	14.0	16.8	5.4

[a] Monthly temperatures = temperature records were only available for 23 days prior to fish collection.

Table 3- Relation between study location water temperature measurements preceding fish collection and estimated threshold temperatures (increased protein synthesis over basal levels) for heat shock protein 70 (hsp70). The 95% confidence interval for the estimated threshold temperature at $LogEC_{50}$ was determined with GraphPad Prism where EC_{50} represents 50% of the hsp70 values greater than 0.5 in liver (hsp70) tissue, and hsp72(hsp70) and hsp73(hsc70) in white muscle (WM) tissue.. Juvenile redband steelhead (*Oncorhynchus mykiss gairdneri*) trout were collected from 23-24 July 2005. NA= Relation is a third order polynomial, not a sigmoid curve (see results section).

					Standard error of curve		
Tissue type	Temperature measurement	Dose response/ Polynomial equation	Estimated threshold temperature (°C)	r^2	bottom	middle	top
Liver: hsp70	24-h average	y=0.788/(1+10^17.57-x)	16.9 to 18.21	0.708	0.037	0.320	0.023
Liver: hsp70	24-h maximum	y=0.788/(1+10^(20.59-x)	20.28 to 20.91	0.775	0.033	0.159	0.019
Liver: hsp70	pre-visit max	y=0.788/(1+10^21.31-x)	20.99 to 21.62	0.774	0.030	0.157	0.019
Liver: hsp70	MWAT	y=-1.2 – 0.16x + 0.03^2 – 0.00009x^3	NA	0.703	NA	NA	NA
Liver: hsp70	MWMT	y=0.794/(1+10^20.77-x)	20.47 to 21.07	0.769	0.030	0.149	0.020
Liver: hsp70	MMAT	y=0.796/(1+10^15.90-x)	15.63 to 16.17	0.744	0.032	0.137	0.022
Liver: hsp70	MMTmax	y=0.772/(1+10^19.18-x)	18.81 to 19.55	0.640	0.038	0.187	0.026
WM: hsp73 (hsc70)	24-h average	y=0.821/(1+10^18.22-x)	17.97 to 18.46	0.848	0.029	0.123	0.023
WM: hsp73 (hsc70)	24-h maximum	y=0.857/(1+10^22.81-x)	22.59 to 23.03	0.862	0.026	0.109	0.026
WM: hsp73 (hsc70)	pre-visit max	y=0.845/(1+10^22.76-x)	22.54 to 22.98	0.863	0.026	0.110	0.024
WM: hsp73 (hsc70)	MWAT	y=0.821/(1+10^17.12-x)	16.94 to 17.29	0.867	0.031	0.089	0.022
WM: hsp73 (hsc70)	MWMT	y=0.825/(1+10^21.55-x)	21.33 to 21.78	0.867	0.028	0.113	0.023
WM: hsp73 (hsc70)	MMAT	y=0.821/(1+10^16.32-x)	16.14 to 16.49	0.867	0.031	0.089	0.022
WM: hsp73 (hsc70)	MMTmax	y=0.8790/(1+10^21.58-x)	21.01 to 22.14	0.728	0.032	0.282	0.047
WM: hsp72(hsp70)	24-h average	y=1.548/(1+10^18.23-x)	17.93 to 18.54	0.780	0.057	0.154	0.047
WM: hsp72(hsp70)	24-h maximum	y=1.629/(1+10^22.92-x)	22.66 to 23.18	0.801	0.052	0.131	0.053
WM: hsp72(hsp70)	pre-visit max	y=1.604/(1+10^22.87-x)	22.61 to 23.14	0.800	0.053	0.133	0.050
WM: hsp72(hsp70)	MWAT	y=1.549/(1+10^17.17-x)	16.93 to 17.42	0.787	0.065	0.121	0.046
WM: hsp72(hsp70)	MWMT	y=1.566/(1+10^21.56-x)	21.37 to 21.94	0.795	0.057	0.143	0.047
WM: hsp72(hsp70)	MMAT	y=1.550/(1+10^16.38-x)	16.13 to 16.62	0.787	0.065	0.121	0.046
WM: hsp72(hsp70)	MMTmax	y=1.542/(1+10^19.85-x)	19.57 to 20.14	0.712	0.077	0.145	0.053

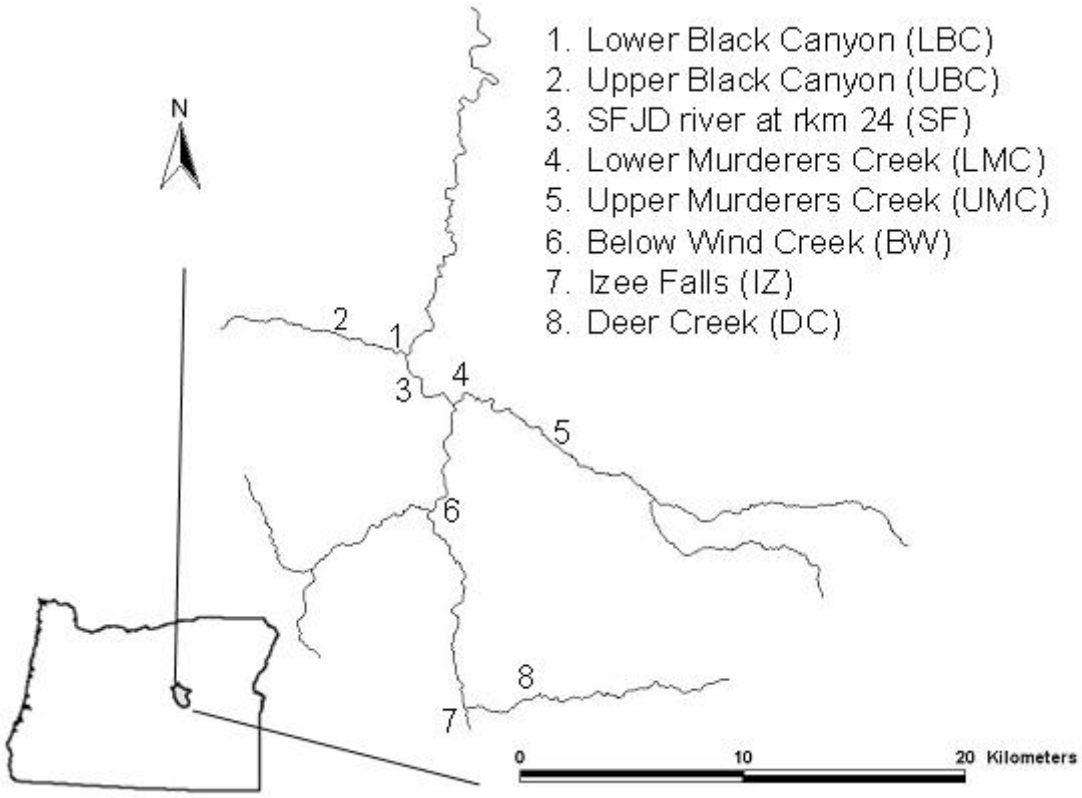

Figure 3- Map of juvenile redband steelhead trout sampling in the South Fork John Day River catchment, Grant County, Oregon.

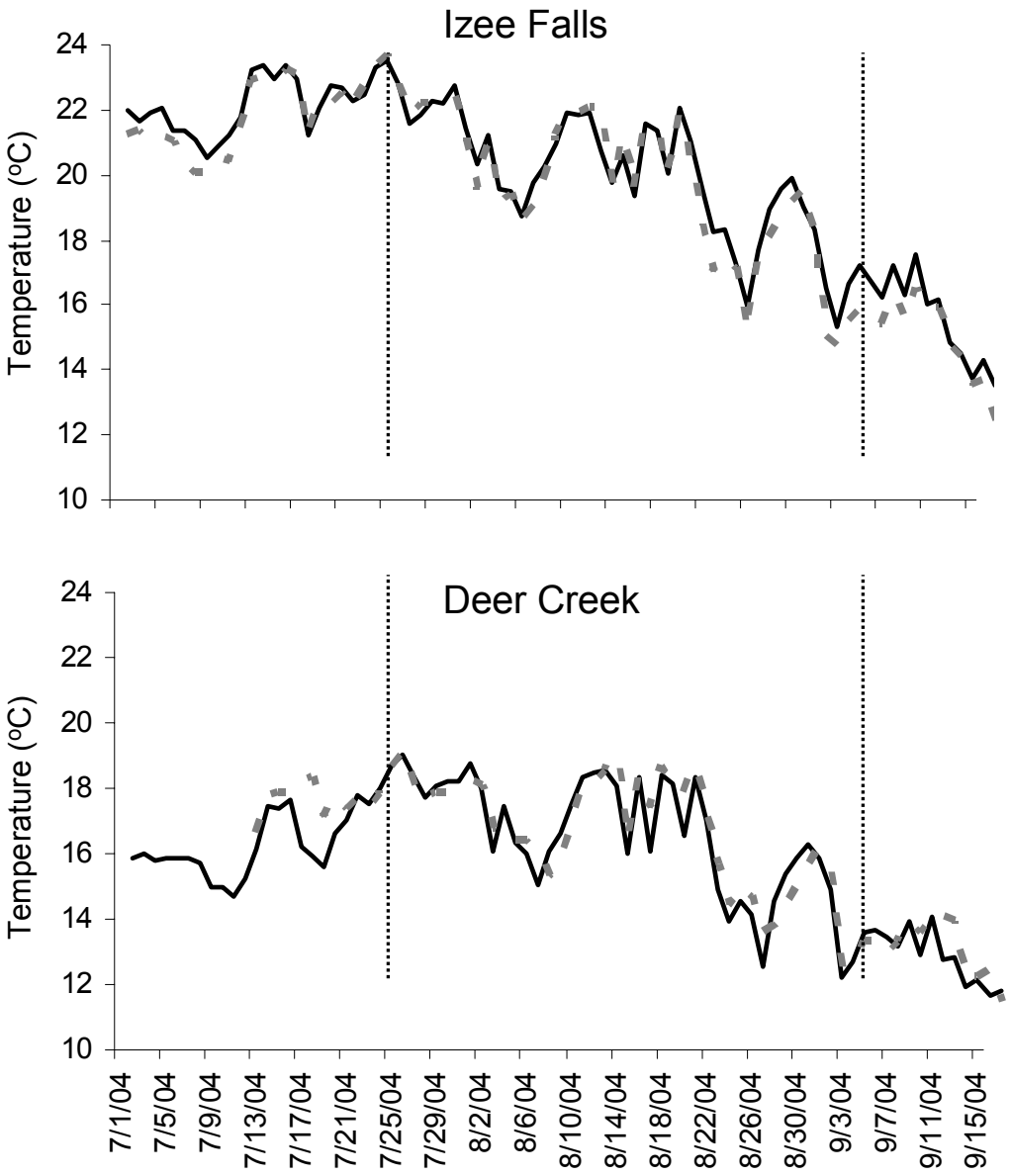

Figure 4- Maximum daily water temperatures (°C) recorded by an instream temperature logger (dashed line) and the Bureau of Reclamation gauging stations (solid line) at Izee falls and Deer Creek from 1 July-15 September 2004. Vertical lines indicate the 29 July and 11 September 2004 sampling dates.

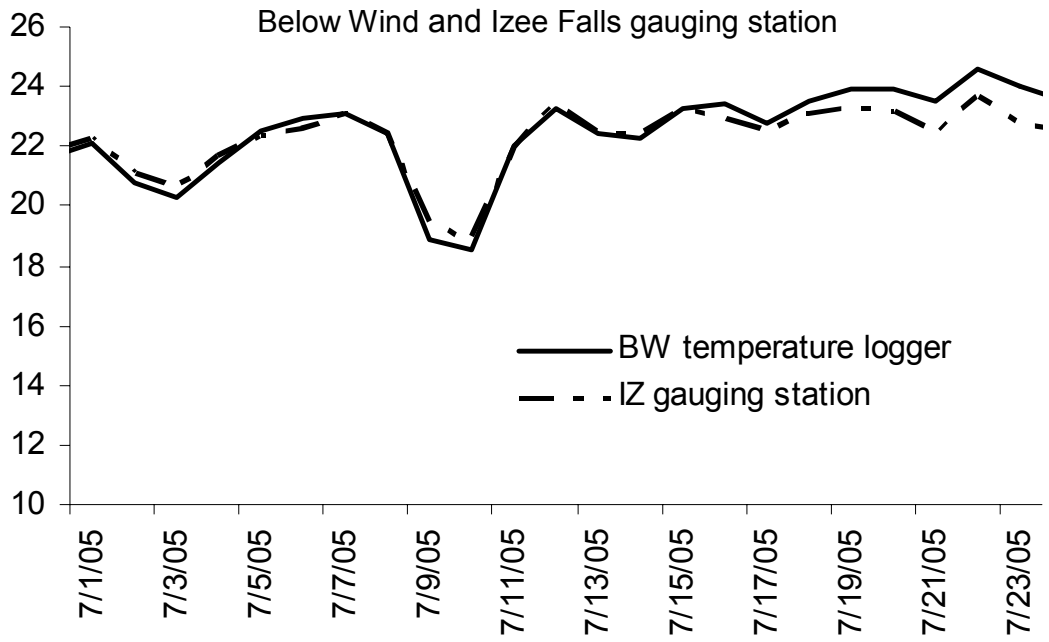

Figure 5- Maximum daily water temperatures (^{o}C) recorded by an instream temperature logger at the Below Wind study location and the Bureau of Reclamation gauging station (solid line) at Izee falls from 1 to 23 July 2005.

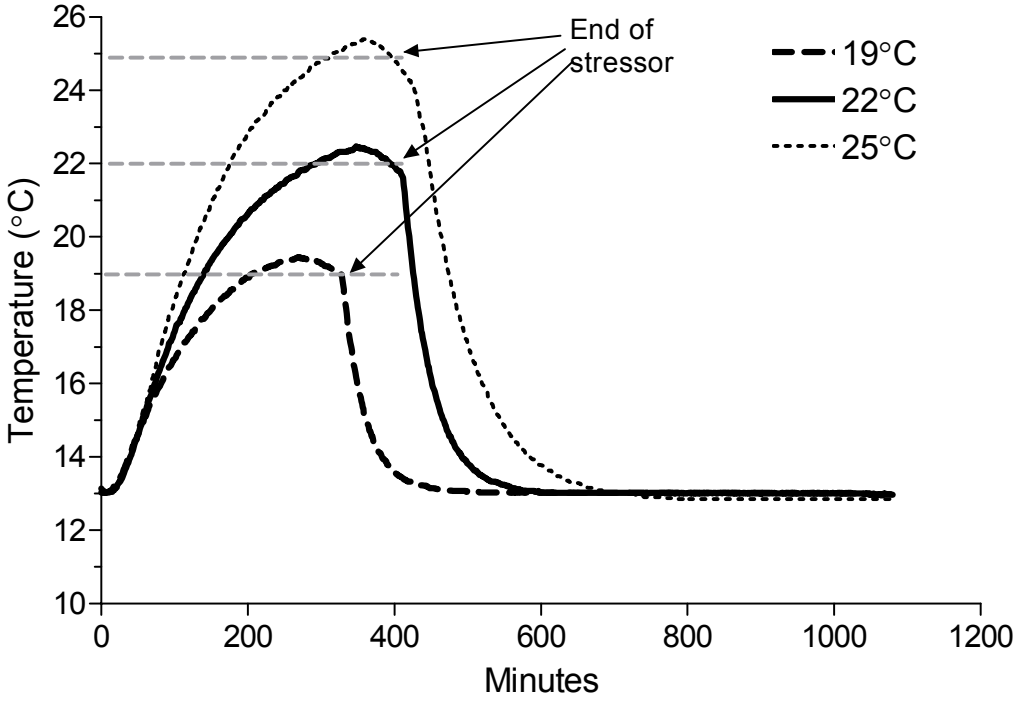

Figure 6- Thermal profile of the 19, 22, and 25ºC temperature stressors. The ambient water temperature (13ºC) was raised to target stressors of 19, 22, or 25ºC and maintained above the target temperature for 90-120 minutes. After the water temperature in the tank dropped below the target stressor, ambient water was added at > 5 liters/minute. Fish were lethally sampled 12 hours after reintroduction of 13ºC water.

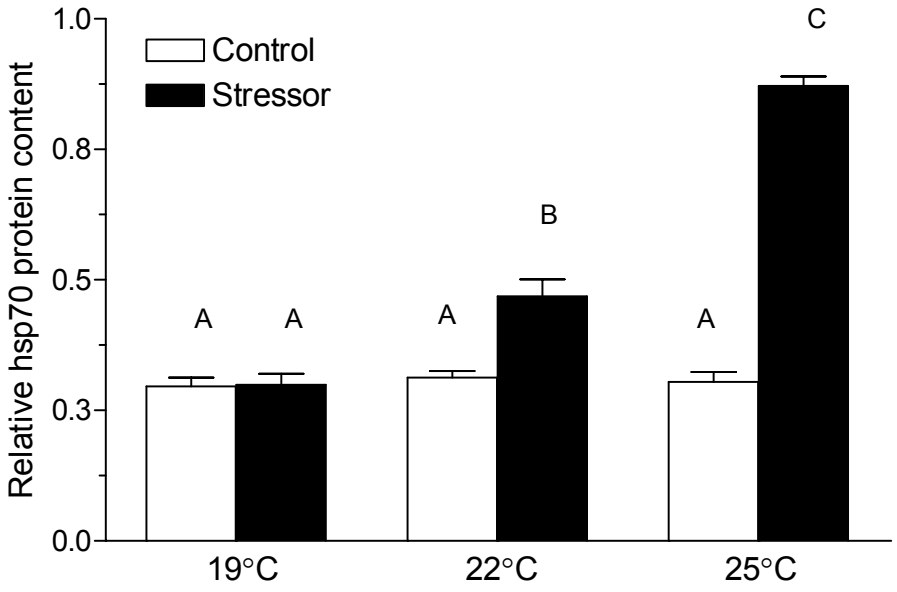

Figure 7- Relative heat shock protein 70 (hsp70) content in liver tissue from rainbow trout (*Oncorhynchus mykiss*) acclimated to 13ºC and exposed to an acute temperature stressor of 19, 22, or 25ºC followed by a 12-hour recovery at 13ºC before sampling. Open bars represent fish sampled before the temperature stressor (control) and dark bars represent fish exposed to the stressor. Values represent mean densitometry values of protein bands (± 1 standard error of the mean) detected by Western blotting; $n = 18$ for each bar. Shared letters indicate a non-significant difference ($P > 0.05$) in hsp70 values.

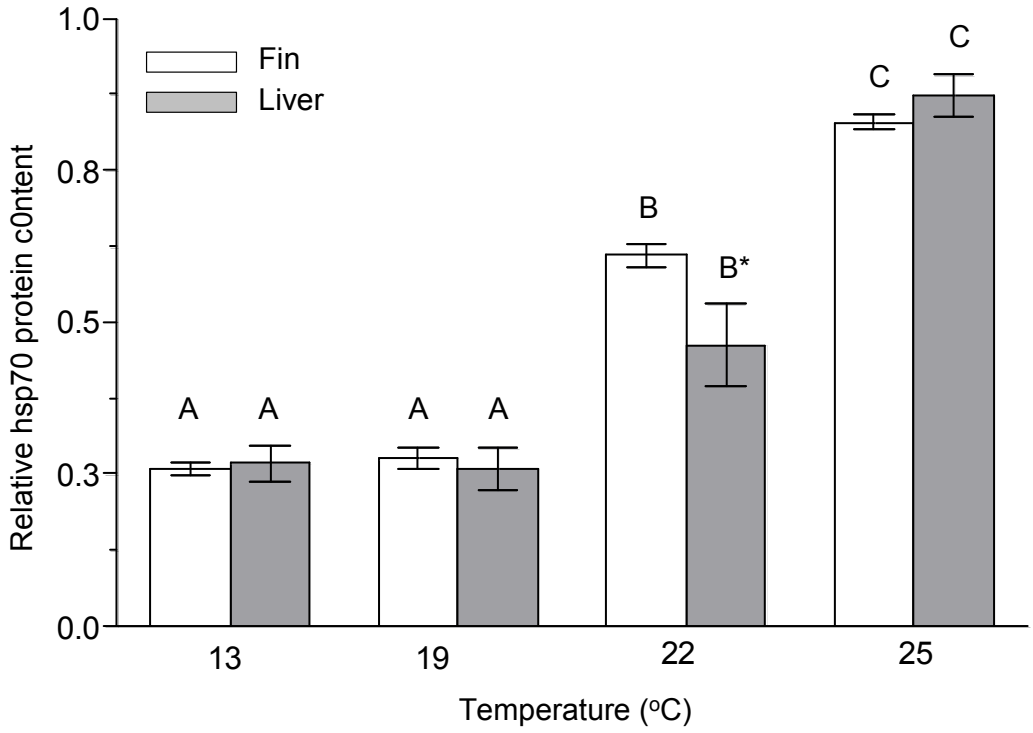

Figure 8- Relative heat shock protein 70 (hsp70) content in caudal fin and liver tissue from rainbow trout (*Oncorhynchus mykiss*) acclimated to ambient water temperature (13ºC) and exposed to an acute temperature stress of 19, 22, or 25ºC followed by a 12-hour recovery at 13ºC. Values represent mean densitometry values of protein bands (± 1 standard error of the mean) detected by Western blotting; $n = 8$ for each bar. Shared letters indicate a non-significant difference ($P > 0.05$) in hsp70 values within a tissue type. The asterisk indicates a significant difference between fin and liver tissue at 22ºC (paired t-test; $P = 0.043$).

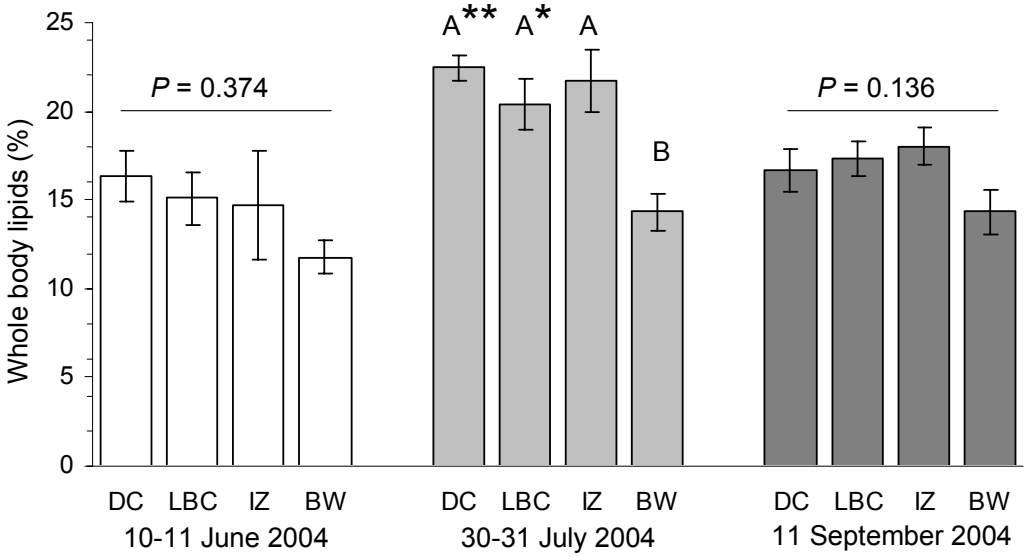

Figure 9- Whole body lipids (mean %; ± 1 standard error of the mean) in juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) collected from the South Fork John Day River. Fish were collected from 10-11 June, 30-31 July, and 11 September 2004 at Deer Creek (DC), lower Black Canyon (LBC), Izee Falls (IZ), and below Wind Creek (BW). $n = 7$ for each bar in June; $n = 9$ in for each bar in July (except BW, $n = 8$, in July) and September. Shared letters indicate a non-significant difference ($P > 0.05$). A single asterisk represents a significant difference ($P < 0.05$) from June to July. A double asterisk represents a significant difference from June to July and a significant difference from July to September.

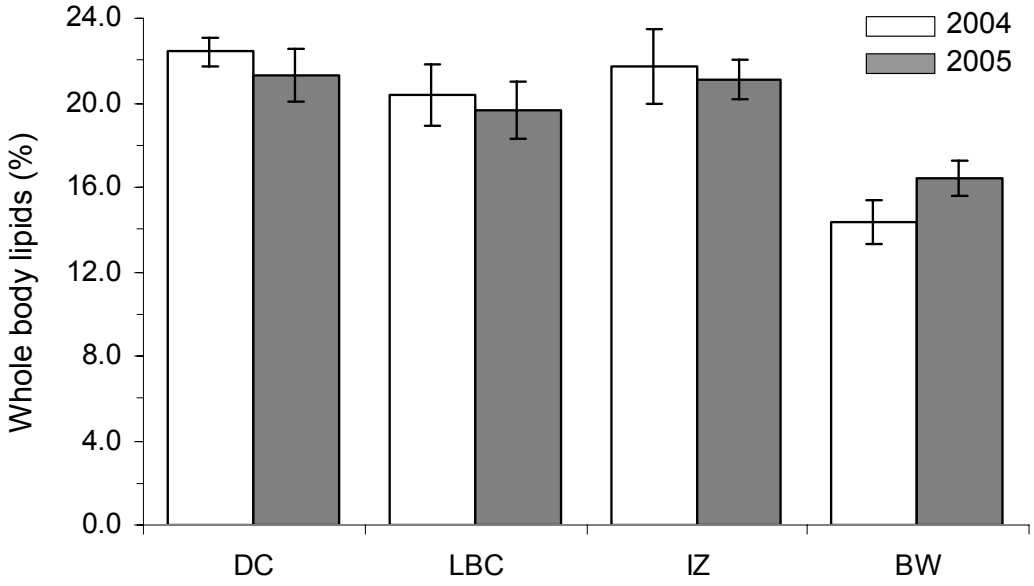

Figure 10- Comparison between years of whole body lipids (mean %; ± 1 standard error of the mean) in juvenile rainbow trout (*Oncorhynchus mykiss gairdneri*) collected from the South Fork John Day River. Fish were collected on 30-31 July 2004 (white bars) and 23-24 July 2005 (grey bars) at Deer Creek (DC), lower Black Canyon (LBC), Izee Falls (IZ), and below Wind Creek (BW). In 2004, each bar represents $n = 9$, except BW $n = 8$. In 2005, each bar represents $n = 9$.

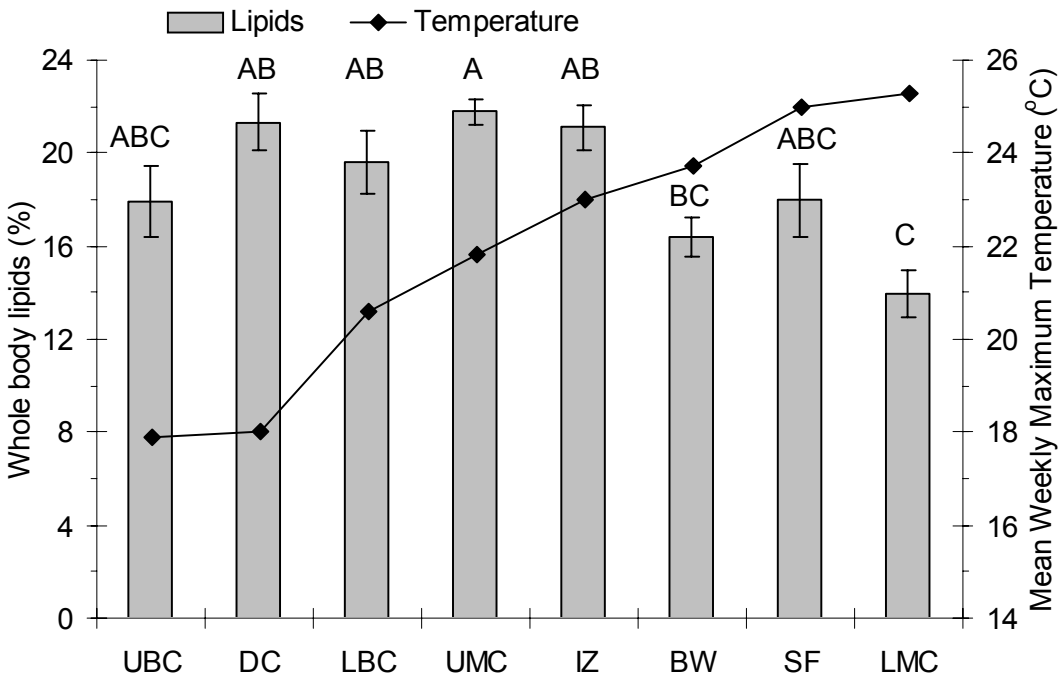

Figure 11- Mean weekly maximum temperature (ºC) compared to whole body lipids (mean %; ± 1 standard error of the mean) in juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) collected from the South Fork John Day River on 24-25 July 2005. Fish were collected at upper and lower Black Canyon (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). Shared letters indicate a non-significant difference at $P > 0.05$. Each bar represents $n = 10$.

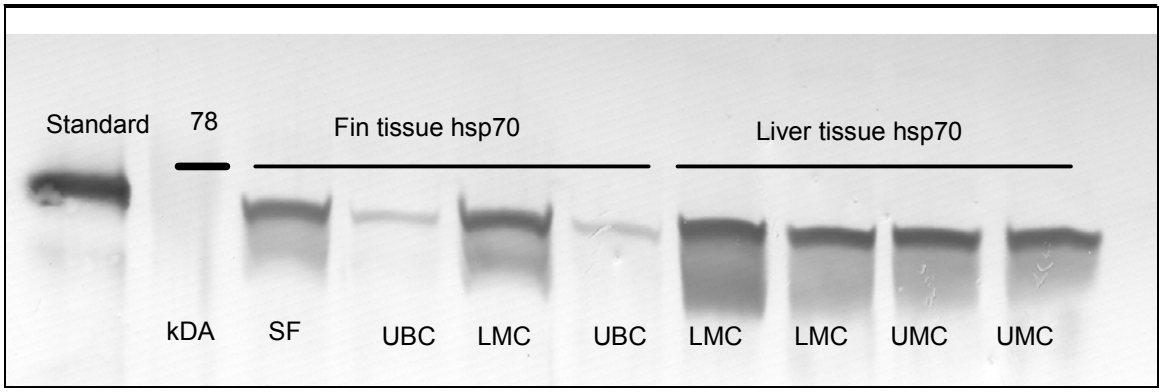

Figure 12- Representative Western blot of hsp70 protein detected in liver and fin tissue of juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) collected on 23-24 July 2005. Samples represented here were collected from fish sampled from the SFJD at river kilometer 24 (SF), upper Black Canyon (UBC), and lower and upper Murderers Creek (LMC, UMC). Proteins were visualized colorimetrically using an alkaline phosphatase conjugate substrate (Biorad). A recombinant chinook salmon hsp70 protein (Standard; StressGen Biotechnologies Corp) was applied to each gel to serve as an internal standard for molecular weight determination and blotting efficiency. Band density readings were adjusted for background.

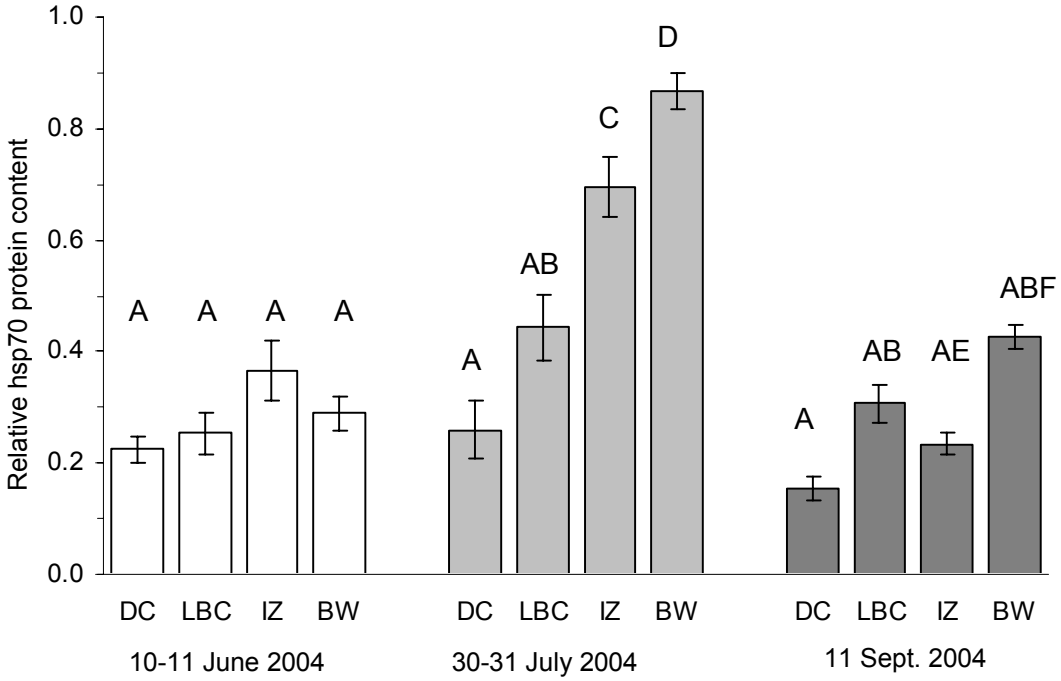

Figure 13- Relative heat shock protein 70 (hsp70) band density (mean densitometry value ± 1 standard error of the mean) in liver tissue from redband rainbow trout (*Oncorhynchus mykiss gairdneri*). Fish were collected from the South Fork John Day River on 10-11 June, 30-31 July, and 11 September 2004 at Deer Creek (DC), lower Black Canyon (LBC), Izee Falls (IZ), and below Wind Creek (BW). $n = 10$ for each bar, except $n = 9$ for LBC and IZ in July, and $n = 9$ for IZ. Shared letters indicate a non-significant difference ($P > 0.05$) in hsp70 values.

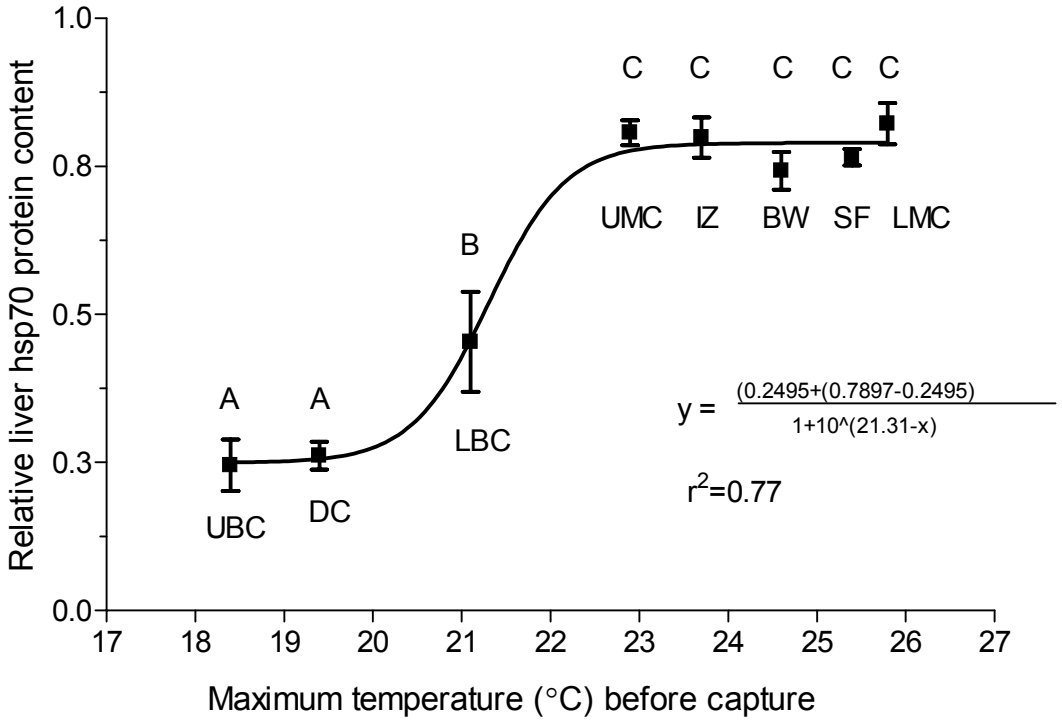

Figure 14- Relation between relative heat shock protein (hsp70) band density (mean densitometry value ± 1 standard error of the mean) in liver tissue of redband rainbow trout (*Oncorhynchus mykiss gairdneri*) and the maximum temperature within 5 days of capture. Fish were collected in the South Fork John Day River 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). Shared letters indicate a non-significant difference at $P > 0.05$. Each symbol represents $n = 10$ except UBC and BW ($n = 9$) and UMC ($n = 8$).

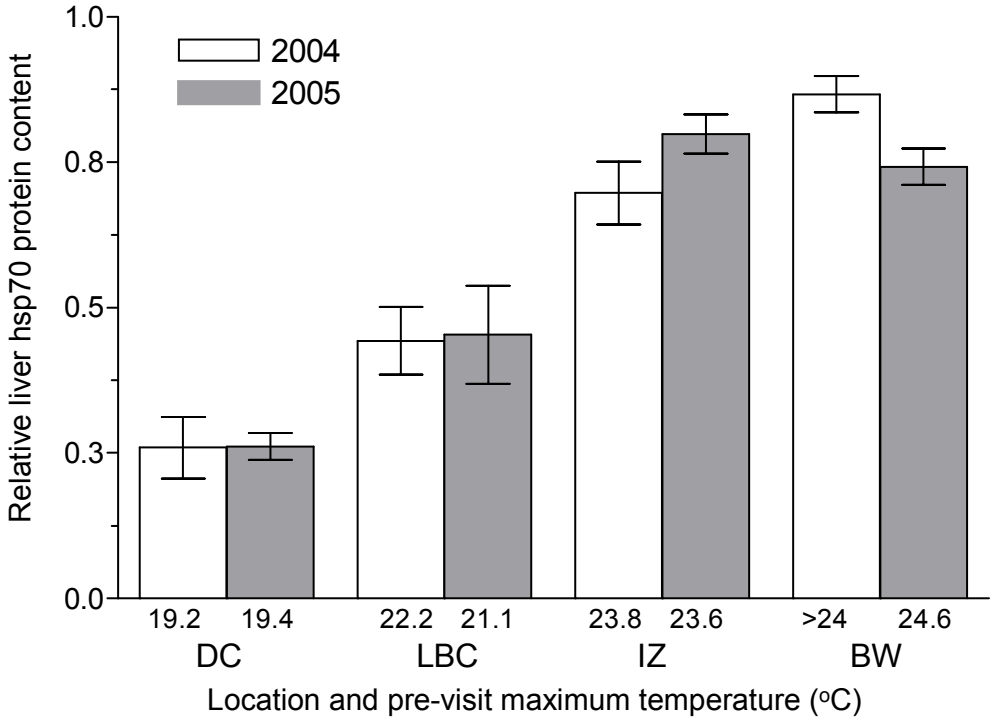

Figure 15- Relative heat shock protein 70 (hsp70) band density (mean densitometry value ± 1 standard error of the mean) in liver tissue from redband rainbow trout (*Oncorhynchus mykiss gairdneri*). Fish were collected from the South Fork John Day River on 30-31 July 2004 (white bars) and 24-25 July 2005 (grey bars) at Deer Creek (DC), lower Black Canyon (LBC), Izee Falls (IZ), and below Wind Creek (BW). Each bar represents $n = 10$ except LBC and IZ in 2004 ($n = 9$) and BW in 2005 ($n = 9$).

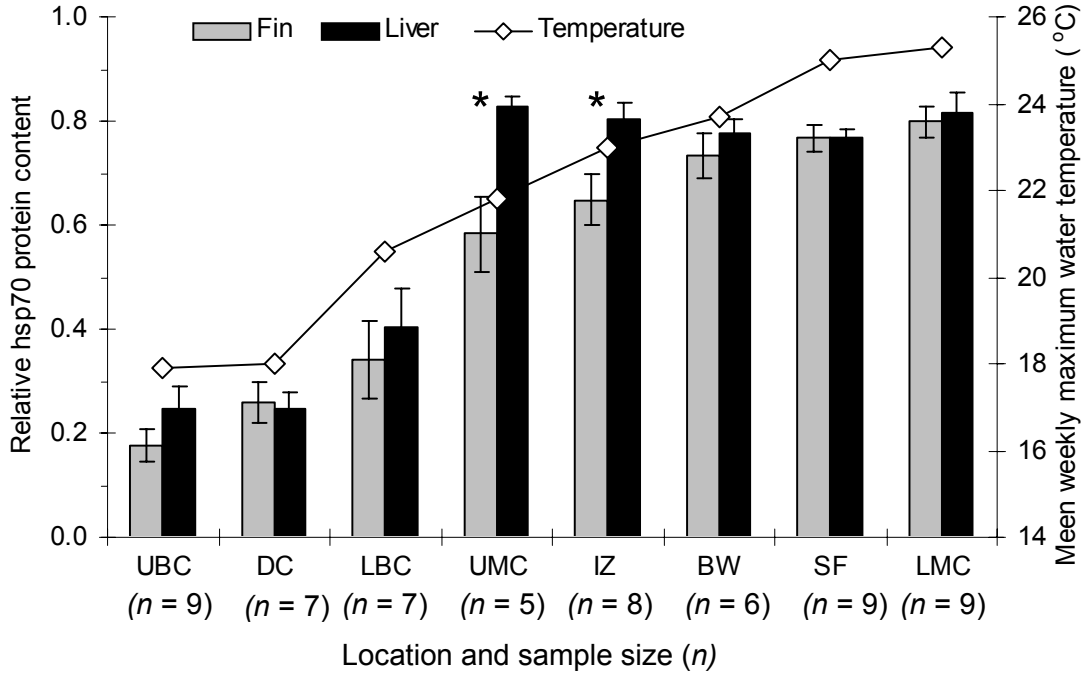

Figure 16- Relative heat shock protein 70 (hsp70) band density (mean densitometry value ± 1 standard error of the mean) in liver (black bars) and fin (grey bars) from redband rainbow trout (*Oncorhynchus mykiss gairdneri*). Fish were collected from the South Fork John Day River (SFJD) on 23-24 July 2005 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 represent the maximum water temperature (^{o}C) within 5 days of fish collection. The asterisk represents a significant difference (paired t-test, $P < 0.05$) in hsp70 band density between liver and fin tissue.

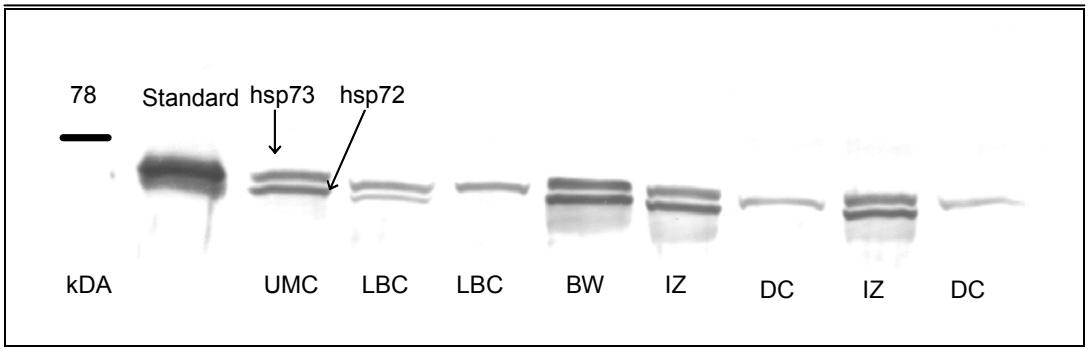

Figure 17- Representative Western blot of hsp72 and hsp73 protein detected in white muscle tissue of juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) collected on 23-24 July 2005. Samples represented here were collected from fish sampled at upper Murderers Creek (UMC), lower Black Canyon (LBC), below Wind Creek (BW), Izee falls (IZ), and Deer Creek (DC). Proteins were visualized colorimetrically using an alkaline phosphatase conjugate substrate (Biorad). A recombinant chinook salmon hsp70 protein (Standard; StressGen Biotechnologies Corp) was applied to each gel to serve as an internal standard for molecular weight determination and blotting efficiency. Band density readings were adjusted for background.

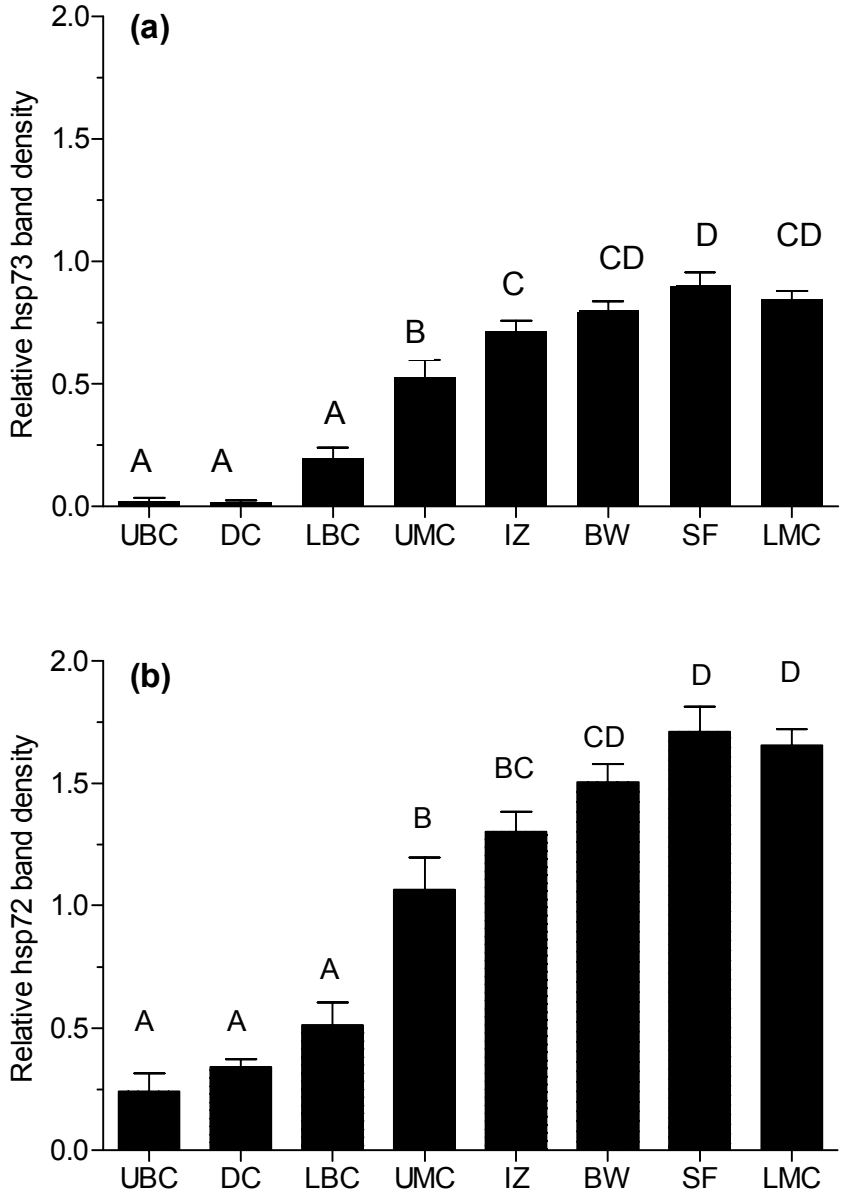

Figure 18- Relative expression of (a) hsp72 (hsp70) and (b) hsp73 (hsc70) in white muscle tissue from redband rainbow trout (*Oncorhynchus mykiss gairdneri*). Fish were collected on 23-24 July 2005 from upper and lower Black Canyon (UBC, LBC), Deer Creek (DC), upper and lower Murderers Creek (UMC, LMC), Izee falls (IZ), below Wind Creek (BW), and the South Fork John Day River at river kilometer 24 (SF). Hsp70 values represent mean densitometry values of protein bands (± 1 standard error of the mean). Shared letters indicate a non-significant difference ($P > 0.05$). Each bar represents $n = 10$, except UBC ($n = 9$).

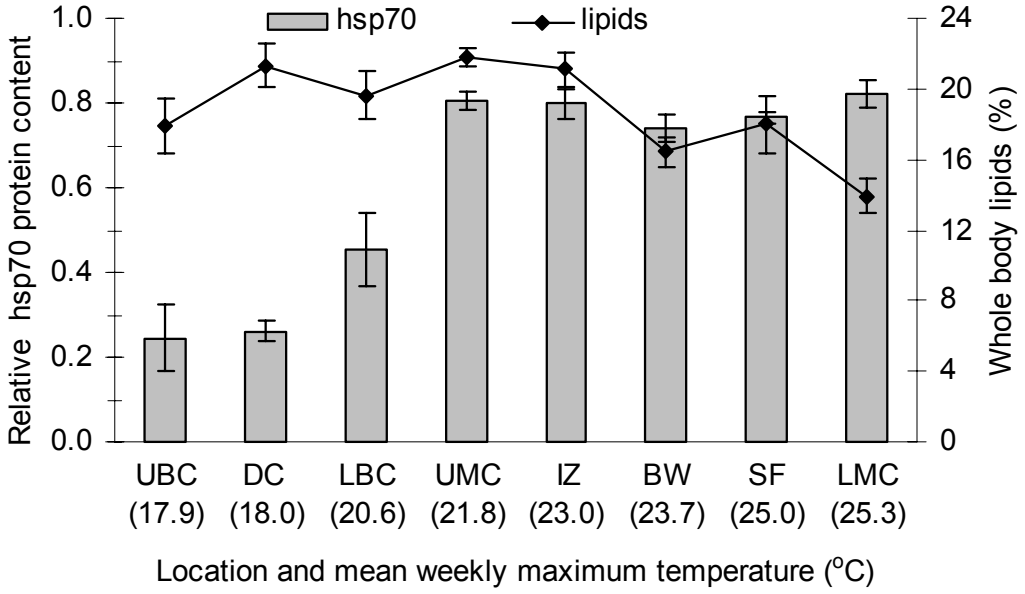

Figure 19- The mean weekly maximum temperature (MWMT) compared to whole body lipids (%) and relative hsp70 band density in liver tissue of juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*). Fish were collected from the South Fork John Day River on 23-24 July 2005 at upper and lower Black Canyon (UBC, LBC), upper and lower Murderers Creek (UMC, LMC), Deer Creek (DC), Izee Falls (IZ), and below Wind Creek (BW). Whole body lipid values represent the mean (± 1 standard error of the mean; n = 10 for each symbol) determined through proximate analysis. 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).

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Chapter 4: General Discussion

In this thesis, I examined the utility of heat shock proteins (hsp70 family) and whole body lipids as indices of fish condition as a method for determining physiologically stressful water temperatures in juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*). Understanding physiological temperature limits is important for defining and classifying summer thermal habitat requirements. I also documented the effect of handling and temperature stressors on hsp70 levels in laboratory studies. Results from the laboratory temperature manipulation study helped explain the patterns of hsp70 expression observed in fish sampled from the South Fork John Day (SFJD) River (chapter 3). Results from the PIT tag experiment (Chapter 2) also provided information about short term effects of PIT tagging on plasma cortisol and hepatic hsp70 levels. This research also examined ecological and management implications for using heat shock proteins as an indicator of thermal stress.

Results of the PIT tag experiment (Chapter 2) show that hsp70 levels are not affected by PIT tag handling stress. Furthermore, there were no long term (> 24 h) increases in plasma cortisol levels in PIT tagged fish. There was an increase in plasma cortisol levels following the initial tagging procedure at Time 2h, but PIT tagging was no more stressful than handling. There was some evidence that PIT tagged fish had higher plasma cortisol levels at 6 hours post-tagging than control fish, but the variability in plasma cortisol and the small sample size leave this conclusion open to debate. Moreover, changes in plasma cortisol levels did not measurably alter hsp70 expression. This finding is important because PIT tags are used throughout the Columbia River Basin to track movement and habitat use of endangered salmonids. Tagging methods that have negative impacts on performance of endangered salmonids should be discouraged. Since PIT tagging does not alter hepatic hsp70 levels, and the effects of PIT tagging on plasma cortisol levels are minimal, it should be possible to use PIT tags in combination with lab or field manipulations to examine the effect of specific or multiple stressors on hepatic hsp70 levels.

A major finding of this research was that juvenile redband steelhead trout in the SFJD experience summertime water temperatures that increase cellular hsp70

levels in liver, white muscle, and fin tissue. The finding that hsp70 can be measured in fin tissue is important because it provides a non-lethal procedure for measuring hsp70 levels. To my knowledge, this is the first documented use of fin clips as a non-lethal technique for quantifying cellular hsp70 levels in wild populations of rainbow trout.

The general pattern of hsp70 expression in relation to temperature was similar in fin, liver and white muscle tissue. However, there was some evidence of a difference in cellular hsp70 levels between these three tissues. For example, in the laboratory study, fin tissue had greater hsp70 expression following the 22ºC temperature stress than liver tissue. A similar finding was found for fish collected from the SFJD River at the UMC and IZ study location when MWMT were between 21 and 23ºC. However, at UMC and IZ, hsp70 levels were higher in liver tissue than in fin tissue. It is also important to recognize that estimated threshold temperatures (i.e. temperature that induce hsp70 synthesis) were higher in white muscle tissue than liver tissue. Differential hsp70 expression in tissues is an important consideration because it provides an example of the danger in comparing the hsp70 response among studies using different tissues. The variability in estimated induction temperature using different temperature summaries, and different tissues, is important because it demonstrates how different temperature summaries lead to different conclusions about sublethal temperatures causing protein damage.

There was also a general relation between increased levels of hsp70 and decreased body condition (whole body lipids). It is not clear which physiological mechanisms best explains the general decrease in body lipids when MWMT exceed 23ºC. One possibility is that metabolic costs associated with maintaining an elevated hsp70 response for a prolonged period of time (e.g., > 1 week) contribute to a net energy loss. Other explanations for decreased lipid levels include differences in food availability among study locations (e.g., community level impacts of water temperature), increased metabolic rates (e.g., Q_{10}), behavioral modification (e.g., appetite suppression), or a combination of effects.

There might be a seasonal component that helps explains hsp70 levels. However, the relation between water temperature and hsp70 expression in this study

make it difficult to determine if the effect of season acts independently of temperature effects. For example, for fish collected from SFJD River, there is greater variation in hsp70 levels among locations (Figure 13) and temperatures (Table 2) on 10-11 June 2004, than there is in Deer Creek from June to September. These findings suggest future research needs to carefully consider variation within a season and among different locations, and the effects of past thermal history on hsp70 expression.

The combination of hsp70 measurements and whole body lipid content is an example of how physiology can be used to help define habitat criteria for fish. However, indices used to define physiologically suitable habitat should be carefully considered. It is important to develop physiological indicators of fish performance that can be used to rapidly evaluate a fish's condition in a given habitat. Large quantities of money are being spent on habitat restoration projects. In general, the goal of habitat restoration efforts is to see an increase in the fish population that can be attributed to the restoration activity. Unfortunately, waiting for a population level response to evaluate the success of a given restoration activity might take years. If there is a change in the fish population, can it be proven that the change was a function of the restoration activity? Other explanations for the population change might include changes in ocean conditions that modify ocean survival of juvenile fish, or some other downstream modification (e.g., removal or building a dam) that effected survival. By using an index of population health, such as measurements of hsp70 to determine if fish are experiencing thermal stress, the impact of recovery and restoration efforts can be evaluated on a much shorter time scale. Another benefit of physiological indicators is that the effects of the restoration activity can be evaluated within the restoration area. For example, in Murderers Creek in the South Fork John Day River, the riparian zone has been protected by fencing that keeps cows out of the riparian zone. In the fenced areas, there is more vegetation. One way to evaluate the effect of this restoration activity on the fish community in Murderers creek would be to compare the physiological condition of fish in fenced areas with the condition of fish in unfenced areas.

Although this research suggests juvenile redband rainbow trout in the SFJD River can tolerate summer daily maximum temperatures in excess of 22ºC, there are

physiological costs. The rapid increase in cellular hsp70 levels is an example of a physiological mechanism that allows cells to withstand temperature increases, but this also means cellular processes are being threatened. There is also a difference between cellular stress and stress behaviors associated with distress. It is premature to presume that cellular stress is a superior or inferior indicator of physiological status if distress– which can have behavioral consequences (e.g., increased predation risk) –acts through mechanisms that do not involve cellular alterations in hsp70 levels. Compared to streams and rivers flowing through cities, the most prominent stressor in the SFJD River is temperature. Would juvenile trout still be able to cope with temperatures in excess of 22ºC if trout were exposed to multiple or sequential stressors (e.g., heat stress + pesticides + disease)?

The positive or negative impacts of altering hsp levels and the subsequent changes in the physiological status of fish are poorly understood. For example, how does thermal stress influence growth and development, long term survival, or life history strategies? At what point do hsp70 levels become detrimental to cellular processes? Although the ability to use hsps as a biomarker of "stress" is still questionable, carefully directed research measuring hsps in combination with other physiological indicators of cellular stress (e.g., ubiquitin) show promise for advancing the understanding of how temperature affects physiological processes in fish.

In conclusion, juvenile redband rainbow trout in the SFJD River are experiencing thermal stress during the summer. Although these fish are surviving summertime water temperatures that approach 26°C, this research does not prove or disprove the hypothesis that redband rainbow trout in the SFJD River are better suited to warm water than any other rainbow trout subpopulation. The definition of suitable thermal habitat for juvenile trout requires an understanding of physiological temperature limits, and a differences of 1 or 2°C is an important distinction when defining "suitable" and "unsuitable" habitat.

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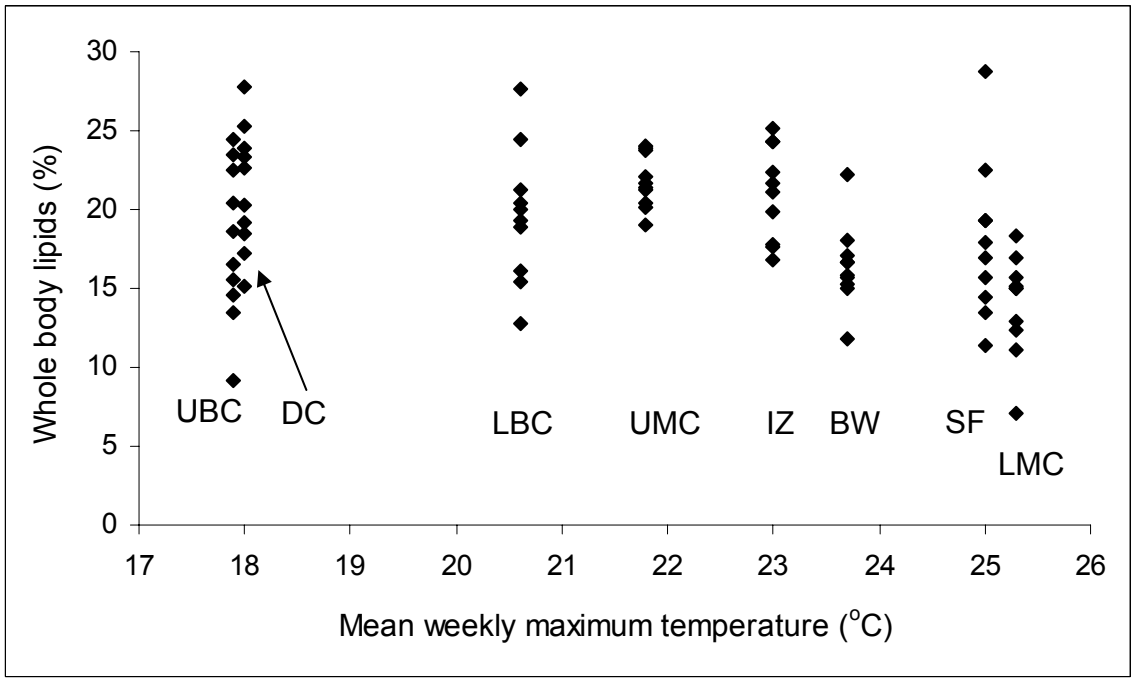
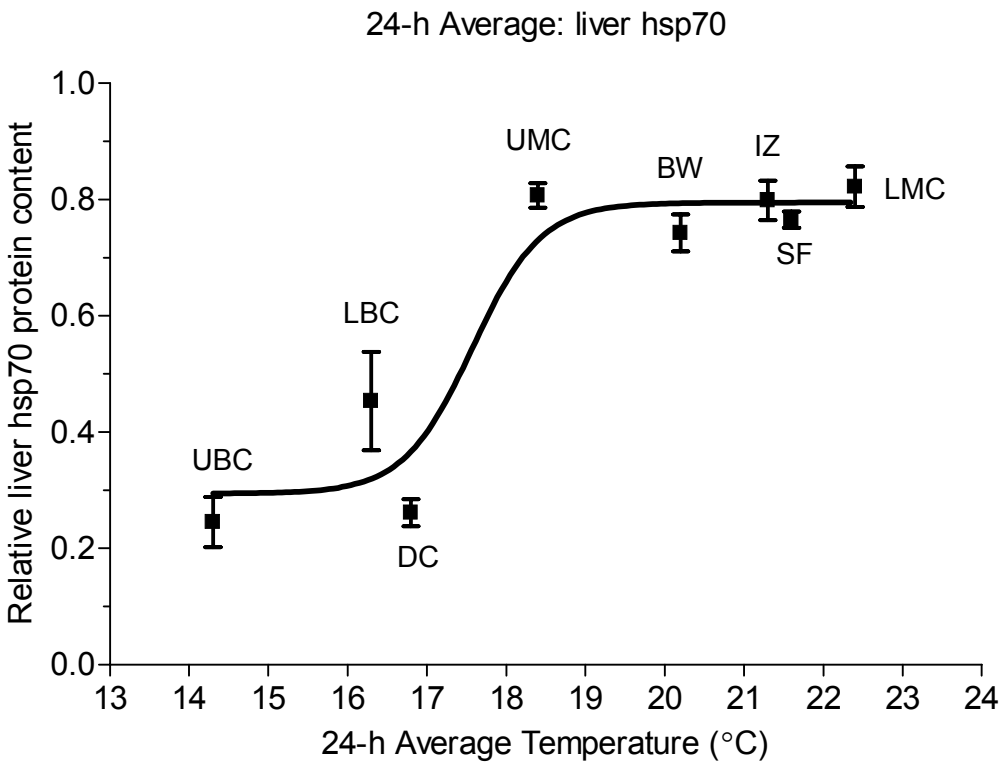
APPENDICES

Appendix A-Summary of mean whole body lipids (%) and water content (%) of juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) collected from the South Fork John Day River. Fish were collected from 10-11 June, 30-31 July, and 11 September 2004, and 23-24 July 2005 at lower and upper Black canyon (LBC, UBC), the South Fork John Day at river kilometer 24 (SF), lower and upper Murderers creek (LMC, UMC), below Wind creek (BW), Deer Creek (DC), and Izee falls (IZ).

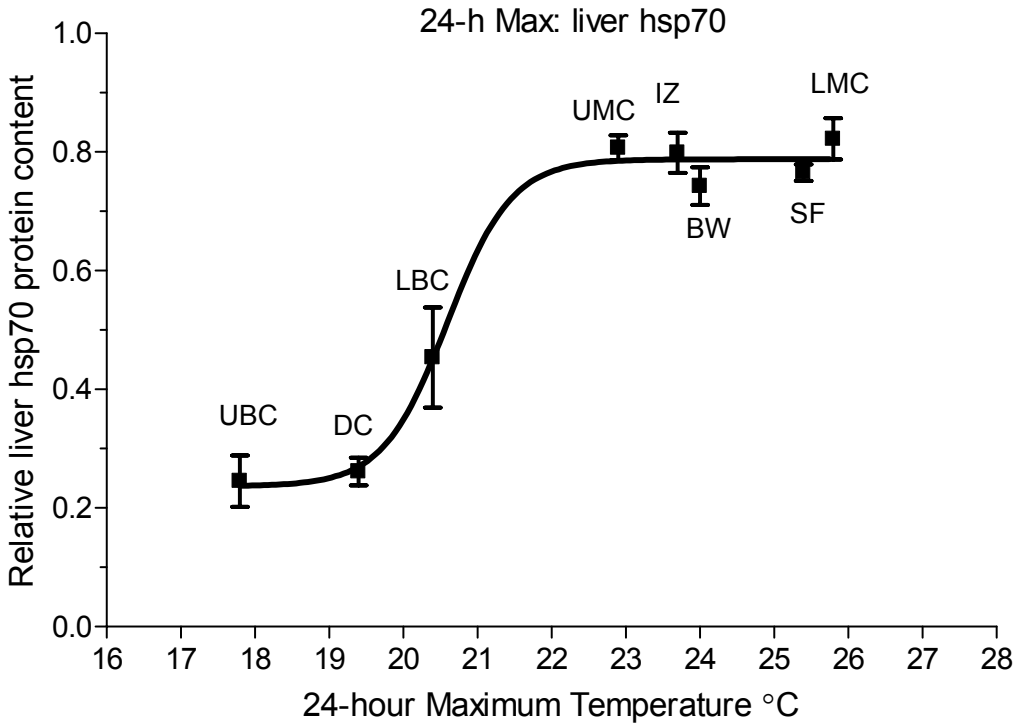
			Whole Body Lipids (%)		Water content (%)	
Sampling Location	Sampling date	*n*	Mean	SE	Mean	SE
Lower Black Canyon	10 June 04	7	15.10	1.50	75.92	0.59
Lower Black Canyon	31 July 04	9	20.39	1.45	73.76	0.47
Lower Black Canyon	11 Sept. 04	9	17.28	0.99	73.76	0.47
Below Wind	11 June 04	7	11.78	0.89	76.87	0.46
Below Wind	31 July 04	8	14.35	1.04	75.79	0.33
Below Wind	11 Sept. 04	9	14.31	1.27	75.79	0.33
Izee Falls	11 June 04	7	14.73	3.06	75.91	0.87
Izee Falls	30 July 04	9	21.72	1.78	72.16	0.64
Izee Falls	11 Sept. 04	9	18.04	1.08	72.87	0.27
Deer Creek	10 June 04	7	16.35	1.43	75.02	0.54
Deer Creek	30 July 04	9	22.44	0.68	73.85	0.37
Deer Creek	11 Sept. 04	9	16.7	1.22	73.54	0.37
Upper Black Canyon	25 July 05	10	17.98	1.58	75.21	0.5
Lower Black Canyon	25 July 05	10	19.62	1.37	75.17	0.6
South Fork	25 July -05	10	17.98	1.58	75.21	0.5
Upper Murderers Creek	24 July 05	10	21.76	0.54	73.52	0.23
Lower Murderers Creek	24 July 05	10	13.94	1.02	75.62	0.32
Below Wind	25 July 05	10	16.42	0.84	76.03	0.58
Izee Falls	24 July 05	10	21.1	0.1	73.84	0.37
Deer Creek	24 July 05	10	21.32	1.24	74.16	0.35
Total		N = 172				



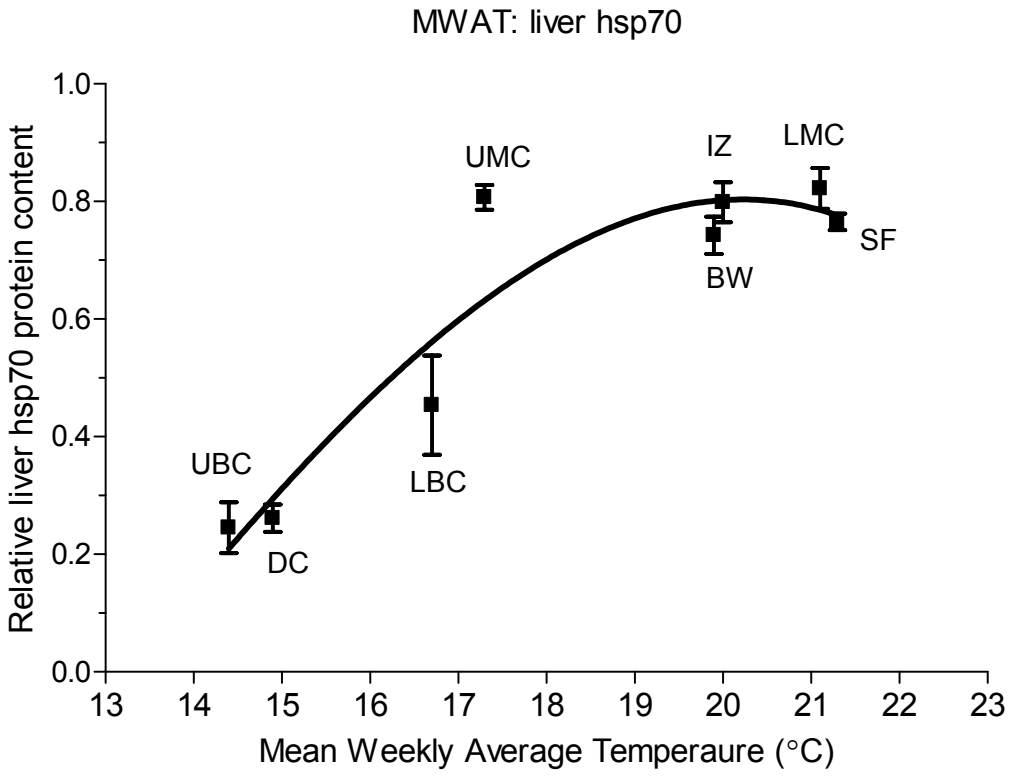
Appendix B- Scatter plot of whole body lipids (%) compared to the mean weekly maximum temperature (°C) before capture for juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) collected on 23-24 July 2005. Fish were collected at lower and upper Black canyon (LBC, UBC), the South Fork John Day at river kilometer 24 (SF), lower and upper Murderers creek (LMC, UMC), below Wind creek (BW), Deer Creek (DC), and Izee falls (IZ).



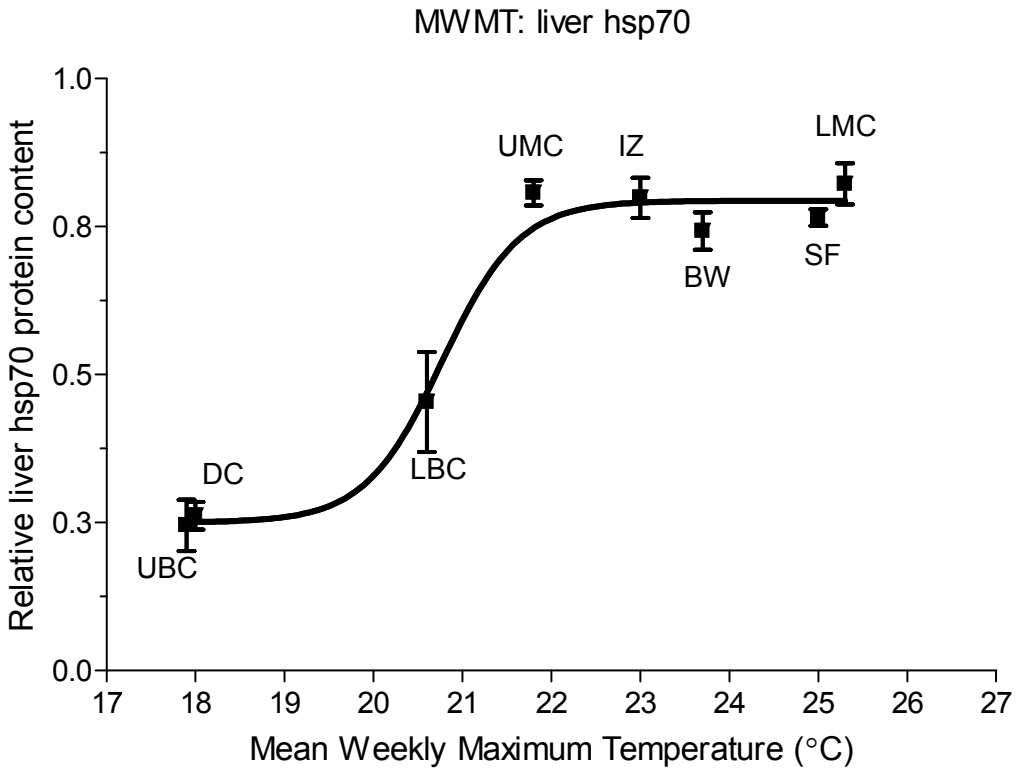
Appendix C- Relation between liver tissue hsp70 protein band density (mean densitometry value ± 1 standard error of the mean) and the 24-hour average daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



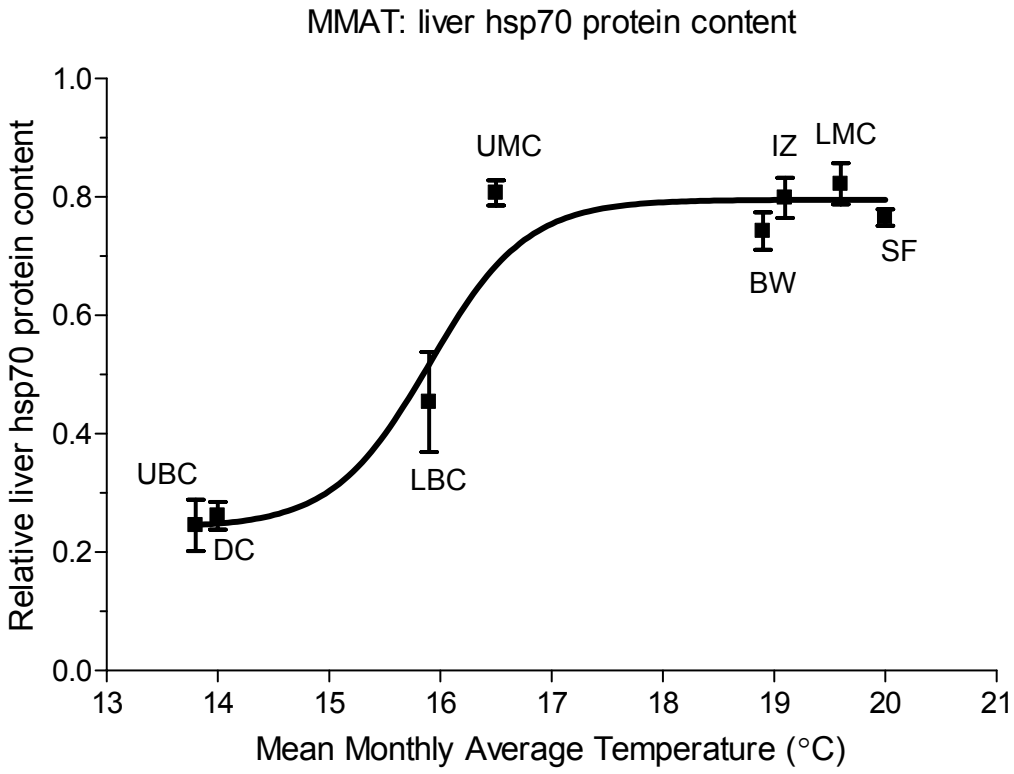
Appendix D- Relation between liver tissue hsp70 protein band density (mean densitometry value ± 1 standard error of the mean) and the 24-hour maximum daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



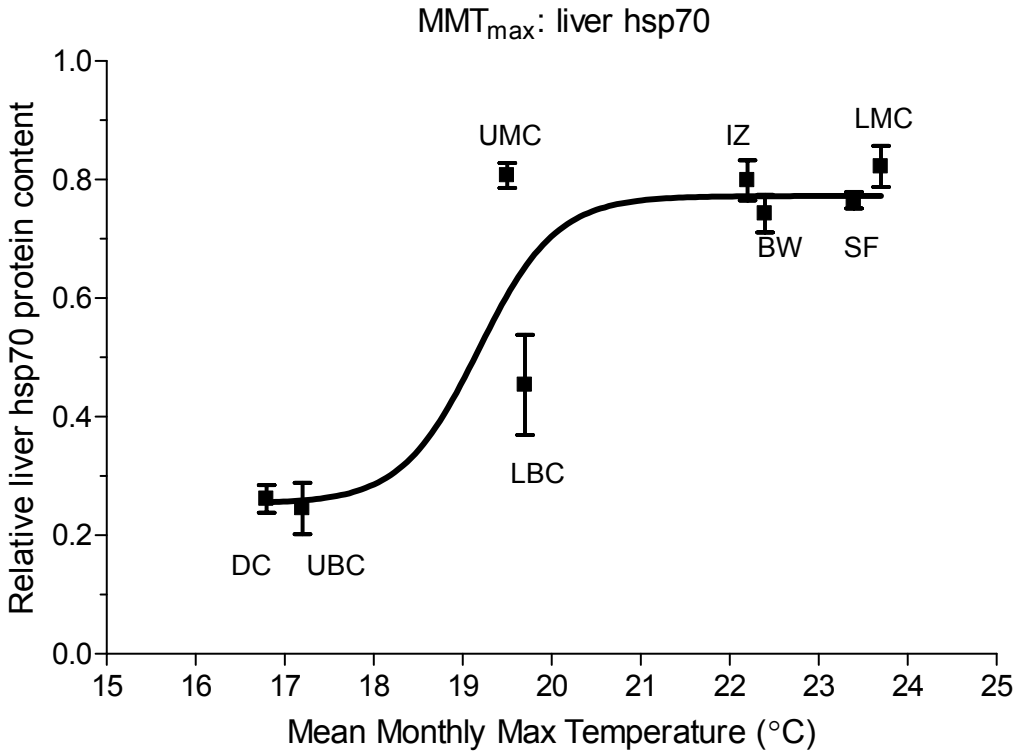
Appendix E- Relation between liver tissue hsp70 protein band density (mean densitometry value ± 1 standard error of the mean) and the mean weekly average daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



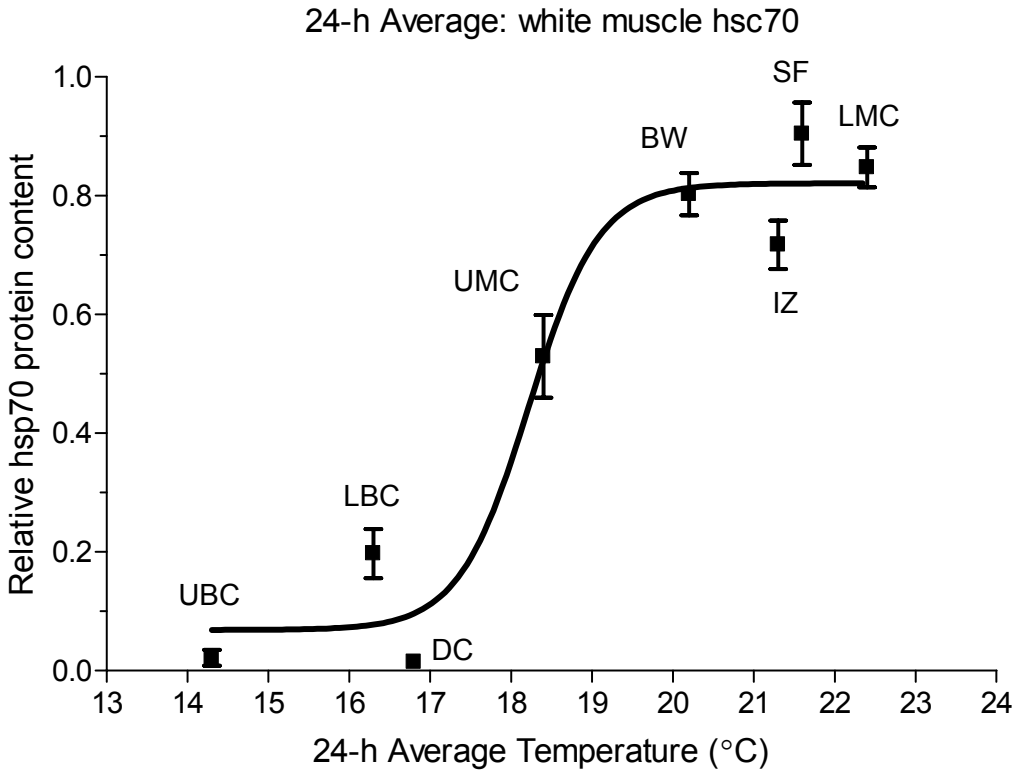
Appendix F- Relation between liver tissue hsp70 protein band density (mean densitometry value ± 1 standard error of the mean) and the mean weekly maximum daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



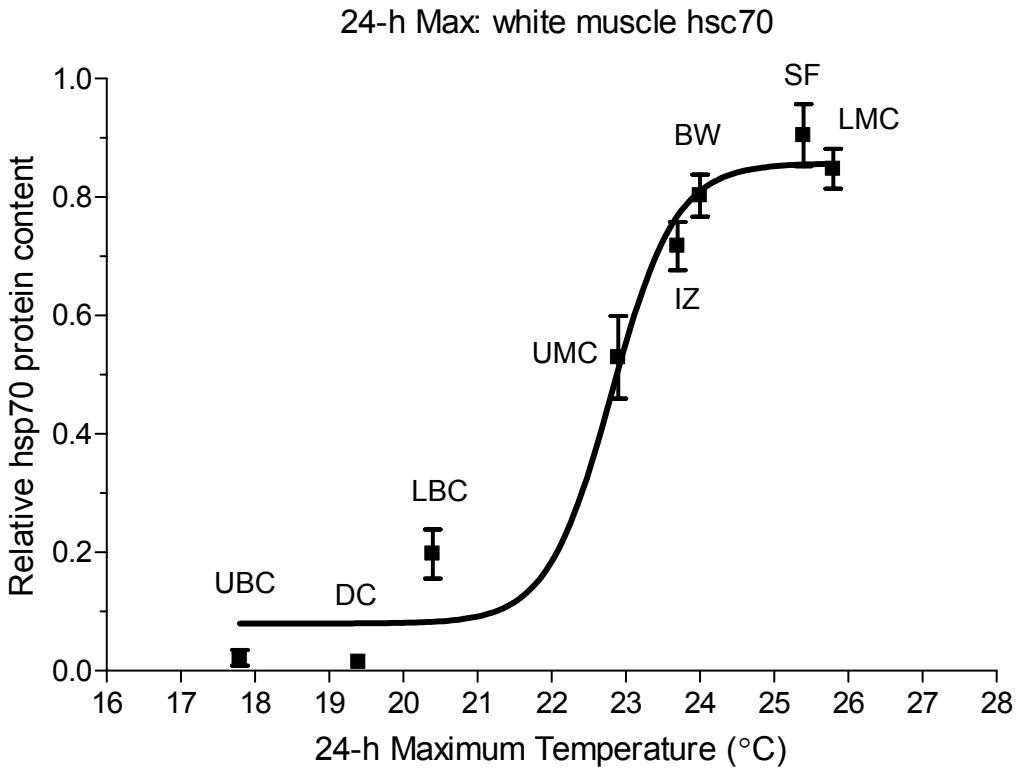
Appendix G- Relation between liver tissue hsp70 protein band density (mean densitometry value ± 1 standard error of the mean) and the mean monthly average daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



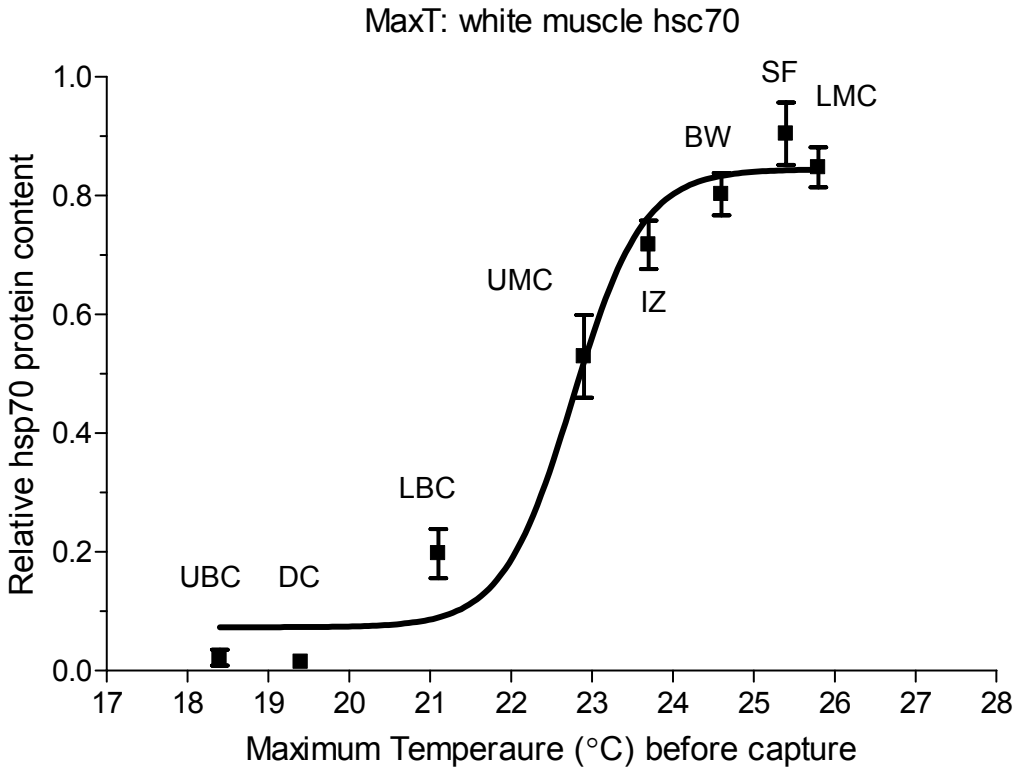
Appendix H- Relation between liver tissue hsp70 protein band density (mean densitometry value ± 1 standard error of the mean) and the mean monthly maximum daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



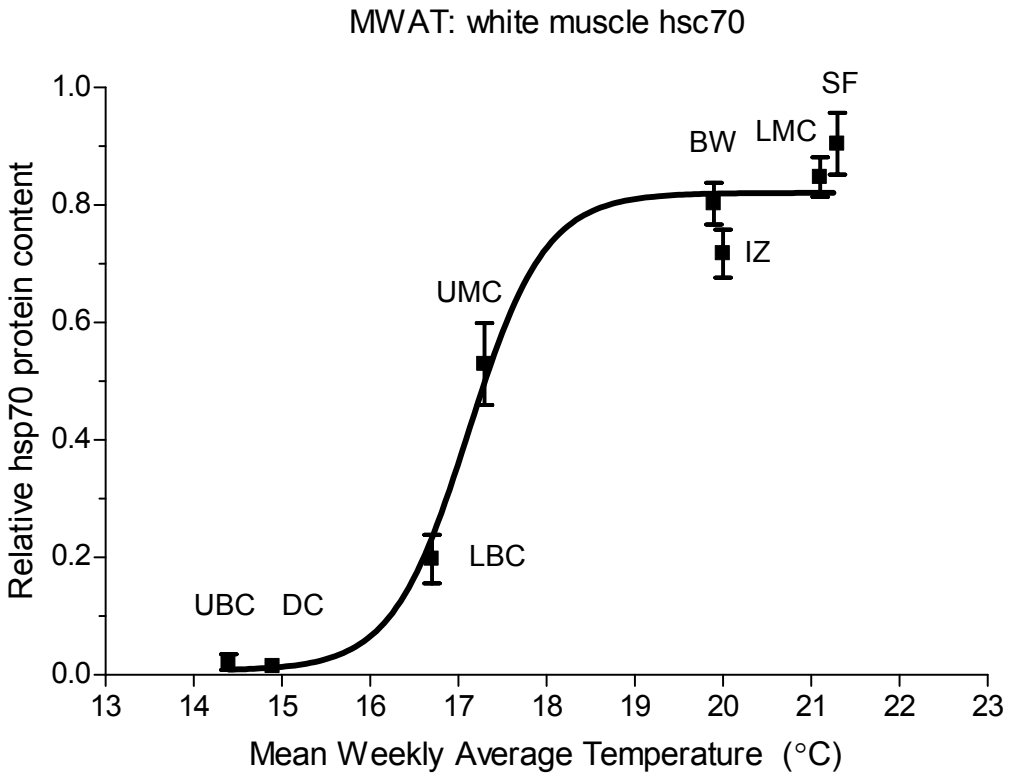
Appendix I- Relation between white muscle tissue inducible hsp73 (hsc70) protein band density (mean densitometry value ± 1 standard error of the mean) and the 24-hour average daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



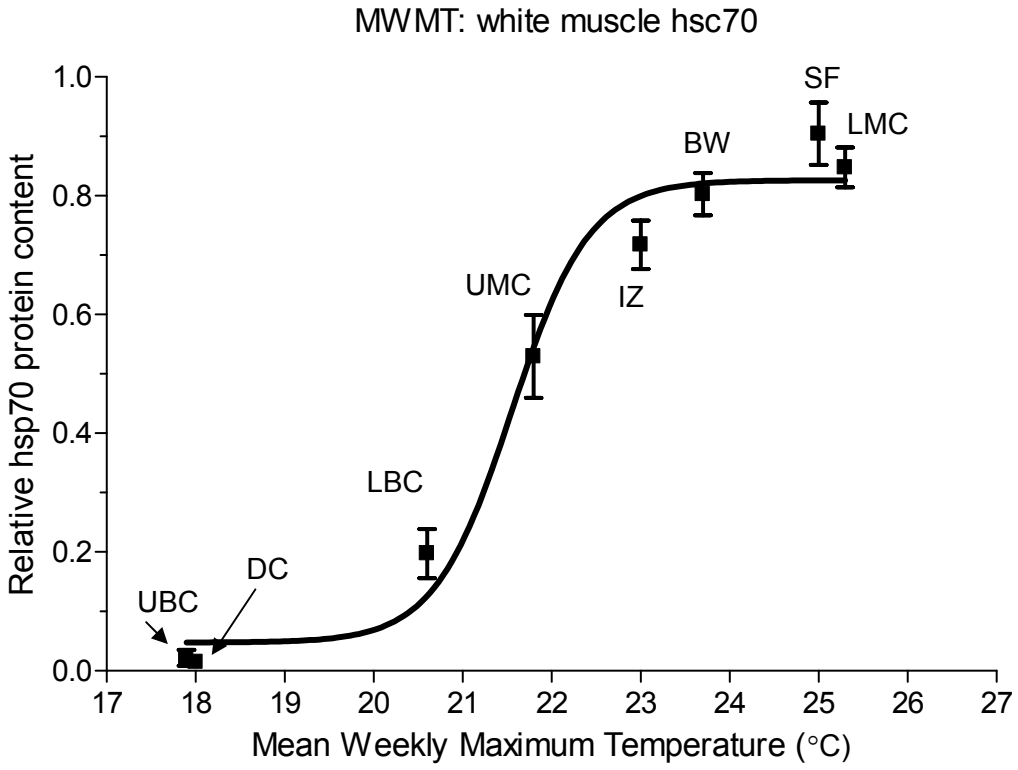
Appendix J- Relation between white muscle tissue inducible hsp73 (hsc70) protein band density (mean densitometry value ± 1 standard error of the mean) and the 24-hour maximum daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



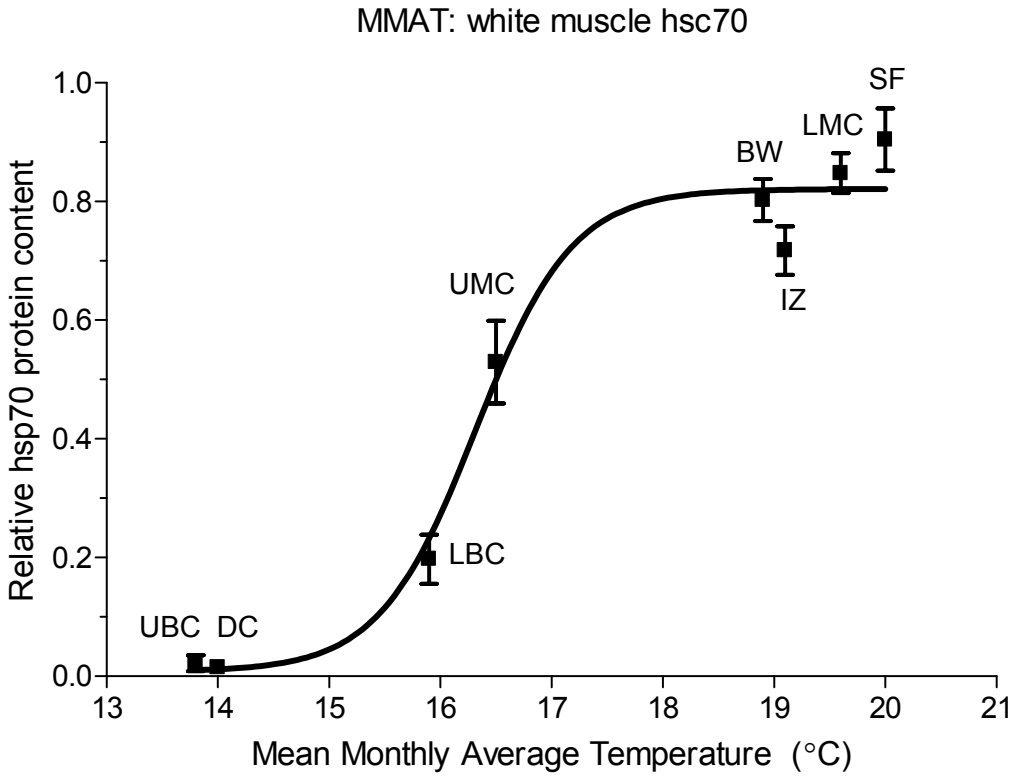
Appendix K- Relation between white muscle tissue inducible hsp73 (hsc70) protein band density (mean densitometry value ± 1 standard error of the mean) and the maximum water temperature (ºC) within five days of capture. Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



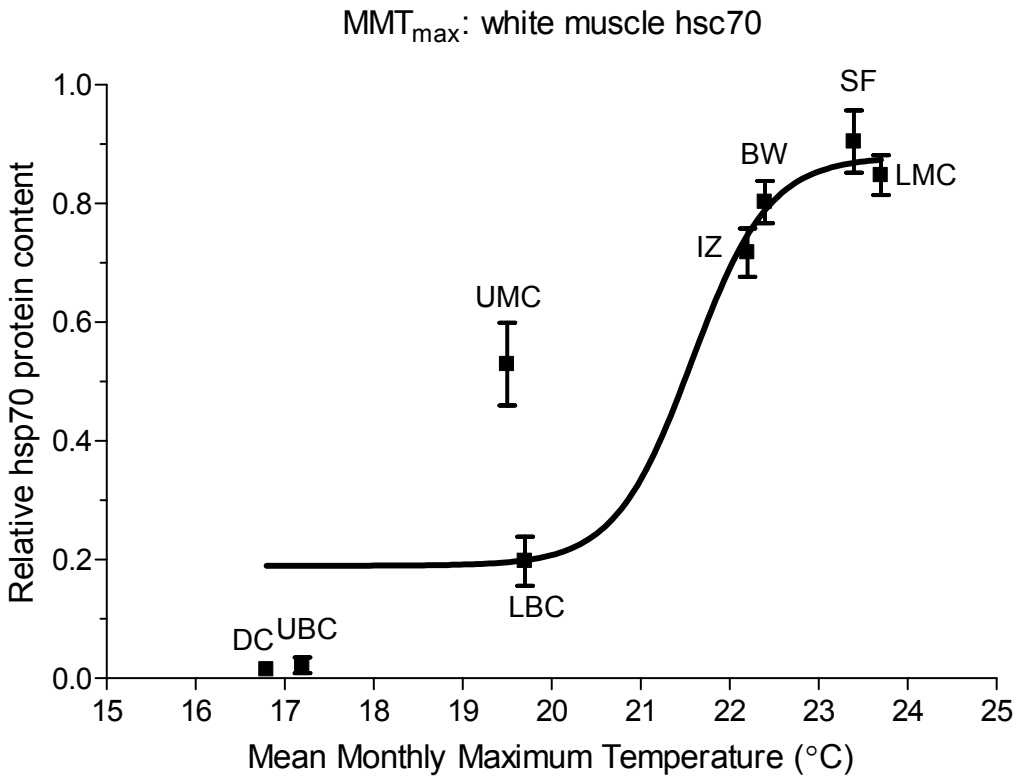
Appendix L- Relation between white muscle tissue inducible hsp73 (hsc70) protein band density (mean densitometry value ± 1 standard error of the mean) and the mean weekly daily average water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



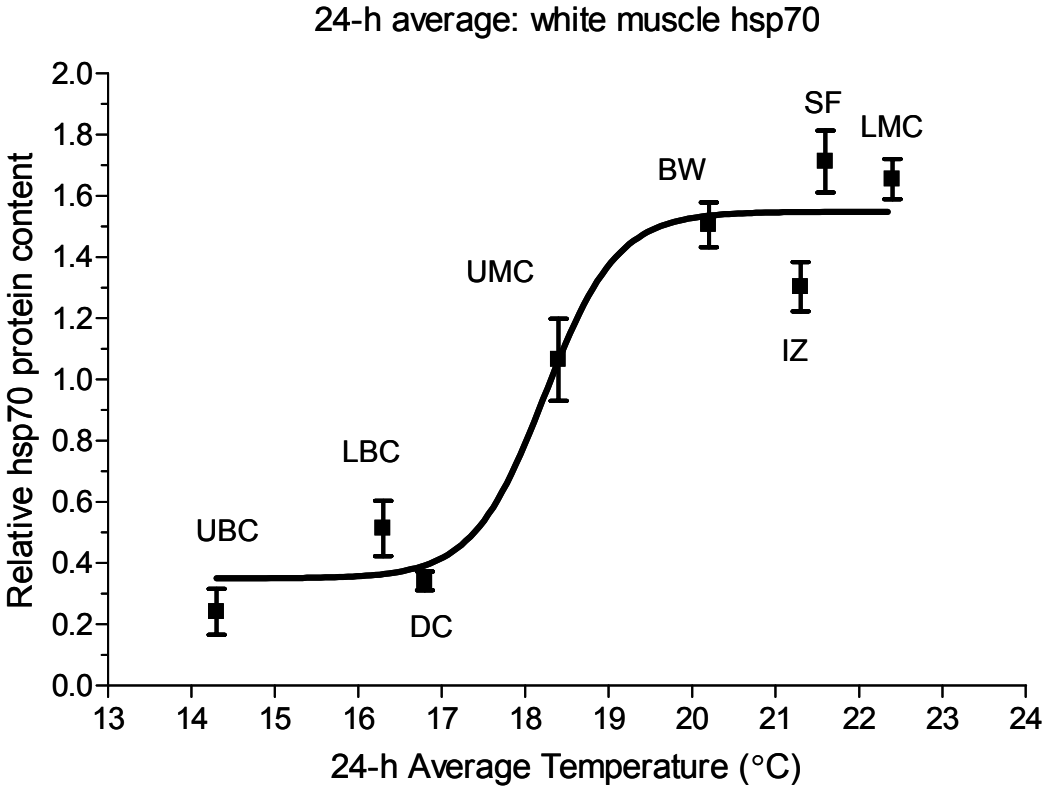
Appendix M- Relation between white muscle tissue inducible hsp73 (hsc70) protein band density (mean densitometry value ± 1 standard error of the mean) and the mean weekly maximum daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



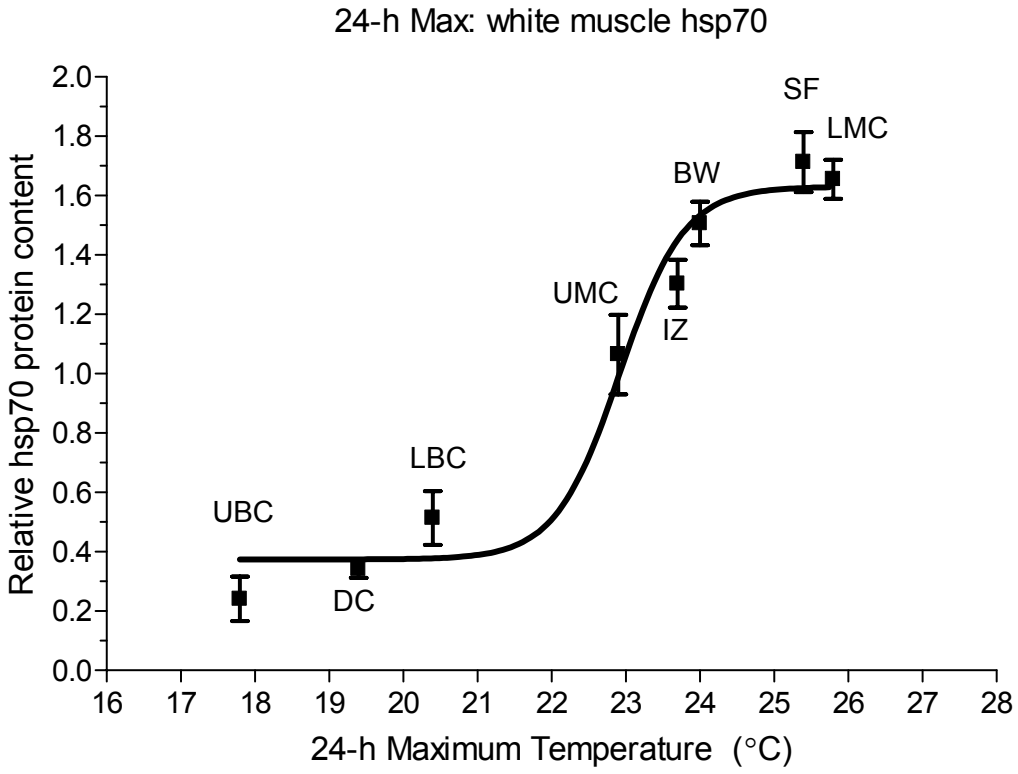
Appendix N- Relation between white muscle tissue inducible hsp73 (hsc70) protein band density (mean densitometry value ± 1 standard error of the mean) and the mean monthly average daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



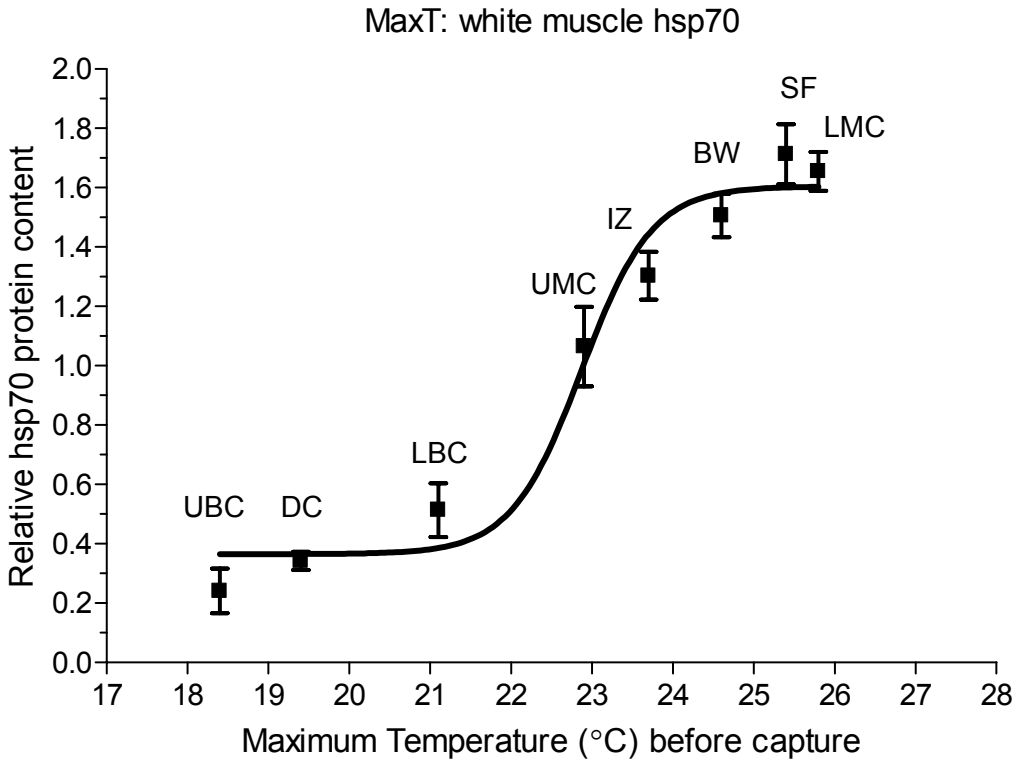
Appendix O- Relation between white muscle tissue inducible hsp73 (hsc70) protein band density (mean densitometry value ± 1 standard error of the mean) and the mean monthly maximum daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



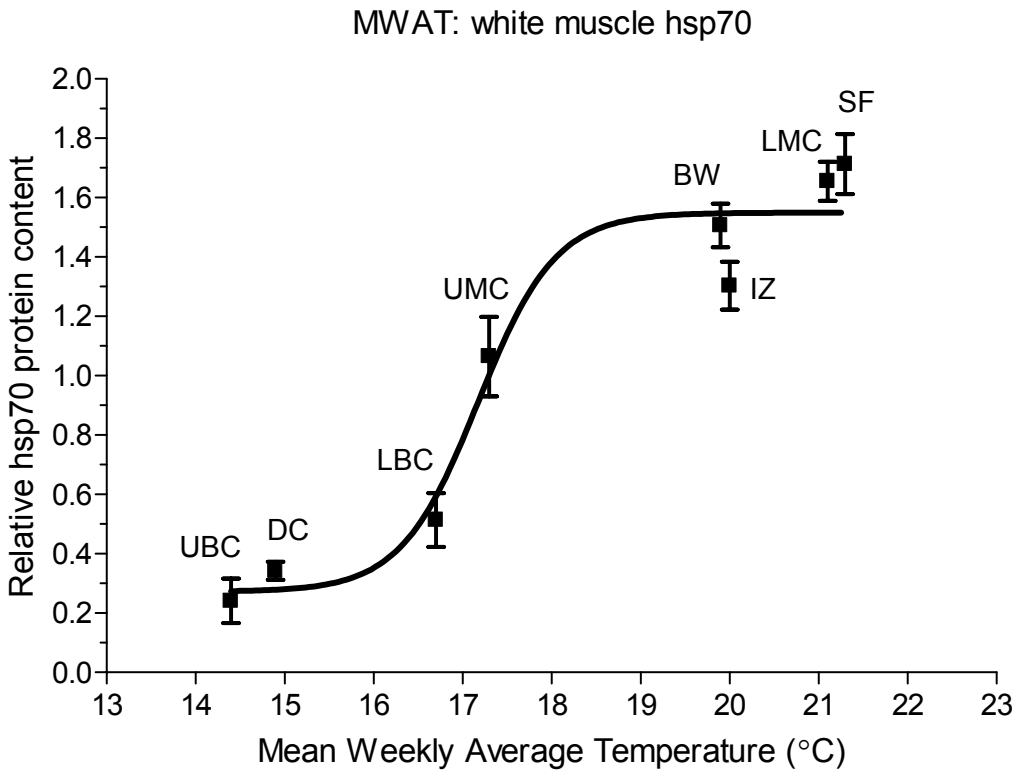
Appendix P- Relation between white muscle tissue inducible hsp72 (hsp70) protein band density (mean densitometry value ± 1 standard error of the mean) and the 24-hour average daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



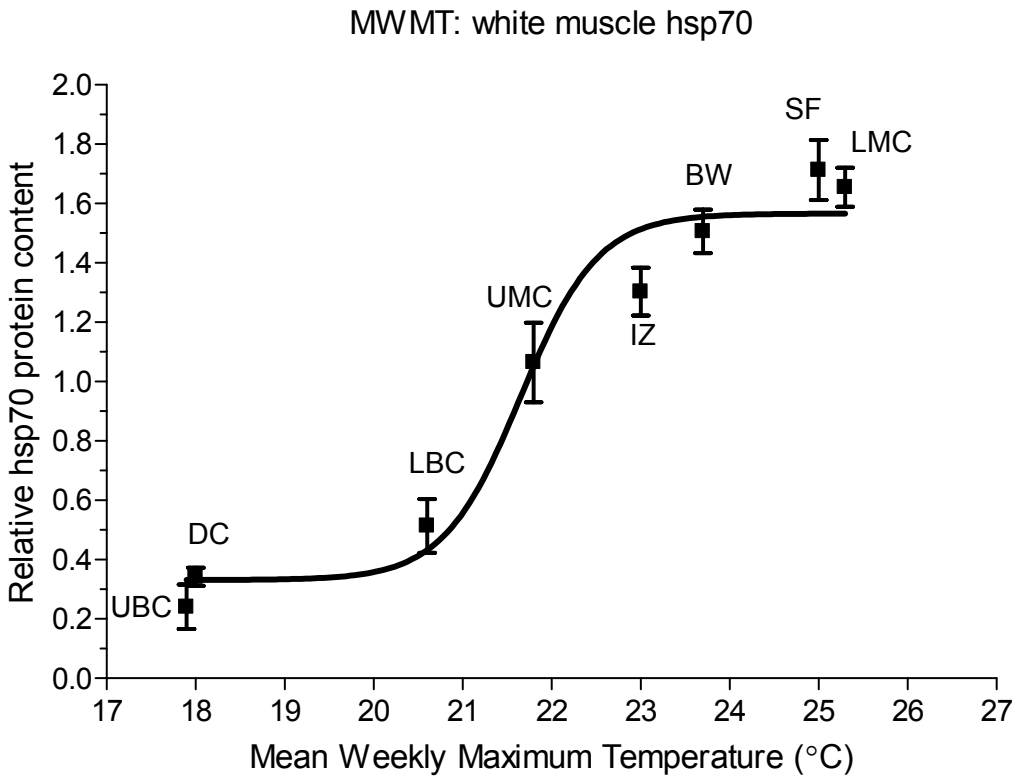
Appendix Q- Relation between white muscle tissue inducible hsp72 (hsp70) protein band density (mean densitometry value ± 1 standard error of the mean) and the 24-hour maximum daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



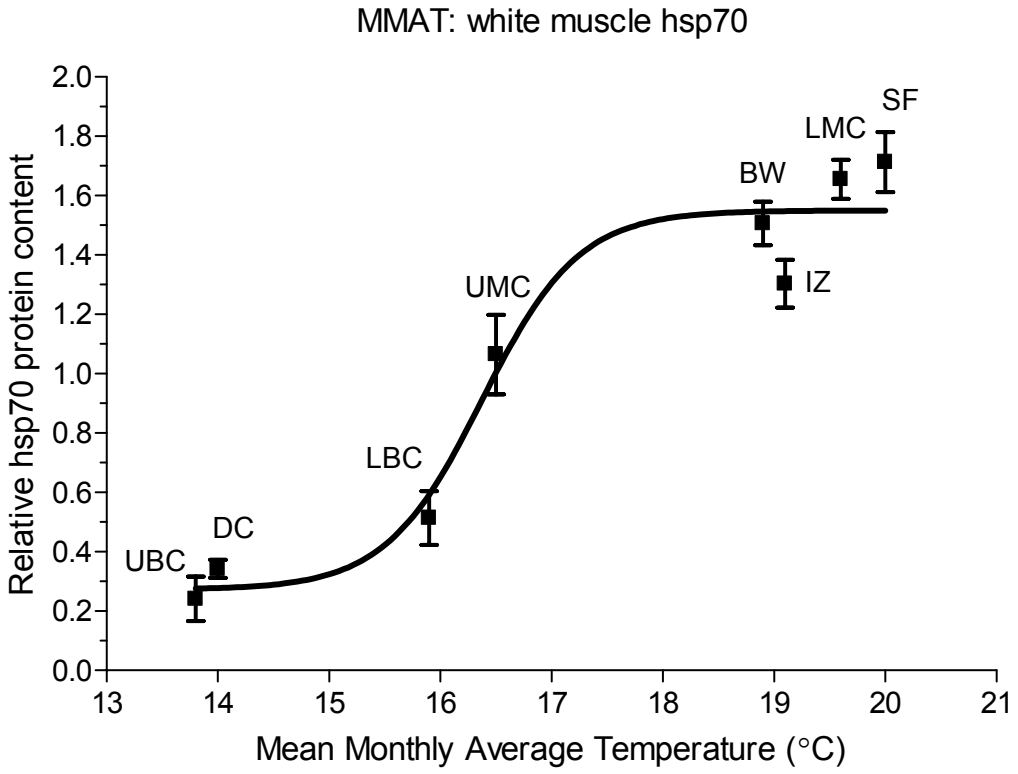
Appendix R- Relation between white muscle tissue inducible hsp72 (hsp70) protein band density (mean densitometry value ± 1 standard error of the mean) and the maximum water temperature (ºC) within five days of capture. Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



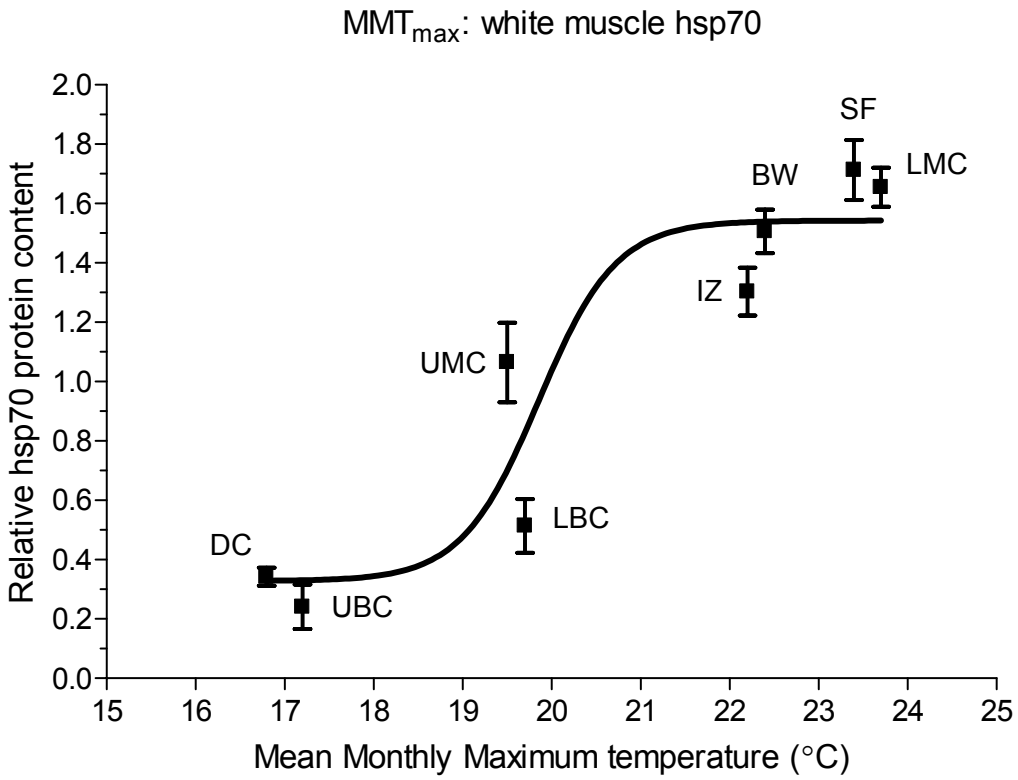
Appendix S- Relation between white muscle tissue inducible hsp72 (hsp70) protein band density (mean densitometry value ± 1 standard error of the mean) and the mean weekly daily average water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



Appendix T- Relation between white muscle tissue inducible hsp72 (hsp70) protein band density (mean densitometry value ± 1 standard error of the mean) and the mean weekly daily maximum water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



Appendix U- Relation between white muscle tissue inducible hsp72 (hsp70) protein band density (mean densitometry value ± 1 standard error of the mean) and the mean monthly average daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).



Appendix V- Relation between white muscle tissue inducible hsp72 (hsp70) protein band density (mean densitometry value ± 1 standard error of the mean) and mean monthly maximum daily water temperature (ºC). Juvenile redband rainbow trout (*Oncorhynchus mykiss gairdneri*) were collected from the South Fork John Day River on 23-24 July 2005 in the South Fork John Day River 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).