Stress-Mediated Disease Susceptibility and Thermal Physiology of Burbot *Lota lota maculosa*

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Authorization to Submit Thesis

This thesis of Marc M. Terrazas, submitted for the degree of Master of Science with a Major in Natural Resources and titled "**Stress-Mediated Disease Susceptibility and Thermal Physiology of Burbot *Lota lota maculosa***," has been reviewed in final form. Permissions, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

A series of experiments were intiated at the University of Idaho to evaluate the influence of stress on Burbot *Lota lota maculosa*. Stress, as it links to pathogenic bacteria and thermal stress, was evaluated as it pertains to initial captive rearing, continued persistence, and habitat evaluation of Burbot. Periodic epizootics due to unknown pathogens during stress events at the University of Idaho provided bacterial isolates potentially linked to stress-mediated Burbot mortality. The thermal conditions in tributaries of the Kootenai River considered important Burbot habitat have created questions of habitat suitability. Burbot were injected and immersed in bacterial isolates from previous epizootics and mortality was evaluated as the response. Burbot were also subjected to fluctuating water temperatures creating high diel peaks similar to those observed in natural systems. Pathogenic isolates of interest include two *Aeromonas* spp. that are both novel pathogens to Burbot and potentially novel species. The thermal response of Burbot was found to be resilient to diel increases in temperature.

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Dedication

They say it takes a village to raise a child, I feel that is very true in my case. I also feel like it took a village to help me through my graduate degree. I want to thank all of my incredible friends and family for the support they have given me in all walks of life but especially that their support did not diminish during graduate school. In particular, I received a tremendous amount of support from Charlotte Milling during the completion of my master's degree. I have also received support throughout my life from my immediate family, which I may not acknowledge as often as I should. Thank you Robert, Colleen, Joseph, Paul, Baylee, and Rosa Terrazas, I love you.

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Chapter 2: Determining juvenile Burbot *Lota lota maculosa* susceptibility to stress-mediated pathogens

Chapter 3: Effects of Diel Temperature Fluctuation on Growth, Stress Response, and Immune Function of Burbot

General Introduction

Burbot *Lota lota* are the only freshwater members of the family Gadidae. Commonly referred to as ling or eel pout, this species can be found in both lentic and lotic systems across its distribution (McPhail 1995). The two subspecies, European Burbot *L. l. lota* and North American Burbot *L. l. maculosa*, have a circumpolar distribution at northern latitudes, with the exception of Greenland, where they are absent (Scott and Crossman 1998; Berra 2001). In North America, the native distribution of Burbot ranges from the Seward Peninsula of Alaska (McPhail and Lindsey 1970) to the Atlantic coast of New Brunswick (Scott and Crossman 1973). In recent years, population declines across many parts of their distribution have become a cause for concern. Pullianen et al. (1992) postulated that environmental contamination was a factor in the declining numbers of Burbot in Sweden. Fisher (2000) discussed stream channelization as a relevant factor in Burbot population declines because of backwater utilization by young Burbot in the Mississippi River system.

In the case of the Kootenai River basin including Kootenay Lake in British Columbia, Burbot provided an important sport, commercial, subsistence, and tribal fishery prior to a dramatic population decline (Ireland and Perry 2008). Subsequent fishing restrictions and closures were implemented in 1992 for the Kootenai River, Idaho and in 1997 in the Kootenay River and Kootenay Lake, British Columbia (Paragamian 2000; Paragamian et al. 2000; Ahrens and Korman 2002; Paragamian and Hoyle 2003). The decline of this population has been well documented and it is speculated that hydrologic changes due to the completion of Libby Dam in 1974 is the cause. Construction of the dam created higher winter flows with warmer water temperatures and low summer flows (Partridge 1983; Paragamian and Whitman 1998). Altered hydrograph and thermographs are compounded in the Kootenai River system due to Lake Koocanusa, the impoundment created by Libby Dam, acting as a nutrient sink and reducing productivity in the river downstream (Richards 1996; Snyder and Minshall 1996). Even with reduced creel limits and fishery closures, the Burbot population has failed to rebound (Paragamian 2000; Ahrens and Korman 2002).

To prevent extirpation and eventually produce an active fishery in the Kootenai River system, remnant wild populations are now being augmented with artificially propagated Burbot. Despite such efforts, Burbot culture is still novel and methods are just now being optimized. In Europe, Burbot propagation studies have been conducted (Harzevili et al. 2003)

and reports related to Burbot aquaculture have come from the Czech Republic, Hungary, Finland and Belgium (Adamek 2000; Pääkkönen et al. 2000; Harzevili et al. 2003, 2004; Vught et al. 2008). Studies on Burbot culture have also been ongoing in North America and have documented captive spawning (Jensen et al. 2007), characterized temperature regimes for larval Burbot (Barron et al. 2012), determined egg incubation and larval feeding strategies (Jensen et al. 2008), tested fungicide treatment for Burbot eggs (Polinski et al.2010a), and noted a range of initial pathogen susceptibility for juveniles (Polinski et al. 2010b). There are still substantial gaps in Burbot culture methods compared to other species. In recent years, stress-mediated mortality events have occurred and were linked to potential bacterial pathogens. At this time, infectious agents that affect Burbot and are linked to culture conditions are unknown. As part of this current conservation program, we have identified the need to determine specific pathogens capable of causing disease in Burbot following a stress event (such as density or poor water quality) during various culture periods.

Additionally, stocking locations have been established in tributaries to the Kootenai River which have much different thermal profiles than the main stem river. The success of tributary stocking locations are currently being assessed (Zach Beard, University of Idaho, personal communication). The suitability of some tributary stocking locations has come under scrutiny partly because of high summer temperatures in some locations. The high summer temperatures of tributaries used for Burbot stocking has been hypothesized as a factor in survival after release. Therefore, this study will focus on two distinct objectives: (1) determining the disease causing agent(s) associated with previous Burbot mortality events observed at the University of Idaho and establishing juvenile susceptibility to these pathogens, and (2) evaluating the growth, thermal stress response, and immune response of Burbot exposed to high diel temperatures. Each of these objectives will be discussed individually in the following chapters.

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CHAPTER ONE

Review of Literature

1. Burbot

1.1 Life History

Burbot (*Lota lota)* are the only freshwater members of the family Gadidae (cod) (Cohen et al. 1990, McPhail 1995). There are two subspecies referred to as European (*lota*) and North American (*maculosa*) (Hubbs and Schultz 1941) that have a circumpolar distribution at northern latitudes, generally occurring above 40°N (Scott and Crossman 1998; Berra 2001; Van Houdt et al. 2003). The European subspecies occurs across the northern portions of Europe and Asia to the Great Slave Lake in North America (Van Houdt et al. 2003). The North American subspecies is found south of the Great Slave Lake across the northern protion of North America. In all, Burbot range from the Seward Peninsula of Alaska (McPhail and Lindsey 1970) to the Atlantic coast of New Brunswick (Scott and Crossman 1973) in North America. Mitochondrial DNA suggests three clades of subspecies *Lota lota maculosa*, (Mississippi, Missouri, and Pacific) in accordance with major North American basins shaped by continental glaciations (Van Houdt et al. 2003; Elmer et al. 2008; Powell et al. 2008). Also commonly referred to as ling or eel pout this species can be found in both lentic and lotic systems across its range (McPhail 1995). Although tolerant to brackish water (Jaeger et al. 1981; Pulliainen and Korhnonen 1990) the absense of this species in Greenland, Iceland, the Canadian Archipelago, and areas of northern Eurasia (Van Houdt et al. 2003) suggests divergence from saltwater life long ago, thought to be 10 million years, and the lack of suitable freshwater habitats because of ice sheets covering these locations (Stapanian 2010). Phylogenetic divergence of burbot from other gadids is relatively minimal as observed by the Cytochrome oxidase subunit I gene in mitochondrial DNA (Ratnasingham and Herbert 2007; Powell et al. 2008).

Burbot have a unique life history. Often considered cold-water stenotherms (Hofmann and Fisher 2002; Tiitu and Vornanen 2002), they form spawning aggregations with a few females in the center surrounded by males (Cahn 1936) in the winter, often times under ice, when water is near 4°C (Jaeger et al. 1981). Fecundity is highly variable but they can release up to 3.5 million eggs per spawning event (Roach and Evenson 1993), or as few as 6,300 eggs (Miller 1970). This spawning style produces buoyant to semi-buoyant eggs that are very small

for freshwater fish (0.88 to 1.14 cm diameter; McPhail and Paragamian 2000) and follow water currents with little movement required for dispersal. The eggs are thought to be highly sensitive to temperature and low temperatures are required. Burbot eggs have incubation intervals of 80-180 degree days (Bjorn 1939; McPhail and Paragamian 2000; Jensen et al. 2008a; Jensen et al. 2008b) and temperatures of 0-7°C have been observed with reproductive success (McPhail and Paragamian 2000; Taylor and McPhail 2000). After hatch, embryos are extremely small, less than 2mm in length (Jensen et al. 2008a) and further development is required prior to exogenous feeding at 4.4-5.5mm (Ghan and Sprules 1993; Fischer 1999; Jensen et al. 2008). Larval burbot exhibit planktonic behavior and prey upon phytoplankton and small zooplankton (Ryder and Pesendorfer 1992; Ghan and Sprules 1993; Harzevilli et al. 2003; Jensen 2006). Metamorphosis in Burbot from larvae to juveniles includes the development of external morphological features equivalent to those in adult fish. Changes in burbot appearance during metamorphosis include fin differentiation, formation of a single chin barbel, and full pigmentation (Donner and Eckmann 2011). During this time a benthic orientation is adopted from previously pelagic larval Burbot, which is also termed negative photaxic behavior (Girsa 1972; Ryder and Pesendorfer 1992; Fischer 1999; McPhail and Paragamian 2000).

Sexual maturity of Burbot in the southern parts of their distribution has been observed at 3-4 years of age (Robins and Deubler 1955; Lelek 1980; Boag 1989). Adult Burbot require cold water temperatures even during non-spawning months and prefer temperatures between 10-14°C (Hofmann and Fischer 2002). Adults are negatively photaxic (Girsa 1972), are preferentially benthic (Fischer 1999), and emerge at night to feed (Carl 1995), often on fish (Chisholm et al. 1989). They have low swimming endurance and are considered ambush predators (Jones et al. 1974). Spawning migrations of up to 400 km have been observed in large lakes, reservoirs, and rivers (Sorokin 1971; Stephenson et al. 2013). Spawning grounds typically consist of shallow water, typically covered by ice, in lakes, rivers, and streams with low water velocity and suitable substrates for egg incubation (Sorokin 1971; McPhail and Paragamian 2000). Burbot age can vary by latitude with life span as low as 7 years (Magnin ad Fradette 1977) but can reach a maximum age of 20–22 years (Hatfield et al. 1972; Nelichik 1978; Guinn and Hallberg 1990) and commonly reach sizes from 1-3 kg at lengths of 300-600 mm (McPhail and Paragamian 2000).

2. Culture

Atlantic Cod *Gadus morhua*, a close relative of Burbot, have been studied extensively. Hatchery methods for Cod originated around 1984 with initial descriptions of live feed for cultivation of Atlantic Cod (Howell 1984). The efforts at describing Cod culture continued with descriptions on growth rate and protein turnover (Houlihan et al. 1988). As early as 1990 it was thought that Atlantic Cod could become suitable for commercial farming (Tilseth 1990) and much of the literature following reflects this effort. Studies focused on growth continued with the effect of feed type, starvation, and fish size (Folkvord 1991); effects of initial size distribution on growth and survival (Folkvord and Ottera 1993); nutritional regime on morphological and biochemical indicators of growth (Foster et al. 1993); growth rate at different salinity levels (Lambert et al. 1994); effects of food consumption and temperature on growth parameters (Peck et al. 2003); effect of ammonia exposure on growth rates (Foss et al 2004); the effect of feed composition and feeding frequency on growth (Rosenlund et al. 2004); and diet comparisons on growth (Imsland et al. 2006) to name a few. Further studies have evaluated variation in annual egg production (Kjesbu 1996), growth rate and water quality at different levels of water reuse, and stocking density (Foss et al. 2006). A worldwide status and prospective of Gadoid culture has been recently published (Rosenlund and Skretting 2006) as has an evaluation of sustainability of Cod farming (Standal and Utne 2007).

The first reported attempts to culture Burbot comes from Bjorn (1939), but this effort did not result in standardized culture methods. The next documented attempt at culturing Burbot was McCrimmon (1959), which was somewhat successful at incubating Burbot eggs similar to Whitefish *Coregonus clupeaformis* eggs by using low flow because the eggs are buoyant (McCrimmon 1959). Manual stripping of gametes has been reported without attempts to subsequently culture the progeny (Kouril et al. 1985). More recently there has been increased interest in Burbot culture. In Europe, there have been efforts regarding cryopreservation of Burbot sperm (Lahnsteiner et al.1997; 2002); controlled studies regarding feeding mixtures (Adamek 2000); feeding rates at different temperatures (Pääkkönen and Marjomäki 2000); growth and survival at different temperatures (Wolnicki et al. 2001; 2002); investigations of starter live starter feeds (Harzevili et al. 2003); first feeding due to different light and temperature conditions (Harzevili et al. 2004); captive spawning and larviculture

(Vught et al. 2008); and optimal feed rates and light conditions for growth and survival (Trechel et al. 2013).

Research conducted at the University of Idaho has further evaluated the effect of cryopreservation on fertilization, developed egg incubation protocols, characterized captive spawning, stocking density, temperature preferences, and further developed captive aquaculture methods for Burbot (Jensen 2006; Jensen et al. 2008a; Jensen et al. 2008b; Jensen et al. 2008c; Barron et al. 2012; Barron et al. 2013a; Barron et al. 2013b). Among these methods are incubation strategies using jar or cone incubators and initial feeding of Burbot incorporating live prey diets, transitioning to dry artificial feeds, and using grading to minimize cannibalism (Jensen 2006; Barron et al. 2013a). Some effort has also been spent on pond culture of Burbot with survival being higher on ponds that are pre-fed with live feed prior to pond stocking (Vught et al. 2008) and a stocking density of 50 and 100 larvae/m^2 providing the best survival and predicted maximum yield, respectively, in semi-intensive burbot culture (Barron et al. 2013b). Additionally, Burbot pond culture in live cages produced significant effects of year and density in another study (Paragamian et al. 2011) with low and medium densities (approximately 20 and 40 fish/m^3) producing higher survival and pre-feeding fish before cage stocking having an effect on the medium and high density (approximately 40 and 100 fish/m^3) cage survival.

2.1 Disease Susceptibility

The United States Department of Agriculture has listed Burbot as a species susceptible infection from viral hemorrhagic septicemia virus (VHSv) genotype IVb found in the Great Lakes region of the United States and Canada (USDA 2007) with no information regarding the ability of Burbot to act as a carrier of VHSv or Burbot mortality from VHSv. Without apparent disease manifestation, the bacterial pathogen *Yersinia ruckeri*, the causative agent for enteric redmouth disease, was isolated from wild burbot in the Mackenzie River, Canada (Dwilow et al. 1987). Burbot have tested positive for *Aeromonas salmonicida* by bacterial plate culture and confirmed by DNA diagnostic PCR, a very sensitive test, in fish from Washington as part of the wild fish survey conducted by the U. S. Fish and Wildlife Service (USFWS-WFS 2015). *Mycobacterium salmoniphilum* has been isolated by bacterial culture and confirmed with molecular techniques from Burbot in two locations in Norway (Zerihun et al. 2011). Lab challenge studies have also confirmed Burbot susceptibility at different life

stages to *Aeromonas salmonicida* and *Aeromonas hydrophila* (Natrah et al. 2012). Polinski et al. (2010) conducted an in depth study of Burbot susceptibility to many common salmonid pathogens including: infectious pancreatic necrosis virus, infectious hematapoietic necrosis virus, *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, and *Renibacterium salmoninarum*. The results from Polinski et al. (2010) indicate variable sensitivity of Burbot to the different pathogens including greater than 50% mortality from: immersion in infectious hematapoietic necrosis virus (IHN) and infectious pancreatic necrosis virus (IPN), injection of IPN, and injection of *Aeromonas salmonicida*. However, the results of Polinski et al. (2010) are somewhat confounded by the seemingly high mortality in some of the control treatments so the IHN immersion and the *Aeromonas salmonicida* were the only treatments to have significantly higher mortality in the treatments than the controls.

2.2 Stress-Mediated Pathogens

Stress has been implicated as a contributing factor to a number of diseases in fish, one of which is White Sturgeon *Acipenser transmontanus* iridovirus (WSIV) as outlined by LaPatra et al. (1994). LaPatra et al. (1994) suggested that a stressor other than density (i.e., temperature, handling, or transportation), is responsible for enhancing the manifestation of WSIV on juvenile White Sturgeon. Georgiadis et al. (2001) found that a stressful event was a likely trigger to WSIV symptoms showing up in juvenile White Sturgeon. A study on grouper *Epinephelus* sp. fry suggested that environmental stress, in this case heavy metals contamination, caused the pathogenicity of a low pathogenicity strain of infectious pancreatic necrosis virus to increase (Chou et al. 1999). Biophysical and environmental stressors have been shown to affect the survival and condition of Largemouth Bass *Micropterus salmoides* infected with largemouth bass virus (Inendino et al. 2005). There have been many studies looking specifically at density as a contributor to mortality and pathogenicity of fish diseases. In addition to LaPatra et al. (1996), there have been studies on density effects involving Chinook Salmon *Oncorhynchus tshawytsha* (Mazur et al. 1993; Banks 1994) and Coho Salmon *O. kisutch* (CDFO et al. 1984). Specifically, the effects of fingerling mortality due to bacterial kidney disease *Renibacterium salmoninarum* were evaluated based on rearing densities (Banks 1994), as well as the effects of bacterial kidney disease on fish after transfer to salt water at different densities (Mazur et al. 1993).

3. Temperature

3.1 Thermal Requirements

Temperature is often considered the most important abiotic factor for fishes, given that it affects all biochemical processes (Hardewig et al. 2004). Burbot have been termed cold-water stenotherms (Hofmann and Fisher 2002, Tiitu and Vornanen 2002) and also temperate mesotherms (Hokanson 1977). These differing terms do not detract from the preference of adult Burbot to occupy habitats with temperatures of 10-14°C (Cooper and Fuller 1945; Hackney 1973; Hofmann and Fischer 2002). Thermal maximum temperatures have been reported at 26.8-31.7°C based on different acclimation temperature (Hofmann and Fischer 2002). Burbot eggs and larval Burbot do not have the same temperature requirements with temperatures of only 0-7°C observed with reproductive success (McPhail and Paragamian 2000; Taylor and McPhail 2000). Habitat preferences of Bubot have been well documented with larvae typically pelagic (Ghan and Sprules 1993; Wang and Appenzeller 1998; Fischer 1999), although documentation in the littoral zone has occurred (Kjellman et al. 2000); juveniles inhabiting the littoral zone (Lawler 1963; Scott and Crossman 1973; Ryder and Pesendorfer 1992; Carl 1995; Hofmann and Fischer 2001), and adults often using the cold profundal zone (Lawler 1963; Kieckhäfer 1972; Scott and Crossman 1973). The temperature preferendum for large (20-30 cm) Burbot has been reported as 11.4°C (Hofmann and Fischer 2002). Dixon and Vokoun (2009) found that temperature was the second most important predictor of Burbot occurrence, after gradient, at the reach scale in their habitat selection study. With Burbot culture becoming increasingly popular, there have been numerous studies focusing on the temperature preferences of Burbot at different life stages and for different purposes. Adult Burbot have been shown to decrease relative daily food intake from 10.8°C to 23.4°C in laboratory feeding trials (Pääkkönen et al. 2000). Growth rates of age-0 Burbot have been shown to increase with increasing surface water temperatures from 4-18°C, whereas older age class growth was less temperature dependent (Kjellman and Elanoranta 2002). Donner and Eckmann (2011) found that juvenile burbot performance was best in a simulated experiment where they would use daily vertical migrations and utilize the hypolimnion and epilimnion for growth advantages. Growth of juvenile Burbot has been reported as highest at temperatures of 19, 20, and 21°C (Donner and Eckmann 2011, Barron et al. 2012; Wolnicki et al. 2001). Temperatures of 15°C (Wolnicki et al. 2001) and 16°C

(Hofmann and Fischer 2003; Harzevili et al. 2004) have been also reported as having the highest growth or best performance based on multiple factors such as growth and survival. The results of previous research suggests that fish reared in temperatures of 15-20°C may grow optimally only if the feeding regime provides adequate energy input to keep pace with heightened metabolism (Barron et al. 2012) and mitigates other factors such as cannibalism.

3.2 Physiology

Various aspects of Atlantic Cod, (another Gadiform) respiration have been investigated including effects of size, feeding status, handling, crowding, and induced hypoxia (Saunders 1963). Atlantic Cod have been evaluated for responses to starvation and re-feeding (Kamra 1966); physiological responses to hypoxia (Herbert and Steffensen 2005); temperature (Claireaux et al. 1995a, Pérez-Casanova et al. 2008); the thermal niche including the thermal limits, tolerance, and optimal temperatures (Righton et al. 2010); salinity and oxygenation conditions (Claireaux et al. 1995b); and seasonal variations in the physiological response to salinity (Dutil 1993). The metabolism of cod has been studied including effects of temperature, hypoxia, and exercise on metabolic rate (Shurmann and Steffensen 1997), and the effects of body mass and feeding on metabolic rate (Herbing and White 2002).

Physiology of Burbot outside of the constraints of temperature effects on individual performance is rarely studied in the peer-reviewed literature. Gomazkov (1959) studied the enzyme activity at different temperatures and found isolated enzymes to be active at temperatures near 0°C, but they become inefficient (especially the proteases) around 20°C (as interpreted by Nikčević et al. 2000). Muscle lactate dehydrogenase from white muscle of Burbot have been shown to be significantly more resistant to inactivation by 3 molar urea than similar preparations from Brown Trout *Salmo trutta*, but not significantly different than Danube Salmon *Hucho hucho* (Nikčević et al. 2000). Tiitu and Vornanen (2002) found that the hearts of Burbot depend heavily on sarcoplasmic reticulum stores of Ca^{2+} for contractile activation at low temperatures (1°C). The metabolic rate of Burbot has been shown to be depressed at temperatures used to mimic summertime highs; this is because enzyme activity in the white muscle and liver for metabolic function is thought to be down-regulated during periods of increased water temperatures (Hardewig et al. 2004). Binner et al. (2008) found that Burbot have seasonally dependent metabolic adaptations that allow them to withstand metabolism-activating summer temperatures and manage winter temperatures.

3.3 Cortisol and heat-shock proteins

Stress is not necessarily detrimental to fish, but it is an adaptive mechanism that allows re-establishment of homeostasis through a complex suite of adaptive responses (Chrousos 1998; Barton 2002). Plasma cortisol (hydrocortisone) secretion in fish is under control of the hypothalamus-pituitary-interrenal axis and cortisol is directly secreted by the interrenal tissue of the head kidney (Mommsen et al. 1999). Plasma cortisol concentrations reflect the net effect of production and of plasma clearance of cortisol (Mommsen et al. 1999). Many studies assume that elevated plasma cortisol concentrations during stress is deleterious to fish, however, cause and effect relationships attributable to cortisol have not been established (Barton and Iwama 1991). During chronic stress plasma cortisol can fall back to resting levels even through continued stress (Vijayan and Leatherland 1990). The role of cortisol as an adaptive response during stress has support in the literature, including: the role of stress as it pertains to immune function (Bonga 1997), ionic and osmotic regulation (McCormick 1995), and metabolism (Vijayan et al. 1994, 1996, 1997).

Heat shock proteins (HSP) are a family of proteins with high identity at the amino acid level and are expressed by stressors other than heat (Iwama et al. 1998). Heat shock proteins are also commonly referred to as “stress” proteins and are expressed in cells to maintain critical cellular processes (Iwama et al. 1998). The groups of HSP are identified by molecular weight (Iwama et al. 1998) and have been shown to be vital in protein assembly, folding, translocations, and regulating interactions between hormones and receptors (Welch 1993). Heat shock proteins likely confer thermotolerance in fish along with aiding cellular integrity when faced with other challenges such as environmental toxicants and non-thermal stressors (Iwama et al. 1999). Physiological effects of thermal stress have been investigated in Atlantic Cod by Aursnes et al. (2011), which found increased gene expression of *hsp70* and *hsp90* due to hyperthermia, even though no detectable increases in hsp70 levels where evident in other studies (Zakhartsev et al. 2005). Persistent organic pollutant exposure has been shown to increase gene expression of many different metabolic pathways including *hsp70* expression in Burbot (Olsvik et al. 2013). The hsp70 response has been demonstrated in fish facing other stressors including: heavy metals exposure (Sanders 1993), Rainbow Trout *Oncorhynchus mykiss* and Chinook Salmon exposure to industrial effluents including bleached kraft pulp mill effluent and sodium dodecylsulfate (Vijayan et al. 1997), Nile Tilapia *Oreochromis*

niloticus exposure to the herbicide oxyfluorfen (Hassanein et al. 1999), Coho Salmon experimentally infected with bacterial kidney disease (Forsyth et al. 1997), Green Sturgeon *Acipenser medirostris* and White Sturgeon exposure to cold shock, air exposure, and food deprivation (Wang et al. 2013).

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CHAPTER TWO

Juvenile Burbot *Lota lota maculosa* susceptibility to stress-mediated pathogens

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Running head: Burbot stress-mediated pathogens
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Abstract

Population declines of Burbot *Lota lota maculosa* in the Kootenai River, Idaho have initiated a conservation program for this population. Burbot have limited susceptibility to many pathogens; however, stess and water quality have been hypothesized as the cause of stress-mediated disease outbreaks during the captive rearing of juveniles. Epizootics during periods of poor water quality and high density occurred in 2012 and 2013 at the University of Idaho's Aquaculture Research Institute. Initial clinical diagnostics and sampling resulted in the isolation of numerous potential bacterial pathogens. To determine which bacterial species were the most likely causative agents of these epizootics, juvenile burbot were initially

screened against potential pathogens by intraperitoneally (IP) injection of 14 different bacterial isolates in log phase growth. Mortality associated with 2 specific isolates, (141 and 159) was documented. A more comprehensive study was designed and fish were challenged by IP and immersion methods. The challenges resulted in significantly higher mortalities in our IP groups ($P = 0.01$, 35% for isolate 159; $P < 0.01$, 80% for isolate 141) compared to controls (0%) and no differences were observed in mortality for immersion groups ($P = 0.42$) compared to controls. A lethal dose 50 (LD_{50}) challenge was also conducted for each isolate and this showed that isolate 141 was more virulent ($LD_{50} = 10^{5.09}$) than isolate 159 ($LD_{50} = 10^{6.79}$). Phylogenetic analysis of both isolates indicates they are most closely related to *Aeromonas veronii* compared to similar sequences in GenBank. This represents the first report of an isolated bacterial pathogen causing motile aeromonad like disease in Burbot. This Gram negative pathogen is most closely related to *Aeromonas veronii*, is pathogenic to Burbot and may be linked to stress-mediated disease outbreaks for Burbot.

Introduction

Disease control and prevention is essential to maximize aquaculture production and requires continued attention and modification. Disease issues at aquaculture facilities include but are not limited to: loss of production, movement of pathogens to new facilities, introduction of pathogens to wild fish or the environment, and potential amplification of pathogens in a watershed where specific facilities reside (Winton 2001). Prevention of disease events requires knowledge of the susceptibility of stocks to various pathogens, and implementation of control or prevention measures to mitigate losses from such outbreaks.

Disease susceptibility of Burbot for many common fish pathogens is not clear. *Yersinia ruckeri*, the causative agent of enteric redmouth disease, has been isolated from the internal organs of MacKenzie River, NT Burbot, but fish never exhibited disease symptoms (Dwilow et al. 1987). *Mycobacterium salmoniphilum* has been isolated by bacterial culture and confirmed with molecular techniques from Burbot in two locations in Norway (Zerihun et al. 2011). Lab challenge studies have also confirmed Burbot larval susceptibility to *Aeromonas salmonicida* and *A. hydrophila* (Natrah et al. 2012), but Polinski et al. (2010) found juvenile were also susceptibile to *A. salmonicida*. Polinski et al. (2010) characterized Burbot susceptibility to many common salmonid pathogens, including infectious

hematapoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), *A. salmonicida*, *Flavobacterium psychrophilum*, and *Renibacterium salmoninarum*. Polinski et al. (2010) reported susceptibility of Burbot to IHNV by immersion and *A. salmonicida* by injection, but overall Burbot were less susceptible compared to Rainbow Trout *Oncorhynchus mykiss*. Considering Burbot culture is still relatively novel, further work is required to determine disease susceptibility risks, especially to opportunistic pathogens that may be common in culture systems or the natural environment.

Burbot have been produced and reared at the University of Idaho Aquaculture Research Institute (ARI), with the goal of expediting population growth and recovery in the Kootenai River (Ireland and Perry 2008). Although always associated with a stress event, Burbot reared at the University of Idaho have experienced periodic disease outbreaks during rearing (Ken Cain, personal communication). The outbreaks and resulting bacterial isolates are considered to be a direct result of the stress associated with rearing conditions prior to the outbreak. Clinical signs of individuals during mortality events commonly included bloating, ascites, lethargy, and both internal and external hemorrhages. Upon necropsy, the internal organs of sick fish were often fluid filled and had lost much of their structural integrity. Bacterial isolates were isolated from internal organs, ascites, and the brain of moribund fish, and cryo-preserved for future use and identification. We hypothesized that specific bacterial isolates from such sampling events were linked to the observed mortality events, and would cause higher mortality in challenge studies than controls. Therefore, the goal of this study was to determine specific bacterial isolates capable of inducing disease and identify their role in previous stress-mediated disease outbreaks in Burbot.

Methods

Bacterial reference stocks

Bacteria were isolated from captive Burbot experiencing mortality events at the University of Idaho ARI either as a cohort or as individuals during 2012 and 2013. Bacteria were isolated from internal organs and ascites of moribund fish on tryptic soy agar (TSA) and incubated at 15°C. Tryptone yeast extract with salts (TYES) agar was also used, but no bacteria were isolated on this media. Bacteria were then cryo-preserved following Cain and LaFrentz (2007) for future research needs. Presumptive identification of specific bacterial

isolates was accomplished by direct sequencing, assumed to identify to genus. Selection of the most probable disease agents for use in a screening study was determined by frequency of occurrence in moribund fish, consistency of location (isolated from the same tissue repeatedly), and know pathogenicity of genus members to fish. Selected bacteria were then used in challenge experiments with juvenile Burbot by intraperitoneal (IP) injection of live bacteria or immersion of fish in solution containing live bacteria during log-phase growth in tryptic soy broth (TSB; Difco, Becton, Dickinson and Company, Sparks, MD). Burbot susceptibility was evaluated for each isolate based solely on survival following challenge, because neither clinical symptoms nor morbidity were observed in challenge survivors.

Bacterial identification

Bacterial cultures were grown from cryopreservation in TSB at 15°C and then streaked on tryptic soy agar (TSA; Difco) at 15°C for colony isolation. Individual colonies were then sub-cultured in TSB to ensure pure colonies were present. Isolates were cultured for 48 hours and DNA was extracted using a DNeasy kit (Qiagen, Valencia, CA). Upon completion, DNA was amplified using polymerase chain reaction (PCR) with universal 16S primers fD1 and Rp2 (Weisburg et al. 1991). Controls of sterile water were used to ensure no contamination of PCR reagents. Once amplified, some isolates were sent to a commercial vendor for clean up and sequencing (Amplicon Express, Pullman, WA). The remaining isolates were treated as follows: the PCR product was cleaned using 2µL EXOSap-it (Affymetrix, Santa Clara, CA) and 5µL PCR product in a thermocycler at 37°C for 15 min followed by 80°C for 15 min. The sequencing reaction consisted of 0.2 pM/µL PCR primer with Big Dye Terminator v3.1 (Life Technologies, Grand Island, NY) and EXOSap-it product each at 20% of final cocktail. The sequencer was an Applied Biosystems 3130*XL* (Life Technologies, Grand Island, NY). Analysis of sequences was done using Sequence Analysis 5.2 (Applied Biosystems, Foster City, CA) and Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI). Once sequences were analyzed and cut into useable length, a Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) search of GenBank (NCBI, National Institutes of Health, Bethesda, MD) for presumptive identification of bacterial isolates was performed. Bacteria were identified to the closest possible match by BLAST search with search results and search criteria reported (Table 1). Initial sequencing results provided a presumptive identification, and all isolates used in challenge studies were selected from the

best matches for that genus, as provided by the sequence length, sequence match, and e-value of the BLAST results.

After the bacterial challenge, isolates 141 and 159 were sent to Washington Animal Disease Diagnostic Lab, Washington State University, Pullman, WA for amplification and sequencing using more specific primers for increased resolution and further identification of isolates previously identified to genus. Briefly, DNA was extracted, the *rpoB* gene amplified using PCR with universal bacterial primers (Mollet et al. 1997) and the resulting PCR product was direct sequenced. The sequences were identified and compared to known sequences using a BLAST search of GenBank. The sequences had low correspondence to currently identified species and were submitted to GenBank; accession numbers are provided in the results. Twenty one of the most compatible search matches were chosen for phylogenetic analysis. Our nucleotide sequences were mapped to the retrieved sequences, extracted, aligned and edited (sequences were unambiguous) using Geneious 7.0.4 (http://www.geneious.com; Kearse et al. 2012). Models of sequence evolution were estimated in DT-ModSel (Minnin et al. 2003) and a maximum likelihood search performed in PAUP* (Swofford 2002) to estimate a maximum likelihood gene tree and nodal support for the resulting tree was evaluated by performing 100 bootstrap replicates.

Bacterial virulence screening

A preliminary screening study was conducted using isolates determined to be potential candidates of previous epizootics, by total number of isolations and number of fish infected per genera or species. Fish were netted in groups of 10 (2.4-2.6 g) and placed in a 5L bucket containing 2L of water with 100mg/L tricane methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA). Once fish had been sufficiently anesthetized they were injected with a 25µL dose of the challenge isolate, or sterile PBS for controls, using a 30-gauge needle on a 1mL syringe. Fish were challenged by intra-peritoneal injection (IP) administered in the left, posterior end of the peritoneal cavity. Challenge bacteria were grown to approximately equivalent CFU, determined by growth curves for individual isolates using optical density and confirmed using the drop plate method for plate counts (Chen et al. 2003). During preliminary screening studies, ten fish per treatment were injected with 4.0×10^5 to 7.3×10^6 CFU per fish (25µL) of TSB active culture broth. After injection, fish were placed in a 5L recovery bucket containing 2L of water with aeration and once recovered were placed into 19L holding

tanks with lids and observed for 28 days. The holding tanks were supplied with pathogen free de-chlorinated municipal water and supplemental compressed air through air stones. Photoperiod was set at 12 hours light and 12 hours darkness. Flow rates were approximately 1.2L per minute for each tank, and temperatures were maintained at 14-15°C. Fish were fed to apparent satiation and mortalities removed once daily. With one loss from cannibalism and another suspected, we altered the feeding strategy of fish in the preliminary screening to apparent satiation as opposed to providing a percent bodyweight calculation based on previous years juvenile feeding rates at the ARI (Joseph Evavold, personal communication). Future experiments also implemented a grading procedure, in addition to feeding to apparent satiation, as an attempt to avoid situations of size discrepancy and cannibalism. Following this preliminary screening, we adjusted the number of isolates of interest for future challenge studies according to total mortality induced during the screening.

Bacterial challenge

Data from the preliminary screening were utilized to design a challenge experiment with fish of similar size (2.4–2.6g) following weaning from live feed onto a commercial diet (Gemma; Skretting, North Tooele, UT). Using fish of 2.4–2.6g allowed targeting of fish at a size and age that previously experienced epizootics, potentially by the isolates initially collected from moribund fish. The injection bacterial challenge followed the procedure outlined previously. Briefly, 25 Burbot in triplicate were used per treatment and fish were injected with approximately equivalent CFU/fish. The injection dose was 25µL of TSB culture broth in log-phase growth or 25µL sterile TSB for controls. Two isolates (141 and 159) were selected as putative pathogenic isolates and used for IP and immersion challenge of Burbot with an average weight of 2.4g. Isolate 141 fish were injected with 7.8×10^6 CFU and isolate 159 were injected with 9.5×10^6 CFU. The immersion treatments used 20 Burbot in triplicate and one liter of water that was innoculated with culture broth from each isolate or control (TSB) prior to adding fish. The buckets used for immersion challenge were supplied with supplemental compressed air for the duration of the one hour static immersion. The two isolates used for challenge did not have equal CFU/mL in their broth growth, as determined by OD values and previous growth curves for each isolate, so different volumes of broth were added for each treatment to equalize the number of bacteria/mL in each treatment. For the control treatment, sterile TSB was added at the same volume (115mL) as the 141 treatment,

both of which had a higher added volume than the 159 treatment (45mL). The 141 immersions had a final exposure dose of 3.6×10^7 CFU per mL and the 159 immersions 1.7×10^7 CFU per mL. Both immersion and injection treatments were carried out on the same day. Following treatment, fish were held in 19L tanks, fed to apparent satiation, mortalities removed daily, and monitored for 28 days.

Attempts were made to re-isolate bacteria from at least 20% of daily mortalities from each challenge tank. Bacterial isolation was attempted from the kidney, spleen, liver, and brain of mortalities. Inoculum was streaked on to TSA and incubated at 15°C and observed for colony growth for up to 1 week. Morphology of bacterial isolates on plates was matched to challenge isolates and isolates were further screened using common bacteriological identification tests including a catalase test, and cytochrome oxidase as additional evidence to confirm re-isolated bacteria matched challenge isolates. Any bacteria re-isolated from test fish were considered presumptively positive if they matched the characteristics of the isolate initially used in the challenge.

LD_{50} determination

Burbot were challenged with serial dilutions of isolate 141 and 159 in an attempt to achieve doses with mortality above and below 50% following the lethal dose 50 (LD_{50}) method of Reed and Müench (1938) using proportional distances between dosages. LD_{50} determination was carried out following the same procedure as earlier challenges. Burbot were administered challenge dose by IP injection, held in 19L tanks at ~14°C, fed to apparent satiation twice daily, mortalities were removed once daily, and screened by bacterial culture for correspondence to challenge isolates.

Statistical analysis

Survival curves were generated using nonparametric Kaplan-Meier survival analysis. Kaplan-Meier survival analysis from the survival package (Therneau 2015), analyzes survival time, survival proportions, and determines any significant differences in survival rates between treatments. ANOVA with Tukey's honest significant difference post hoc test was used to analyze pair-wise differences in cumulative percent mortality between treatments. Linear regression was used to evaluate the effect of treatment in the immersion challenge. All analyses were conducted in R statistical programming language (R core team 2015) with $\alpha = 0.05$.

Results

Bacterial selection

Sequencing results from initial isolated bacteria are provided in Table 2.1, with reference numbers 96-118 sequenced by a commercial vendor (Amplicon Express, Pullman, WA) and numbers 119-176 as well as a repeat of 104 sequenced at the University of Idaho following the procedure listed above. We were unable to re-grow several isolates following multiple thawing attempts, isolates (110, 111, 122, and 132) were considered non-viable. We were unable to amplify some isolates (167, 172, and 174), therefore; these isolates were not identified or used for further analysis. A breakdown of isolates, numbers of fish infected by each isolate, total numbers of each isolate and BLAST search percent match is summarized in Table 2.2. Definitive identification to species level was difficult for many isolates based on sequencing and (or) biochemical results, and initial sequencing was considered a presumptive identification.

The list was trimmed to a manageable number of isolates with some criteria used to get the most probable isolate of interest (Table 2.2). If an isolate was only detected once, it was excluded from consideration. Two isolates were identified as *Pedobacter steynii*, a common soil and water bacteria. No previous reports could be found linking this bacterium to fish and the low number of fish infected excluded these isolates from further investigation.

Isolates were selected as challenge candidates if previous documentation suggested they were associated with clinical infection in fish, and if multiple isolates were obtained. Selected isolates were: *Aeromonas* sp., *Carnobacterium* sp., *Chryseobacterium* sp., *Flavobacterium* sp., *Shewanella* sp., *Sphingobacterium* sp., and *Yersinia* sp.

Sequencing results following amplification of the *rpoB* gene for isolate 141 and isolate 159 showed closest matches to members of *Aeromonas* sp. when compared to sequences in GenBank. Sequences from both isolates (which differed from each other by only three base pairs) were a 96% match with *A. veronii*, one of the causative agents of motile *Aeromonas* septicemeia. Phylogenetic analyses support isolate 141 and 159 as the most related, with both isolates being next most closely related to *A. veronii* (Figure 2.1).

Bacterial virulence screening

The doses used in the preliminary screening (Table 2.3) were expected to elicit some mortality if an isolate was truly pathogenic. Isolates which caused no mortalities following

injection were omitted from future experiments. Isolates with corresponding optical density values, CFU/mL, CFU injected, and resulting mortalities following preliminary screening are summarized in Table 3. Multiple isolates were not virulent under the challenge conditions tested and included the PBS controls, isolate 119 and isolate 149 (*Yersinia* sp.), and all the *Flavobacterium* sp. isolates. Three isolates caused a single mortality in their screening groups, 121 (*Sphingobacterium* sp.), 139 (*Chryseobacterium* sp.), and 142 (*Shewanella* sp.). An additional mortality occurred in the 139 challenge but was confirmed as cannibalism and was not attributed to the bacterial challenge. The mortality in the 142 challenge was suspected as cannibalism but not confirmed. The isolates (121, 139, and 142) with low mortality (1) were also omitted from future studies because of a low response to a high challenge dose and (or) likelihood of cannibalism being the true cause of death. The most lethal isolate in the initial screening study was isolate 141 (*Aeromonas* sp.) causing 80% mortality. This was followed by isolate 159, preliminarily identified as *Carnobacterium* sp., but subsequently as *Aeromonas* sp., which caused 40% mortality. In addition to causing the greatest mortality, isolate 141 had the earliest onset of mortality, and the most acute mortality. All mortalities occurred within 48 hours post challenge (Figure 2.2).

Bacterial challenge

The three 141 treatments had earlier onset and more acute mortatlity than the other treatments (Figure 2.3). Initial onset of mortality occurred on the day 2 post injection and was very acute, all mortality in this treatment occurred over three days (days 2, 3, and 4 post challenge). The mortality varied across replicates from 16–23 fish, resulting in cumulative mortality varying from 64% to 92% (Figure 2.3). The 159 treatment also had relatively acute mortality but cumulative mortality varied from 28% to 44% and was delayed compared to the 141 treatment. One tank had initial mortality on the third day post challenge with the other two tanks having initial mortality the fourth day post challenge. The majority of mortality occurred over three days for all tanks but one tank experienced mortality out to seven days. Mortality varied across replicates from 7–11 fish (28–44%) mortality respectively (Figure 2.3). There were no mortalities in control groups.

Kaplan-Meier survival analysis showed a significant effect of treatment when the two isolates were compared ($P < 0.001$, Figure 2.3). From the survival curves generated there were significant differences in timing and proportion of mortality across the treatments

(Figure 2.5). The ANOVA results for cumulative percent mortality indicated a significant treatment effect ($P < 0.001$) and the Tukey mean comparisons were all significantly different from each other ($P \leq 0.011$). Fish infected with isolate 141 had both earlier onset and higher mortality compared to the 159 treatment. The controls did not experience mortality.

Re-isolation of bacteria from all 141 mortalities showed correspondence with the challenge isolate when colony morphology, pigmentation, cytochrome oxidase, and catalase tests were compared. Mortalities caused by challenge with isolate 159 showed two distinct colony pigments with corresponding morphology, cytochrome oxidase, and catalase test results to the challenge isolate. Surviving fish showed no symptoms of bacterial infection and bacterial isolation was attempted on at least 50% of survivors but no bacteria were cultured from tissue of survivors.

There was only a single mortality across all immersion challenge groups. It occurred in one of the isolate 159 replicate tanks on day 1 post challenge. Bacteria isolated from this fish were of mixed morphology and biochemical status (not entirely representative of the challenge isolate). No fish died in the other two 159 immersion treatment tanks, the 141 treatment tanks, or the controls. No significant effect of treatment overall was observed ($P = 0.42$) in mortality when all groups of the immersion challenged fish were compared using linear regression.

LD_{50} determination

Four serial dilutions (10 fold) of isolates 141 and 159 were used to determine LD_{50} doses for each strain when administered to juvenile Burbot. Twenty five Burbot ($\bar{x} = 2.9$g) were injected with each dilution and mortality was monitored for 14 days. The high doses of each bacteria resulted in greater than 50% mortality (Table 2.5), with the second highest dose of the isolate 141 challenge also resulting in greater than 50% mortality. All dilutions of both bacteria resulted in some mortality, with the highest dose of isolate 141 having the most (24, 96%), and the lowest dose of isolate 159 the least (5, 20%). The LD_{50} for isolate 141 was between 6.9×10^4 and 1.8×10^6 CFU/fish, but from can be estimated using proportional distances as $10^{5.09}$ CFU/fish (Figure 2.4). Isolate 159 had an LD_{50} between 2.2×10^6 and 9.5×10^6 CFU/fish (Figure 2.4), and was estimated to be $10^{6.79}$ CFU/fish.

Discussion

The preliminary screening and bacterial challenge experiment suggests *Aeromonas* sp. isolated from juvenile Burbot in 2012-13 are the putative cause of previous mortality events. The phylogenetic analysis suggests the closest related species to be *Aeromonas veronii* one of a few recognized causative agents of motile *Aeromonas* septicemia (MAS). The results of the preliminary screening are similar to the bacteriology results of Polinski et al. (2011), where Burbot had low susceptibility to common bacterial pathogens of Rainbow Trout *Oncorhynchus mykiss,* including *Flavobacterium* sp., and *Yersinia* sp., which are closely related to isolates from this study. There were significant differences in survival between all treatments in the injection challenge. The survival differences suggest the bacteria used in the challenges are pathogenic to Burbot. The causative agents of MAS have well documented infectivity for many fish species including Esteve et al. (1993), which reported high mortality of juvenile European Eels *Anguilla anguilla* to injected *Aeromonas hydrophila* and *A. jandaei,* but contrary to Esteve et al. (1993) who also reported high mortality via immersion challenge at similar doses to our study. Motile *Aeromonas* septicemia has also been documented by Groberg et al. (1978) who reported high mortality of experimentally infected Chinook Salmon *O. tshawytscha*, Coho Salmon *O. kisutch*, and Steelhead to *A. hydrophila*. Řehulka (2002) and Neito et al. (1985) had similar findings with motile *Aeromonas* sp. causing high mortality in farmed Rainbow Trout *O. mykiss* at low temperatures, and experimentally infected Rainbow Trout respectively. *A. hydrophila* has also been isolated from Channel Catfish *Ictalurus punctatus* during a disease outbreak (Pridgeon and Klesius 2011). Shotts et al. (1972) found multiple moribund and stressed species from fish traps yielded *Aeromonas* sp. upon examination; Larsen and Jensen (1976) report *Aeromonas* sp. being incriminating in ulcers and septicemia of Atlantic Cod from the Danish coast; Toranzo et al. (1989) reported another MAS species *A. sobria* causing high mortality in wild Gizzard Shad *Dorosoma cepedianum*. *A. veronii* biovar sobria has been implicated as the causative agent of epizootic ulcerative syndrome in fish from Bangladesh (Rahman et al. 2002), snakehead *Ophiocephalus argus* (Zheng et al. 2012), in addition to Common Carp *Cyprinus carpio* from Poland (Kozińska 2004).

The injection results are confounded by the immersion results where there was no significant mortality or statistical differences between treatments. One potential explanation

for lack of mortality in the immersion challenge is the fish in this experiment were not stressed in the way previous cohorts of Burbot have been stressed when epizootics occurred. Previous year classes potentially had other environmental stressors, such as density and water quality problems, which were not replicated outside of pathogen introduction. The pathogenic bacteria potentially need additional stressors, like poor water quality, to elicit mortality of juvenile Burbot via immersion, which would be similar to the results of Mellergaard and Dalsgaard (1987) who reported outbreaks of *A. hydrophila* of European Eels in Denmark were associated with fish grading and poor water quality. Motile *Aeromonas* septicemia is often associated with stressors such as temperature and organic load as causative factors in predisposing events of environmental stress (Hanson et al. 2014).

Burbot culture is still fairly new, and potential pathogens not fully vetted, but the infectivity experiments indicate *Aeromonas* sp. are pathogenic to Burbot. With reports of many fish species showing susceptibility to currently accepted causative agents of motile *Aeromonas* septicemia and closely related species, the susceptibility of Burbot to *Aeromonas* sp. would not be unlikely. The isolates used in our study were cultured from moribund Burbot experiencing mortality events under stressful conditions, caused mortality when Burbot were experimentally infected, and were re-isolated from the resulting mortalities. The LD_{50} results further demonstrate the virulence of the isolated pathogens and the susceptibility of juvenile Burbot to the isolated pathogens.

The isolates used in the challenge study are both potentially unidentified species. The *rpoB* sequence data and phylogenetic analysis suggests both pathogenic isolates are most closely related to *A. veronii*, but have only a 96% match to the GenBank sequences, potentially indicating a novel *Aeromonas* sp. as a disease causing bacteria for Burbot. The relationship between this clade ([isolates 141 and 159] and *A. veronii*) and other *Aeromonas* species included in this analysis are unsupported by the bootstrapping analysis and remain unknown. The *rpoB* sequences from our two isolates are different from each other by 3 base pairs, potentially indicating different species given this region is highly conserved. The mortality differences between the two isolates would be further evidence they are different species. The *rpoB* sequence identification (*Aeromonas* sp.) for isolate 159 is very different from the 16S identification (*Carnobacterium* sp.). There are potential explanations for the discrepancy in sequencing results. One potential explanation is the *rpoB* gene is more

conserved and better separates species than the 16S region (and specifically the region targeted by the primer set we used) as described by Mollet et al. (1997). Mollet et al. (1997) and de Zwaan et al. (2014) have shown that for many species and strains the divergence is larger for *rpoB* sequences than 16S sequences. The lack of divergence between sequences at the 16S region targeted by our primers could have confounded our presumptive identifications. Another explanation is that the 16S primers most suitable for *Aeromonas* sp. identification (Kong et al. 1999) were not used, and provide a sequence of approximately 550 base pairs. The sequence obtained from the 16S primers we used is 1008 base pairs, but only overlaps the best location on the 16S gene for identification of *Aeromonas* sp. by 60 base pairs, and the remaining sequence is more similar to *Carnobacterium* sp. than *Aeromonas* sp. The identification of isolate 159 was further confounded by the results of biochemical tests indicating it was not a Gram positive bacterium.

According to the criteria outlined by Santos et al. (1988), strains exhibiting an $LD_{50} \geq 10^8$ CFU/fish are avirulent, both isolates would be considered virulent to Burbot. Isolate 141 is particularly pathogenic even at the lowest dose (40% mortality; 7.9×10^3 CFU/fish), has a low LD_{50} ($10^{5.09}$ CFU/fish), and only one survivor from the highest dose (1.03×10^7 CFU/fish). By contrast, isolate 159 needed a much higher dose to achieve LD_{50} ($10^{6.79}$ CFU/fish), over a 10-fold increase in CFU injected, and less fish died than at similar doses of isolate 141. The results of our experiment are similar to Esteve et al. (1993; 1995) who found LD_{50} values of $10^{5.4}$ to $10^{7.6}$ CFU/fish for *Aeromonas* sp. to European Eels; Toranzo et al. (1989) who reported an LD_{50} of $10^{5.3}$ for *Aeromonas sobria*, originally isolated from Gizzard Shad, on Rainbow Trout; Nieto et al. (1985) who showed *Aeromonas* spp. with LD_{50} values of $10^{\leq 5.5}$ CFU/fish on Rainbow Trout; and Pridgeon and Klesius (2011) reported LD_{50} values of 10^5–10^7 for *Aeromonas hydrophila* on Channel Catfish. Isolate 141 can be considered more pathogenic to Burbot by the LD_{50} results. In all screenings, isolate 141 caused more mortality than isolate 159.

The initial sequencing results, although considered presumptive, can give some idea of carrier status for different bacteria. Similar to Dwilow et al. (1987), fish in this investigation of Burbot are presumptively carriers of *Yersinia* sp. (likely *Yersinia ruckeri*) but it does not appear to be pathogenic to Burbot by our screening method. There are a number of other potential pathogens that Burbot presumptively carry, including all the isolates that were

investigated in the preliminary screening, such as *Flavobacterium* sp., but did not meet the threshold set for further investigation.

Ultimately the goal of Kootenai River Burbot propagation is population restoration to a naturally producing, harvestable level. Polinski et al. (2010) and Natrah et al. (2012) found juvenile Burbot to be susceptible to *A. salmonicida*, and Natrah et al. (2012) found larval Burbot to also be susceptible to *A. hydrophila*. We have found additional *Aeromonas* sp. originally isolated from Burbot experiencing mortality events, pathogenic to juvenile Burbot. Understanding and recognizing stress-mediated disease susceptibility of Burbot will allow more fish to survive captive rearing. Additional fish can be used to further restoration goals in the Kootenai River. In addition, this study provides evidence of problematic bacteria associated with Burbot propagation and increases our awareness of potential disease issues for Burbot aquaculture.

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Table 2.1. The 74 reference isolates with preliminary sequencing information used as initial screening criteria which incuded percent match, e-value, and sequence length (# base pairs).

Case/sample	#	Top match	Percent match	E-value	Length
092812001 BW	96	*Sphingobacterium* sp.	95%	<1e-179	973
09281201 KP	97	*Shewanella* sp.	95%	<1e-179	910
092812001 KW	98	*Yersinia* sp.	98%	<1e-179	651
092812001 LP	99	*Shewanella* sp.	94%	<1e-179	730
092812001 SP	100	*Shewanella* sp.	92%	<1e-179	532
092812002 BP	101	*Shewanella* sp.	94%	<1e-179	688
092812002 BW	102	*Sphingobacterium* sp.	91%	6e-170	445
092812002 KP	103	*Carnobacterium* sp.	95%	<1e-179	570
092812002 KW	104	*Carnobacterium* sp.	84%/92%	<1e-179	1030
092812002 LW	105	*Shewanella* sp.	89%	<1e-179	720
092812002 LP	106	*Carnobacterium* sp.	94%	<1e-179	451
092812002 SP	107	*Shewanella* sp.	94%	<1e-179	889
092812002 SW	108	*Carnobacterium* sp.	98%	<1e-179	448
092912001 LP	109	*Shewanella* sp.	93%	<1e-179	939
092912001 SP	110	Non-viable	--	--	--
092912002 BP	111	Non-viable	--	--	--
092912002 KP	112	*Shewanella* sp.	93%	<1e-179	929
100112001 BB	113	*Flavobacterium* sp.	91%	<1e-179	881
100112001 BC	114	*Sphingobacterium* sp.	90%	<1e-179	761
100112001 KP	115	*Paenibacillus* sp.	98%	7e-08	963
100112001 KW	116	*Yersinia* sp.	97%	<1e-179	539
100112001 LW	117	*Yersinia* sp.	97%	<1e-179	747
100112001 SP	118	*Shewanella* sp.	97%	<1e-179	690
100112001 SW	119	*Yersinia* sp.	100%	1e-155	302
100112002 BP	120	*Chryseobacterium* sp.	81%	3e-141	1044
100112002 BW	121	*Sphingobacterium* sp.	95%	<1e-179	1009
100112002 KP	122	Non-viable	--	--	--
100112002 KW	123	*Yersinia* sp.	96%	<1e-179	1007
100112003 BW	124	*Sphingobacterium* sp.	95%	<1e-179	991
100112003 KC	125	*Aeromonas* sp.	95%	<1e-179	987
100112003 KW	126	*Carnobacterium* sp.	97%	<1e-179	1024
100112003 SC	127	*Sphingobacterium* sp.	95%	<1e-179	1005
100112003 SW	128	*Flavobacterium* sp.	96%	<1e-179	1004
100112004 BW	129	*Flavobacterium* sp.	96%	<1e-179	1007
100112004 BY	130	*Flavobacterium* sp.	96%	<1e-179	1014
100112004 KB	131	*Enterobacter* sp.	97%	<1e-179	996
100112004 KP	132	Non-viable	--	--	--
100112004 KW	133	*Carnobacterium* sp.	97%	<1e-179	1017
100112004 SB	134	*Carnobacterium* sp.	98%	<1e-179	1008
100112004 SP	135	*Shewanella* sp.	98%	<1e-179	1022

100112004 SW	136	*Lactococcus* sp.	98%	<1e-179	1017
110612001 Fluid	137	*Aeromonas* sp.	97%	<1e-179	1016
110612001 WK	138	*Yersinia* sp.	98%	<1e-179	1011
110612001 OB	139	*Chryseobacterium* sp.	98%	<1e-179	1010
110612002 WB	140	*Pedobacter* sp.	97%	<1e-179	1014
110612003 WS	141	*Aeromonas* sp.	97%	<1e-179	1018
110612003 PS	142	*Shewanella* sp.	97%	<1e-179	1019
110612003 Fluid	143	*Yersinia* sp.	97%	<1e-179	1019
110612003 CB	144	*Pedobacter* sp.	97%	<1e-179	1023
110612003 PL	145	*Shewanella* sp.	97%	<1e-179	1015
110612003 WL	146	*Aeromonas* sp.	98%	<1e-179	1019
110612003 PS	147	*Shewanella* sp.	98%	<1e-179	1025
110612003 PK	148	*Shewanella* sp.	98%	<1e-179	1016
031513001 L1	149	*Yersinia* sp.	98%	<1e-179	1009
031513001 S1	150	*Carnobacterium* sp.	95%	<1e-179	998
031513001 S2	151	*Yersinia* sp.	97%	<1e-179	1031
031513002 B1	152	*Rheinheimera* sp.	97%	<1e-179	1015
031513002 S1	153	*Carnobacterium* sp.	97%	<1e-179	1003
031513002 S2	154	*Carnobacterium* sp.	93%	<1e-179	1021
031513002 S3	155	*Yersinia* sp.	98%	<1e-179	1018
031513003 K1	156	*Aeromonas* sp.	98%	<1e-179	1019
031513003 K2	157	*Carnobacterium* sp.	96%	<1e-179	1028
031513003 L1	158	*Yersinia* sp.	98%	<1e-179	1016
031513003 L2	159	*Carnobacterium* sp.	98%	<1e-179	1008
031513003 O1	160	*Carnobacterium* sp.	95%	<1e-179	1005
031513003 O2	161	*Aeromonas* sp.	94%	<1e-179	1007
031513003 O3	162	*Yersinia* sp.	96%	<1e-179	1027
031513003 S1	163	*Aeromonas* sp.	97%	<1e-179	1026
031513003 S2	164	*Yersinia* sp.	96%	<1e-179	1035
032113001 B1	165	*Salinibacterium* sp.	98%	<1e-179	1026
032113001 B2	166	*Brevundimonas* sp.	97%	<1e-179	1015
032113001 B3	167	DNA not extracted	--	--	--
032113001 B4	168	*Flavobacterium* sp.	100%	3e-161	312
032113003 K1	169	*Flavobacterium* sp.	100%	4e-160	310
032113003 B1	170	*Flavobacterium* sp.	100%	2e-157	305
033013 113-15 S1	171	*Aeromonas* sp.	100%	2e-157	305
090613 SW	172	DNA not extracted	--	--	--
090613 SB	173	*Shewanella* sp.	100%	1e-155	302
091113 AW	174	DNA not extracted	--	--	--
091113 SW	175	*Ewingella* sp.	100%	2e-152	296
091113 LB	176	*Aeromonas* sp.	100%	5e-154	299

Table 2.2. Isolates, presumptive identification, and composition of fish they occurred in during outbreaks of 2012-2013.

Isolate reference number	Sequencing result	% Match	# Isolates	# Fish
97, 99, 100, 101, 105, 107, 109, 112, 118, 135, 142, 145, 147, 148, 173	*Shewanella* sp.	89-100	15	8
103, 104, 106, 108, 126, 133, 134, 150, 153, 154, 157, 159, 160	*Carnobacterium* sp.	92-98	13	7
98, 116, 117, 119, 123, 138, 143, 149, 151, 155, 158, 162, 164	*Yersinia* sp.	96-100	13	8
125, 137, 141, 146, 156, 161, 163, 171, 176	*Aeromonas* sp.	94-100	9	7
113, 128, 129, 130, 168, 169, 170	*Flavobacterium* sp.	91-100	7	5
96, 102, 114, 121, 124, 127	*Sphingobacterium* sp.	90-95	6	5
120, 139	*Chryseobacterium* sp.	81-98	2	2
140, 144	*Pedobacter* sp.	97	2	2
166	*Brevundimonas* sp.	97	1	1
131	*Enterobacter* sp.	97	1	1
175	*Ewingella* sp.	100	1	1
136	*Lactococcus* sp.	98	1	1
115	*Paenibacillus* sp.	98	1	1
152	*Rheinheimera* sp.	97	1	1
165	*Salinibacterium* sp.	98	1	1

Table 2.3. Isolate, optical density values, corresponding bacterial counts, and mortality attributed to each isolate for the preliminary screening. Ten fish were experimentally infected with each isolate.

Preliminary ID	Isolate #	OD (600 nm)	CFU/mL	CFU injected	Mortality
Yersinia sp.	119	0.525	2.5×10^8	6.2×10^6	0
Sphingobacterium sp.	121	0.193	2.5×10^8	6.3×10^6	1
Flavobacterium sp.	128	0.248	4.7×10^7	1.2×10^6	0
Flavobacterium sp.	129	0.318	2.5×10^7	6.3×10^5	0
Flavobacterium sp.	130	0.154	1.1×10^8	2.6×10^6	0
Chryseobacterium sp.	139	0.190	2.3×10^8	5.6×10^6	1
Aeromonas sp.	141	0.446	9.3×10^7	2.3×10^6	8
Shewanella sp.	142	0.129	8.8×10^7	2.2×10^6	1
Yersinia sp.	149	0.377	7.7×10^7	1.9×10^6	0
Aeromonas sp.	156	0.205	5.5×10^7	1.4×10^6	0
Aeromonas sp.	159	0.506	2.9×10^8	7.3×10^6	4
Flavobacterium sp.	168	0.307	5.7×10^7	1.4×10^6	0
Flavobacterium sp.	169	0.241	4.1×10^7	1.0×10^6	0
Flavobacterium sp.	170	0.204	1.1×10^7	6.3×10^5	0

Table 2.4. Isolate, optical density values, CFU/mL, CFU injected, CFU/mL exposed (immersion), tanks, and resulting mortality for the injection and immersion challenges.

Agent	Isolate	OD (600nm)	CFU/mL	CFU injected	CFU/mL exposed	Tanks	Mortality
Aeromonas sp.	141	0.366	3.15×10^8	7.8×10^6	--	4, 6, 15	16, 23, 23
				--	3.6×10^7	1, 2, 10	0
Aeromonas sp.	159	0.441	3.8×10^8	9.5×10^6	--	13, 14, 17	8, 11, 7
				--	1.7×10^7	3, 5, 18	1
Control				0	--	8, 9, 11	0
				--	0	7, 12, 16	0

Table 2.5. Results of the LD_{50} determination following the method outlined by Reed and Muench (1938).

Isolate	OD	CFU/mL	CFU injected	Alive	Dead	Total		Percent Mortality
						Alive	Dead	
Aeromonas sp. (141)	0.000	3.2×10^5	7.9×10^3	15	10	40	10	25
	0.002	2.8×10^6	6.9×10^4	15	10	25	20	44
	0.056	7.2×10^7	1.8×10^6	9	16	10	36	78
	0.439	4.1×10^8	1.0×10^7	1	24	1	60	98
Aeromonas sp. (159)	0.001	2.0×10^6	5.1×10^4	20	5	54	5	8
	0.065	8.7×10^7	2.2×10^6	13	12	34	17	33
	0.452	3.8×10^8	9.5×10^6	14	11	21	28	57
	1.237	2.7×10^9	6.7×10^7	7	18	7	46	87

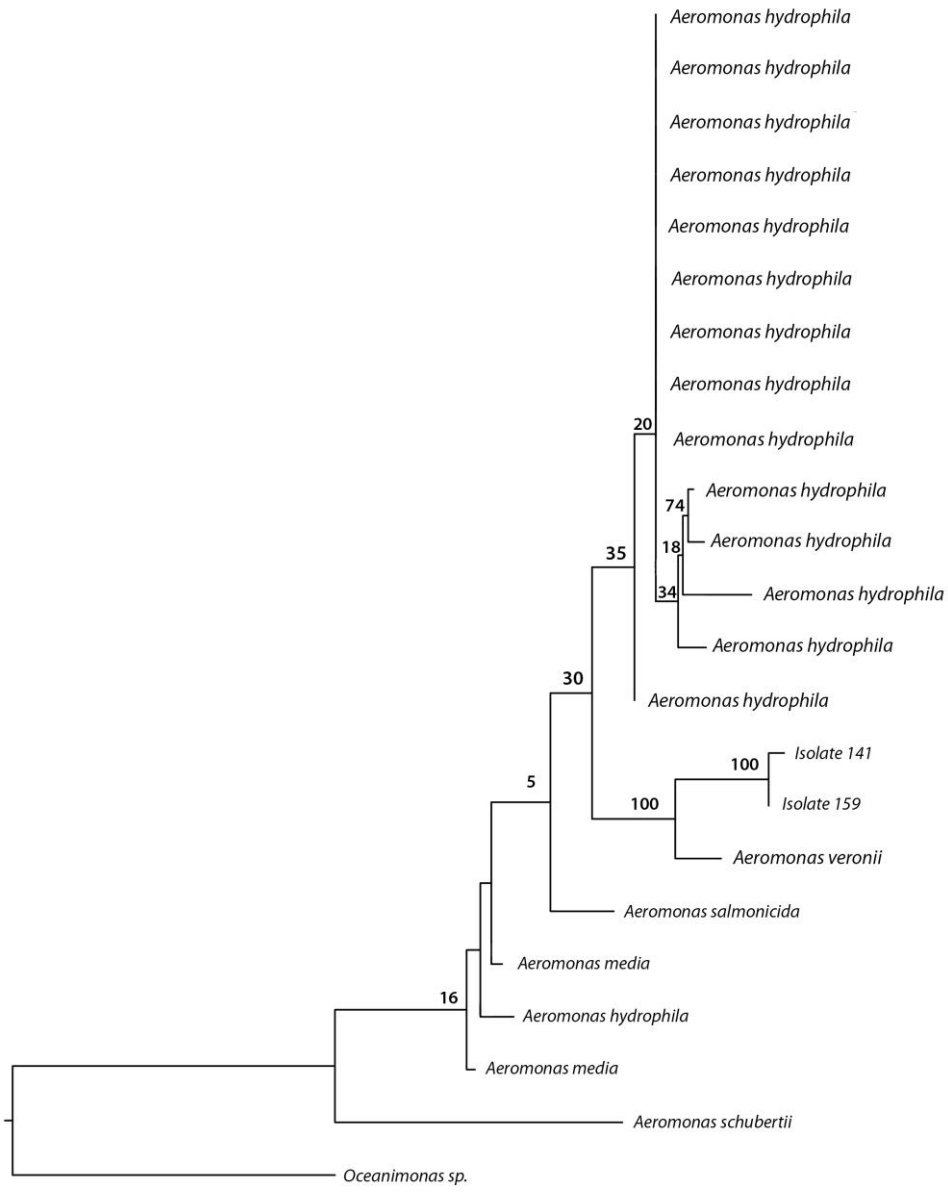

Figure 2.1. Maximum likelihood tree based on close matches by BLAST search of GenBank. Number at nodes indicates number of bootstrap replicates (out of 100) supporting each relationship.

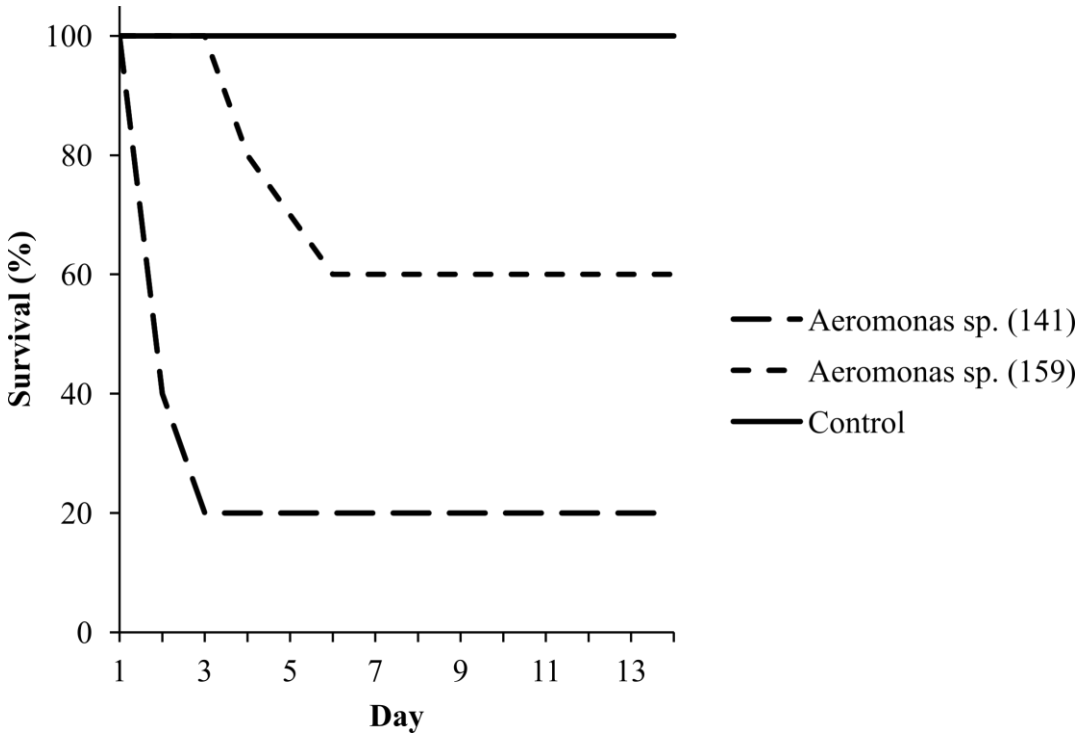

Figure 2.2. Percent survival by day for the two most pathogenic isolates used in the preliminary screening.

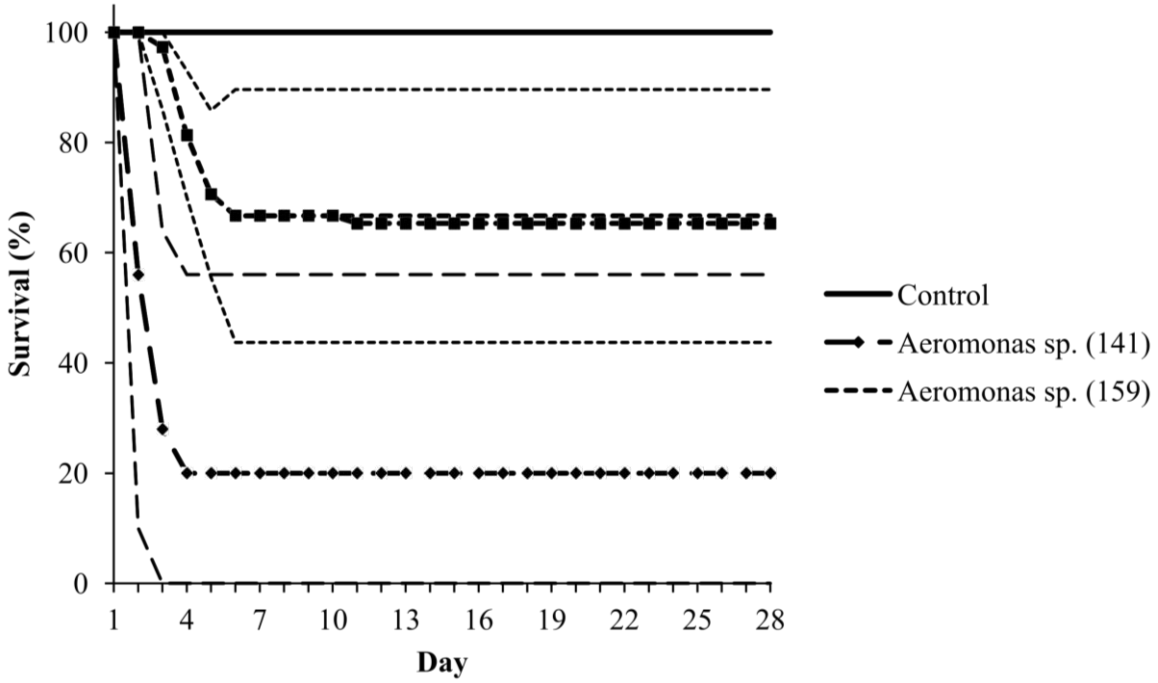

Figure 2.3. Survival results for the injection challenge. Bolder lines indicate means and lighter lines show 95% confidence intervals. Marked lines are predicted survival curves generated by Kaplan-Meier survival estimation; the 141 mean and Kaplan-Meier curve are identical.

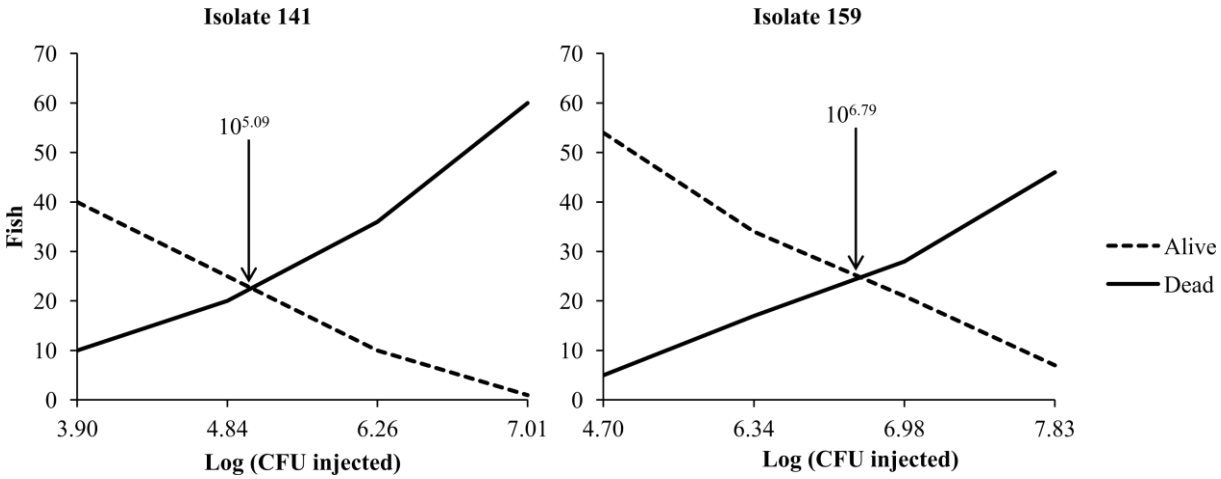

Figure 2.4. Cumulative survival and mortality for LD_{50} of isolates 141 and 159 as calculated by the method outlined in Reed and Muench (1938). The crossing point is the point estimate of LD_{50} by proportional distance estimated to be $10^{5.09}$ for isolate 141 and $10^{6.79}$ for isolate 159.

CHAPTER THREE

Effects of Diel Temperature Fluctuation on Growth, Stress Response, and Immune Function of Burbot

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Running head: Burbot thermal physiology
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Abstract

Burbot *Lota lota maculosa* occupy habitats from deep, cold lakes to prairie streams. Controlled laboratory trials were conducted to evaluate the growth, immune response, and thermal stress physiology of Burbot subjected to fluctuating, high diel temperatures. We tested age-0 Burbot from a captive propagation program associated with population restoration in the Kootenai River, Idaho in temperature cycles that imitated summer conditions observed in regional stream habitats. The diel temperature cycle for treatments varied from 18°C to 27°C and controls were held at a constant temperature of 14°C. We evaluated survival, growth, feed efficiency, heat-shock protein 70 (hsp70) levels, *hsp70* gene expression, serum lysozyme levels, and proximate body composition in samples of fish taken before, during, and at the completion of the trials. Both treatments had high survival, but experienced differences in growth with the control fish having higher growth rates and better

feed conversions than treatment fish. Heat shock protein 70 expression and abundance were higher in tissues from treatment fish but varied between tissues with liver having higher abundance of hsp70 than muscle. Serum lysozyme levels were not different between treatments. We conducted an additional short-term trial to evaluate the response of Burbot to upper lethal diel temperatures where daily maximums approached 32°C. The thermal stress resiliency of Burbot is robust for fish commonly called cold-water stenothermes, with fish from treatment and controls able to withstand temperatures up to and exceeding 31°C.

Introduction

Burbot *Lota lota* are the only freshwater member of the family Gadidae and have a Holarctic or circumpolar distribution (Scott and Crossman 1973; Cohen et al. 1990; McPhail 1995; Berra 2001; Stapanian 2010). Two subspecies of Burbot occur, across Eurasia and North America to the Great Slave Lake *Lota lota lota*, and south of the Great Slave Lake to the Atlantic Coast *Lota lota maculosa* (Scott and Crossman 1973; Van Houdt et al. 2003). In Idaho, Burbot are only native to the northern most drainage of the Kootenai River, where the population is imperiled largely due to anthropogenic habitat alteration (Paragamian et al. 2000). The Kootenai River population has been closed to fishing since 1992 and a population restoration program was implemented to conserve and restore Burbot in the drainage (Paragamian et al. 2002; KVRI 2005; Ireland and Perry 2008). Restoration efforts have employed captive rearing and release of Burbot as an initial strategy to increase density and population size in the Kootenai River (Jensen et al. 2011; Neufeld et al. 2011; Hardy and Paraganiam 2013). The current restoration strategy has been successful, with hoop net CPUE near what they were a decade prior and much higher than pre-stocking (Hardy and Paragamian 2013).

Burbot are spawned from Moyie Lake, British Columbia, which is a tributary drainage to the Kootenai River just upstream of the town of Bonners Ferry, Idaho. The Moyie Lake population has been determined to be a similar phylogenetic group as the Kootenai River Burbot (Powell et al. 2008) and has been considered suitable as a donor stock (Hardy and Paragamian 2013). Stocking of Burbot at age 2 and age 3 has resulted in rapid and widespread dispersal of tagged fish in the Kootenai River system (Neufeld et al. 2011b). The dispersal information suggests that select stocking locations will allow Burbot access to critical habitats

within a short time of release (Neufeld et al. 2011b) and that lake-origin, hatchery-reared Burbot are suitable for stocking in this system (Hardy and Paragamian 2013). Increased catch rates are also a sign that Burbot are surviving after stocking in this system. Increased abundance of Burbot is evidence the restoration program is working and the first example that intensively reared Burbot are surviving in measurable numbers in the wild, as culture and release programs have been unsuccessful in other circumstances (Dillen et al. 2008; Vught et al. 2008). For example, Vught et al. (2008) produced Burbot using different culture conditions that were subsequently stocked as larvae and juveniles into three rivers in Belgium with only low numbers of juveniles recaptured (Dillen et al. 2008).

Impounded upstream by Libby Dam, the Kootenai River has cool, stable temperatures throughout the summer, similar to the control temperatures. The hypolimnetic dominated releases from Libby Dam also keeps the Kootenai River near the upper threshold of 10-14°C, thought to be the ideal temperatures for adult Burbot (Cooper and Fuller 1945; Hackney 1973; Hofmann and Fischer 2002). Several tributaries of the Kootenai River have been selected as release sites to evaluate stocking success of age-0 Burbot. One such tributary, Deep Creek, has a passive integrated transponder array to evaluate Burbot use of the system, and has been stocked with age-0, tagged Burbot (Hardy and Paragamian 2013). Summer water temperatures of up to 27°C have been recorded near release locations in Deep Creek (Ryan Hardy, Idaho Department of Fish and Game, personal communication). The upper thermal limit for 10-20 cm Burbot has been reported to be up to 31.5°C, depending on acclimation temperature (Hofmann and Fischer 2002). Suitability of stocking locations should be further interrogated for fall-stocked age-0 Burbot with regard to over summer temperature exposure at age 1. Evaluating stocking locations is especially important considering a large proportion of fish stocked in this system have been late age 0 (fall stocking date) and the younger age classes do not seem to disperse like the age 2 and older fish after stocking (Zach Beard, Idaho Cooperative Fish and Wildlife Research Unit, personal communication; Hardy and Paragamian 2013).

Many studies focus on temperature in relationship to maximum growth for Burbot (Wolnicki et al. 2001; Kjellman 2002; Hofmann and Fischer 2003; Harzevili et al. 2004; Barron et al. 2012) or have focused on critical thermal maximum and minimum based on acclimation temperature (Hoffmann and Fischer 2002). Understanding limits and ideal

temperatures for certain parameters (e.g. growth) are important to understand with respect to the physiological limits and ideal conditions for Burbot. Even though daily thermal cycles are part of the natural environment, no studies have evaluated at the effect of diel temperature fluctuations on the growth, physiology, and immune response of Burbot.

In this study we determined the effects of thermal stress on growth, physiology, and immune response of age-1 wild-origin Burbot to temperatures equal to observed summer high temperatures encountered after fall release as an age 0. We determined growth, survival, proximate composition of whole fish, heat shock protein-70 (hsp70) production, *hsp70* gene expression, and plasma lysozyme activity. Heat shock proteins are considered "stress" proteins and are expressed to maintain cellular processes, especially during thermal stress (Iwama et al. 1998). Lysozyme activity is an indicator of innate immune response (Saurabh and Sahoo 2008). A subset of fish from each treatment were also challenged with select bacterial isolates (isolate 141, *Aeromonas* sp.) to determine effects of pathogen exposure following chronic stress due to high diel temperatures. With the changing weather patterns associated with global climate change, higher water temperatures may become more commonplace across Burbot's southern distribution, and in tributaries of the Kootenai River. This study strives to create relevant data to assess current concerns for local introduction and may reveal physiological effects important to Burbot across their distribution in the face of climate uncertainty.

The objectives of this study were to determine if diel temperature fluctuations affected growth, physiology, and health of Burbot. We hypothesized that growth, survival, proximate tissue composition, hsp70 production, *hsp70* gene expression, and lysozyme activity were negatively affected following diel temperature fluctuations. Higher hsp70 values, increased hsp70 expression, lower growth rates, decreased survival, lower lipid values, lower tissue engery values, and decreased lysozyme production would be due to the detrimental effects of daily high temperatures.

Methods

Fish source and experimental design

Fish were angled through the ice with ripe individuals manually spawned and eggs water hardened following Neufeld et al. (2011a), prior to transport to the University of Idaho.

Fish were grown to a size of 24.9g and 148.4mm prior to starting the experiment at the University of Idaho.

Temperature data from Deep Creek, a primary Burbot stocking site, were provided by Ryan Hardy, Idaho Department of Fish and Game; and Zach Beard, Idaho Cooperative Fish and Wildlife Research Unit, University of Idaho. Field temperature loggers in Deep Creek have been recording temperature data for the past 2 years, and this study simulated severe summer conditions to determine Burbot responses to high water temperatures. Our study was 30-days to simulate peak summer conditions in Deep Creek.

Responses of the Moyie Lake stock were compared between simulated peak summer temperature and controls at 14°C. Triplicate tanks for each treatment were supplied with different quantities of ambient, chilled, and heated water to create the thermal conditions for peak temperature simulation, or control tanks. Water was supplied from specific pathogen-free municipal de-chlorinated water with supplemental compressed air through an air stone for aeration and to break surface tension. Treatments and fish were randomly assigned to 280 L tanks at equal initial weights of 1145 g (46 fish) for a density of 4.1 g/L. Flows were variable due to changing water temperatures but airflow remained constant. Dissolved oxygen was checked prior to feeding in the morning and treatment tanks were similar to control tanks. Daily water temperatures were increased from baseline low (18°C) until mid-day; after 2h at peak temperatures (27°C) water temperatures were lowered back to baseline low. Water temperatures were logged in each tank at 15min intervals with Hobo data loggers (Onset Computer Corporation, Bourne, MA). To evaluate the magnitude of the daily temperature exposure, the daily temperature units were computed by adding the temperatures at the beginning of each hour. Temperature units were calculated compared between experimental temperatures and two field sites on Deep Creek: McArthur Lake and Idaho Department of Lands (IDL) from the same summer (2015; Figure 3.1), logged by field data loggers in Deep Creek, similar to Cassinelli and Moffitt (2010). A natural photoperiod was used to simulate Deep Creek (48°N) from July 15 to August 14. Fish were fed Gemma size 3.0 (Skretting, North Tooele, UT) once daily at 1% bodyweight/day initially, keeping with their feed rate at the time, but increased to 1.2% seven days into the trial. Uneaten feed was approximated 20 min after feeding daily, for an idea of feeding behavior between treatments and to correct for

feed conversion rates by counting uneaten pellets and sampling dry feed to determine the average weight/pellet similar to Cassinelli and Moffitt (2010).

During the 30-day study, 12 fish were sampled prior to temperature alteration (day 0). Five fish per tank were sampled halfway through the study (day 15), and at the end of the study (day 30). Feed rates were adjusted based on the increase in average weight of all 30 fish sampled at the halfway point. All tanks were fed the same weight of feed each day for the entire experiment. Fish were euthanized in a solution containing tricane methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA) at a concentration of 250 mg/L or greater and buffered to a pH between 7.0 and 7.5. Protocols for euthanasia followed both the American Veterinary Association ethical animal care guidelines as well as the University of Idaho institutional animal care and use guidelines, protocols 2013-97 and 2014-88.

Sampling

Upon euthanasia, fish were weighed to the nearest gram and measured to the nearest millimeter prior to removing gill filaments, liver, and muscle. Absolute measures of weight as well as growth rate were calculated for each tank and compared between treatments. Growth rate was calculated using an average percentage of body weight assimilated per day for fish at day 30 {Specific Growth Rate, SGR; Ricker 1975; Rosenlund et al. 2004; [$100 \times (\ln W_2 - \ln W_1) / T$]}. Feed conversion rate (FCR) was calculated using the formula: feed consumed / weight gain (Rosenlund et al. 2004), and Fulton's condition factor [Ricker 1975; $K = (W/L^3) \times 10^5$] was used as an index of condition and compared between treatments at the end of the experiment. Liver and muscle samples were held on dry ice during collection and stored at −80°C for later analysis. Additional portions of liver, muscle, and gill tissue were collected and stored in RNAlater (Sigma-Aldrich Corp., St. Louis, MO) for analysis of *hsp70* gene expression by real-time quantitative polymerase chain reaction (RT-qPCR). Blood was collected immediately from euthanized individuals by clipping a gill arch, because caudal severance did not produce enough blood, and collecting the blood in heparnized hematocrit tubes (Fisher Scientific, Pittsburg, PA). Hematocrit tubes were centrifuged to separate the blood cells from plasma and plasma was representatively pooled (equal amount per fish) per tank by scoring and breaking the hematocrit tube at the plasma interface with the white blood cells. A 10 μL pipette tip was inserted into the hematocrit tube, allowing for equal collection of plasma, and plasma was stored at −80°C for serum lysozyme analysis. Serum lysozyme

levels were measured in triplicate per tank using a turbidimetric assay modified from Korkea-aho et al. (2011). Briefly, 10 μL of plasma was added to wells in a 96 well plate along with 190μL of lyophilized *Micrococcus lysodeikticus* suspension of 0.2 mg/mL in 0.04 mol sodium phosphate buffer. The suspension was incubated 20 min at 25°C prior to use and the absorbance of wells was read at 490 nm after one and five minutes. Lysozyme activity was expressed as the change in absorbance units × $minute^{-1}$ × mL^{-1}, and wells of 200 μL sodium phosphate buffer served as controls.

Proximate analysis

Proximate body composition was measured from a random sample of 12 fish at the beginning of the study and five fish from each tank at the end of the experiment. Sample fish were euthanized, the hindgut removed to prevent confounding the analysis with any uneaten feed, and stored at -30°C for proximate analysis following Selong et al. (2001). Proximate analysis was performed at the Hagerman Fish Culture Experiment Station (Hagerman, ID) with crude lipid concentration determined by petroleum ether extraction (AOCS Official Procedure Am 5-04) using an Ankom XT15 extractor (Ankom Technology, Macedon, NY). Protein content was calculated for all samples using subtraction (% protein = 100 - % water - % fat - % ash; Hendry et al. 2000). Using feed consumption data, the protein and lipid efficiencies were estimated by dividing the average lipid and protein gain per fish per tank by the average approximate amount of lipids and protein consumed over the duration of the experiment. Proximate analysis of feed took place in parallel with proximate analysis of fish for determination of lipid and protein concentrations in feed (AOAC Official Method 920.36). Additionally, energy values of Burbot white muscle tissue was determined by using a Parr 6300 calorimeter (Moline, Illinois) and was expressed in kilojoules per gram of tissue (kJ/g).

Western blotting

Proteins were extracted from frozen tissues using T-PER tissue protein extraction reagent (Pierce, Rockford, IL) with added protease inhibitor cocktail ("Halt"; Thermo Scientific, Rockford, IL) in disposable 1.5 mL tubes using disposable pellet pestles (Fisher Scientific). Total protein concentration of supernatant was determined using a Qubit 2.0 (Life Technologies, Grand Island, NY) and 25 μg total protein was used in a Western blot. Primary polyclonal antibody (ADI-SPA-757-F; Assay Designs, Ann Arbor, MI) and secondary antibody alkaline phosphate labeled anti-rabbit IgG (A-3687; Sigma-Aldrich Corp., St. Louis,

MO) were used to detect hsp70. Positive controls of hsp70 standard (SPP-758; Assay Designs), at known concentrations, were used in each run of the Western blot for comparison of blot density. Extracted proteins were run on 7.5% precast polyacrylamide Mini-PROTEAN gel (4561026; Bio-Rad Laboratories Inc., Herucules, CA) using SDS-page in running buffer containing 28.9 g glycine, 6.06 g Tris base, 4 mL 20% sodium dodecyl sulfate (SDS) and 726 mL distilled water. Each well of the polyacrylamide gel was loaded with 25 μg of protein combined with 1/5 volume Laemmli buffer (Laemmli 1970). The sample was heated at 100°C for 2 min prior to loading on gel and run at 100 V until the dye band just ran off the gel (~1.25 hours). All gels were loaded with protein standards (PageRuler Plus Pre-stained Protein Ladder; Thermo Scientific) and 5 μL of positive control diluted 1:50 in TBS (68ng hsp70) followed by duplicates of each sample. The proteins were then transferred to a polyvinylidene fluoride blot membrane (Millipore, Billerica, MA) via Western blots to determine blot density in correspondence to 70 kD molecular weight standards. For the Western blots, gels were placed in a "sandwich" consisting of a synthetic pad, a sheet of filter paper, the gel, a transfer membrane soaked in running buffer, another sheet of filter paper, and a synthetic pad all within a plastic cartridge and soaked in transfer buffer (100 mL 10x running buffer, 100 mL methanol, 0.5 mL 20% SDS, and 800 mL distilled water). The transfer was run at 100 V for 1 hour (Bio-Rad Mini-PROTEAN Gel System) after which the membrane is blocked for 30 min in a solution [50 g skim milk powder, 0.5 mL Tween-20, and 1 L 1X Tris-buffered saline (TBS)(1.8 g Trizma Base, 29.2 g NaCl, 1 L deionized water)] on a rocker.

For development, the membrane was incubated for 1.5 hours on a rocker with the primary polyclonal antibody (ADI-SPA-757-F; Assay Designs, Ann Arbor, MI), diluted 1:3000 in 1X TBS, and rinsed three times for 15 min each on a rocker with Tween-20 TBS (0.5 mL Tween-20, 1L 1X TBS). Incubation with the secondary antibody, alkaline phosphate labeled goat anti-rabbit IgG (A-3687; Sigma-Aldrich Corp., St. Louis, MO), used to detect the primary hsp70 antibody, was also 1.5 hours on a rocker and diluted 1:3000 in 1X TBS. After secondary antibody incubation, the membrane was again rinsed with TTBS three times. After rinsing, the membrane was developed with a coloring solution {100 μL 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) stock [15 mg BCIP in 1mL dimethylformamide (DMF)], 100 μL Nitro blue tetrazolium chloride (NBT) stock (0.7 mL DMF, 0.3 mL deionized water, 30 mg NBT), 10 mL bicarbonate buffer (8.4 g sodium bicarbonate, 0.2 g Mg_2Cl-$7H_2O$, 1 L deionized

water)} for 15 min. Then the membrane was rinsed with deionized water for 10 min, dried on a paper towel, and placed in the refrigerator. Quantification of protein was accomplished by scanning the membranes as a digital image prior to importing to Image-J freeware (Rasband 1997) to scan band densities for determination of hsp70 concentration. Image-J allows for background correction for each scanned image, each band is outlined and the "integrated density" is read. This allows for correction compared to the positive control band from the same gel by dividing the sample band density by the positive control band density. The density compared to control can then be multiplied by the quantity of positive control to estimate hsp70 quantity per sample.

RT-qPCR

Tissues for the comparative gene expression analysis were removed from the fish and stored in RNAlater (Sigma-Aldrich Corp., St. Louis, MO) until RNA extraction was performed using an RNeasy kit with added RNase free DNase set (Qiagen, Valencia, CA). Mortar and pestle was used for homogenization of liver and gill, and bead homogenization was used for muscle using 2.8 mm ceramic beads (MO BIO, Carlsbad, CA) and a Tissue Lyser II (Qiagen, Valencia, CA). Total RNA was quantified with a Qubit 2.0 (Life Technologies) and 250 ng total RNA was a template for complimentary DNA (cDNA) using a high capacity RNA-to-cDNA kit (Life Technologies). If the necessary RNA concentration was not attained during RNA extraction the sample was discarded. Reference genes from Atlantic Cod have previously been used in Burbot gene expression studies for exposure to persistent organic polutants (Olsvik et al. 2013). Two reference genes identified as the most stable reference genes in Atlantic Cod during thermal stress, *rplp1* and *ubiq* (Aursnes et al. 2011), were used as reference genes for *hsp70* expression. Primers developed in Atlantic Cod for the *rplp1*, *ubiq*, and *hsp70* genes (Aursnes et al. 2011) were screened for amplification success in Burbot. Primers were run in singleplex and the final reaction contained 5 μL Sybr Green Master Mix (Life Technologies), 0.4 μM of *hsp70*, 0.5 μM of *rplp1*, and 0.7 μM of *ubiq* respectively, and 1 μL cDNA in a 10 μL reaction. Molecular grade water was used as an extraction control through reverse transcription, and additional no-template RT-qPCR cocktail controls were run for each reaction. Thermocycling conditions consisted of 95°C for 10 min followed by 40 cycles of 95°C for 15s and 53°C for 1min. Reactions were run on a QuantStudio 7 (Life Technologies) using QuantStudio 6 and 7 Flex Real-Time PCR System

Software (Life Technologies). Crossing point values (C_p) were obtained from the run output and compared using Relative Expression Software Tool (REST; Pfaffl et al. 2002). Expression stability of reference genes was also compared using C_p values entered into *BestKeeper* (Pfaffl et al. 2004) macro for Excel.

Bacterial challenge

Upon completion of the temperature experiment, fish from control and treatment groups were consolidated to one tank each. Water temperature for fish from the temperature treatment was slowly lowered overnight to 14°C. The following morning, triplicate groups of 18 Burbot from the temperature treatment and controls were challenged by IP injection with 25 μL of *Aeromonas* sp. (isolate 141) grown in trypticase soy broth (TSB). The broth had colony counts of 2.9×10^8 colony forming units (CFU)/mL, determined by drop plate method (Chen et al. 2003); resulting in an injection dose of 7.2×10^6 CFU/fish. A group of control fish from both treatments were mock challenged via injection with 25 μL sterile TSB. Protocols for fish treatment followed the outline in Chapter 1. Briefly, fish were held at 15°C, fed to apparent satiation, survival was monitored for 14 days, and re-isolation of challenge bacteria was to be attempted from at least 20 percent of daily mortalities. Survival differences between temperature and control fish were assessed to determine any.

Thermal maxima

At the end of the 30 day temperature trial 18 fish from both the temperature treatment and control groups were held, separately, to test thermal maxima of each group. Water temperatures were increased each day from 27°C for 6 days until upper lethal temperature was reached. Tanks were frequently (~30 min) inspected during peak temperature each day for mortalities with time and temperature recorded for mortalities. Fish were fed to apparent satiation once daily prior to increasing the temperature.

Statistical analysis

Differences in survival in both the thermal challenge and bacterial challenge were analyzed using Kaplan-Meier non-parametric survival analysis. Response variables of weight, specific growth rate, feed conversion rate, Fulton's condition factor, and lipid and protein efficiencies were compared between treatment and control groups with paired T-tests. Percentage of lipid, protein, and total calories was analyzed for pre and post tissues using ANOVA followed by Tukey's honest significant difference test for mean comparisons. For

hsp70 expression and production a total experiment weighted average was created to account for equal contribution from each sample to the overall results. Variables measured over time (hsp70 expression and production) were analyzed using repeated-measures ANOVA of output (blot density values for Western Blots and C_p values for RT-qPCR) in the car package (Fox and Weisberg 2011) in R statistical programming language (R Core Team 2015). If the assumption of sphericity for repeated measures was not met ($P \leq 0.2$) then Greenhouse-Geisser corrections for departure from sphericity were used for significance tests. Most statistical analyses were conducted in R statistical programming language. Additionally, C_p values were also analyzed using software packages to compare normalized gene expression results (REST), and evaluate reference gene stability (*BestKeeper*). Statistical significance was pre-defined at $\alpha = 0.05$ and p-values are reported.

Results

Diel temperature trial

After fish were distributed to treatment tanks, tanks were gradually increased from 15°C to 18°C overnight. Control tanks were gradually lowered from 15°C to 14°C overnight. One temperature spike occurred on day 7 due to a chiller disruption. The control and treatment tank temperatures increased by 5°C during this period overnight of approximately 9 hours (Figure 3.2). Once the chiller turned on, the control tanks temperatures were lowered to 14–15°C where they remained for the remainder of the experiment. The treatment tanks were increased from an overnight low of 23°C to a normal peak of 27°C like the other days of the experiment; but the fish did not experience normal nighttime temperatures during the chiller outage. One fish died over the duration of the experiment, on day 23 in a treatment tank, but there were no significant differences in the main effect of survival between the treatments ($P = 0.32$) when analyzed using linear regression. The daily temperature units were computed for the duration of the experiment (Figure 3.3) and an increase in the graph from the chiller outage can be seen, but is still comparable to the field location temperature units. The experiment mimicked Deep Creek fairly well over the summer of 2015 with the MacArthur site reaching 28.5°C on June 28 and the IDL site reaching 27°C the same day. The main reason for the discrepancy in daily thermal units between the experiment and the field sites was because the field sites did not get as cool at night as the experiment did. The in-stream

daily highs were rarely above the experimental daily highs, but the nightly lows were often 3°C higher in-stream.

Growth

Sixty fish were sub sampled prior to the experiment for length and weight. Batched wet weight was measured ($\bar{x} = 24.9$), as were individual lengths, which varied from 120 mm to 177mm ($\bar{x}$ = 148.4 ± 1.78 mm). Day 15 fish weight averaged 30.2 ± 1.78 g with lengths varying from 128 mm to 192 mm ($\bar{x} = 160.7$ ± 2.92 mm). Feeding was increased for each tank from 1% bodyweight (BW)/day (11.5 g) to 1.2% BW/day (14 g) on day 7 and further increased (14.8 g) on day 15 based on weight gain until day 15. The increase in feed at day 7 was because fish were utilizing all feed initially, but after day 15 the treatment tanks utilized all feed approximately 50% of the time. On day 30, fish for hsp70 and proximate analysis were weighed and measured individually. All remaining fish, to be used in the bacterial challenge and thermal maxima trials, were batch weighed. Day 30 fish weight averaged 35.5 g across all groups with lengths, taken from fish for hsp70 and proximate analyses, varying from 132 mm to 202 mm ($\bar{x} = 170.9$ ± 2.06 mm).

Comparison of batched average weight/fish for control ($\bar{x} = 38.4$ g) and treatment ($\bar{x} = 32.6$ g) groups was significantly different at the end of the experiment (t = -3.27; df = 6; P = 0.02). Specific growth rate data indicated no significant differences between control ($\bar{x} =$ 1.04 ± 0.18) and treatment ($\bar{x} = 0.90$ ± 0.20) groups (t = 0.50; df = 58; P = 0.62). Feed conversion rate was measured per tank at the end of the experiment using feed consumption data for each tank. The FCR for the control ($\bar{x} = 0.68$ ± 0.08) tanks was significantly different from treatment ($\bar{x} = 1.33$ ± 0.22) tanks (t = -2.81; df = 4, P = 0.05). Fulton's condition factor varied for control fish from 0.60–1.05 ($\bar{x} = 0.71$ ± 0.02) and for treatment fish from 0.49–0.88 ($\bar{x} = 0.65$ ± 0.02). Fulton's condition factor (K) was significantly different between groups (t = 2.71; df = 58; P = 0.01).

Proximate analysis

Lipid concentration of both treatment ($P < 0.01$; $\bar{x} = 5.21$) and control ($P < 0.01$; $\bar{x} =$ 4.92) groups were significantly different from day 0 ($\bar{x} = 4.22$) fish, with day 0 fish having a lower percentage of lipid. Protein and kilocalorie data were square-root transformed to better meet the assumption of normality. Protein concentration for the treatment ($\bar{x} = 15.88$) group was significantly different from the controls ($P < 0.01$; $\bar{x} = 15.36$) and day 0 ($P < 0.01$; $\bar{x} =$

15.4) fish, but kilocalorie/g was not significantly different for any groups ($P > 0.5$). Analyses of lipid ($t = 0.11$; $df = 4$; $P = 0.92$) and protein ($t = -0.19$; $df = 4$; $P = 0.86$) efficiencies were not significantly different between treatment and control groups.

RT-qPCR

Extraction of RNA was attempted for all tissue types and individual samples. Samples that did not meet the 250 ng of total RNA needed following extraction were not utilized. RNA extraction was difficult for day 0 gill tissue, potentially because of small starting quantity of tissue. Muscle tissue was also difficult, even with a bead homogenizer, resulting in unequal sample sizes. Two samples from liver yielded low total RNA.

BestKeeper analysis of the reference genes *rplp1* and *ubiq* revealed both reference genes were equally stable and suitable when compared within tissue type (Table 1). However, when all tissues and both genes were used in the *BestKeeper* index it revealed that *rplp* was a less stable reference gene for gill tissue (Table 3.2) and that muscle was the most stable tissue (Table 3.2). The C_p values for reference genes (Figure 5) show consistent expression across treatments and tissue types. *BestKeeper* analysis also indicated that muscle tissue was the most stable for both reference genes (Table 3.2). The REST analysis of C_p values revealed similar patterns of *hsp70* expression when compared to both reference genes. All treatment tissues had significantly higher *hsp70* expression than controls at day 15 and day 30 when compared to both reference genes (Figure 3.6). At day 15 the increase in normalized *hsp70* expression over reference genes was highest in muscle tissue (252x times controls) and lowest in gill (12x). Gill and liver *hsp70* had lower gene expression and higher variability than muscle at day 15 compared to both reference genes (Figure 3.6), but all tissues have similar variability with high normalized gene expression (277–1131x) compared to reference genes at day 30. Gene expression for muscle *hsp70* is similar for both day 15 and day 30 but the variability at day 30 is smaller than day 15 compared to both reference genes.

Repeated measures ANOVA of raw C_p values revealed similar patterns for individual tissues and the overall weighted average. Liver ($P = 0.07$) and the weighted average ($P = 0.11$) did not meet the assumption of sphericity, but gill ($P = 0.81$), and muscle ($P = 0.80$) did meet the assumption of sphericity. In all analyses the main effects of treatment, time, and the treatment × time interaction were all significant (Table 3). The analysis indicates the C_p values were significantly different between the treatments, over time, and the treatment tissue

changed differently over time for all tissue types and for the overall experiment compared to controls (Figure 7). Generally, treatment and control groups had different expression of *hsp70*, and *hsp70* expression increased the longer Burbot were exposed to high diel temperatures. The exception to this would be muscle tissue, which had high expression at day 15 and similar expression at day 30, but this analysis also indicated significant effects of time, and the treatment × time interaction.

Western blots

The quantity of hsp70 per sample as estimated from band densities was significantly different for both liver and muscle tissue in treatment tanks compared to controls (Figure 8). The repeated measures ANOVA for liver and muscle tissues indicated a violation of the assumption of sphericity. The treatment effect was significantly different and Greenhouse-Geisser significance test corrections indicate time and the treatment × time interaction were not significant for liver and muscle tissue (Table 3). The overall analysis of both tissues did meet the assumption of sphericity, and the treatment effect was significantly different, but the time and treatment × time interaction were not significantly different between the treatments (Table 3; Figure 9).

Thermal maxima

The temperature cycles recorded for the 6 day extreme temperature trial were: 18-27.1; 18-28; 18-24; 18-28.7; 18-29.5; and 18-31.9°C (Figure 3.4). During day 3 there was a structural failure of a valve requiring all water to be shut off during repair. Water was turned off for ~6 hours and therefore a high temperature of only 24°C in both tanks was attained. The failed valve was repaired and the next day temperatures were resumed as originally anticipated. All fish died on day 6 of the extreme temperature trial with control fish dying at temperatures between 31.2–31.6°C and treatment fish dying between temperatures of 31.5–31.9°C.

Serum lysozyme analysis indicated no differences in treatment ($\bar{x} =$ -0.007) and control ($\bar{x} =$ -0.005) groups. Main effects of treatment, time, and an interaction of treatment × time were all not significant ($P \geq 0.12$) with the results suggesting no differences in innate immune function as measured by serum lysozyme activity.

Bacterial challenge

Only two fish died during the challenge, one from control (day 4) and one from treatment (day 9) tanks. There were no significant differences in survival between treatments.

Discussion

Lake origin Burbot were tested in a laboratory for responses to fluctuating diel temperatures. Only one fish died during the experiment, in a treatment tank, but prescribing the mortality to the diel temperatures seems unsupported as the temperature was well below the thermal maximum observed in the current study and others (Hoffman and Fisher 2002). Burbot were resilient to the diel temperature stress created by our experimental thermograph. Our results of thermal capacity and thermal maximum were similar to other studies on Burbot (Hofmann and Fisher 2002), but were conducted in a way to mimic a natural environment and also provide the physiological responses for a better understanding of Burbot physiology and thermal ecology.

Our results show that Burbot survived following repeated daily temperature fluctuations, but it appears the response to higher temperatures comes at a cost compared to controls. Fish in the temperature treatment often did not consume all the feed fed, even though the same quantity was offered as controls. Even with nightly thermal refuge, the exposure to high temperatures decreased the appetite of treatment fish similar to Burbot exposed to higher temperatures (16°C–24°C) (Hardewig et al. 2004); Dolly Varden *Salvelinus malma* and White-Spotted Char *Salvelinus leucomaenis* in increasing temperature (8°C–28°C) conditions (Takami et al. 1997); steelhead *Onchorhychus mykiss* in high temperature treatments (25°C; Kammerer and Heppell 2013b); and Bull Trout *Salvelinus confluentus* under thermal stress (>18°C; Selong et al. 2001). The lower feeding activity likely contributed to the differences in weight between the treatment and control groups with the treatment groups weighing significantly less at the end of the experiment, also similar to steelhead (Kammerer and Heppell 2013b). The weight data is confounded by the SGR data which indicate no significant differences between the groups. So while the control fish gained more weight, they did not do it at a significantly higher rate, similar to what Kjellman and Eloranta (2002) found for age 1 Burbot where growth rate and absolute growth was evaluated. The analysis of FCR and Fulton's K support the data from overall weight with the treatments having both elevated FCR

and lower Fulton's K at the end of the experiment than the controls. The FCR data is similar to steelhead trout exposed to thermal stress (Kammerer and Heppell 2013b), with higher values indicating less allometric growth per gram of feed, and the Fulton's K data supporting the finding. The SGR data is really the outlier in the growth analyses, possibly the rate was calculated across too short a time period to have much divergence in the groups and the daily differences were not much different but the additive result was total weight differences after 30 days. Burbot exposed to high diel temperatures have lower weight gain, elevated FCR, lower Fulton's K, but similar SGR compared to controls.

Proximate analysis of Burbot tissues produced some unexpected results. Whole body lipid concentration of control tissues being greater than day 0 fish is not unexpected. Whole body lipid concentrations of the treatment fish were not significantly different from the controls, but were significantly higher than the day 0 fish. This was unexpected and in contrast to other studies where lipid levels decreased with exposure to higher water temperatures (Kammerer and Heppell 2013a; 2013b). With exposure to the high daily temperatures and the apparent lower appetite of the treatment fish, we expected lipid concentrations to be lower in the treatment fish, but this result is similar to Bull Trout (Selong et al. 2001). The treatment fish had less total lipids, as they had significantly lower weight than the control fish, but similar lipids as a percentage of body composition. Whole body protein for the treatment group being significantly higher than the controls and day 0 fish coupled with lipid levels of treatment fish being similar to controls is confusing. It is likely that muscle and liver enzymes down regulate during high temperatures (Hardewig et al. 2004), and affected the metabolism of Burbot in the treatment tanks which allowed for the maintenance of proximate body composition. The lack of difference in total energy between treatment and control fish is confounded because the common thought is that fish use energy stores to get through stressful periods (Bonga 1997). For Burbot, who rely on coldwater habitat for much of their life history, the stressful period would seem to be summer. The gene expression data suggest the treatment was thermally stressful to juvenile Burbot and they responded by up-regulating *hsp70*, but there was still similar energy reserves per gram of tissue. Lipid and protein efficiencies were not different between groups suggesting that the weight gain (as a percentage) of lipid and protein for each group was similar. Protein and lipid efficiency results are different from Selong et al. (2001), who found decreased lipid and

protein efficiencies of Bull Trout exposed to temperatures above 18°C. Burbot exposed to high diel temperatures have higher protein concentration, similar levels of lipids (energy/g), similar protein efficiencies, and similar lipid efficiencies compared to fish in the controls.

The Atlantic Cod primers appear to work well for all three genes in Burbot. Our results are similar to observations by Olsvik et al. (2013), who also used Atlantic Cod primers for a Burbot gene expression study. Some samples did not provide adequate quantities of RNA needed for downstream applications and unequal sample sizes occurred for some tissues between sampling times. As was the case with Atlantic Cod (Aursnes et al. 2011), the reference genes *rplp1* and *ubiq* appear stable for Burbot across temperatures and tissue types (Figure 5). Across all tissues and both reference genes, it appears *rplp1* may be less stable for gill tissue compared to the others, but this should be biologically irrelevant given the C_p values were nearly identical between gill control and treatment for *rplp1* (Figure 5).

The pattern of expression variability of *hsp70* in gill and liver compared to reference genes was surprising. If anything, gill tissue seems the likely candidate to have high *hsp70* expression and low variability because of the direct exposure to the environment. However, muscle tissue showed this pattern more clearly; indicating high synthesis of hsp70 at an earlier time than gill or liver. There was a significant difference between treatment and control tissue at all time points but the day 15 differences in *hsp70* expression is considerably lower, with higher variability, than expression on day 30 for gill and liver compared to muscle (Figure 6). One potential explanation is the chiller failure on day 6. The increased temperature to the control tanks could have influenced gene expression results, but this failure was 8 days prior to tissue sampling, and likely had minimal influence on the results. Recovery would be expected in our situation following the results of Lund et al. (2003), who found the recovery of *hsp70* expression in Brook Trout *Salvelinus fontinalis* to take 24–48 hrs following acute thermal stress. Gene expression shows that by day 15 an average increase in *hsp70* gene expression occurs (12-251 times controls; Figure 6), but should be more pronounced over time (day 30: 277-1131x; Figure 6) for Burbot. Our results are in contrast to the results of Narum et al. (2010) who reported a spike in *hsp70* at day 1 then a decrease relative expression of *hsp70* in Redband Trout over a 28 day exposure to chronic thermal stress. The pattern seen in normalized gene expression data where gene expression of *hsp70* increased over time (Figure 6) is supported by the repeated measures ANOVA results. The main effects of

treatment, time, and the time × treatment interaction were all significantly different (Table 3). For all tissues and the experimental average, gene expression of *hsp70* for the treatment and control groups were different, they were different across time, and the effect of the treatment became more pronounced (higher *hsp70* expression) from the controls over time (Table 3; Figure 7).

The abundance of hsp70 over the duration of the experiment suggests a sublethal stress on Burbot, but it was different between liver and muscle tissue. Liver hsp70 increased substantially from day 0 to day 15, but then decreased considerably by day 30 (Figure 8). Muscle tissue showed a different pattern with hsp70 increasing at day 15 but increasing further by day 30 compared to day 0 and control tissues (Figure 8). The results are interesting but not altogether surprising given the different perfusion, clearance, and induction rates of different tissue types (Dyer et al. 1991; Dietz and Somero 1993; Williams et al. 1995; Williams et al. 1996), with liver being more perfuse and having faster clearance than muscle. The response of Atlantic Cod to increased temperatures has been shown to increase *hsp70* gene expression (Aursnes et al. 2011) without a measurable increase in hsp70 (Zakhartsev et al. 2005). We detected both an increase in gene expression of *hsp70* and increase in hsp70, but the accumulation of hsp70 in liver was highest at day 15 and was lower by day 30. Muscle hsp70 increased throughout the thermal stress.

The cellular stress response is specific to species, organ, and stressor, with other potential factors influencing hsp70 levels (Iwama et al. 2004) and *hsp70* gene expression. Olsvik et al. (2010) found increased *hsp70* expression in Burbot exposed to persistent organic pollutants. Exposure to other stressors has also altered hsp70 levels in fish including heavy metals (Sanders 1993), industrial effluents (Vijayan et al. 1997), herbicide (Hassanein et al. 1999), bacterial kidney disease (Forsyth et al. 1997), cold shock, air exposure, and food deprivation (Wang et al. 2013).

The live bacterial dose used to challenge fish in this trial was similar to other trials where this isolate was used for injection of challenge juvenile Bubot (Terrazas et al., unpublished data), which caused high mortality. The calculated LD_{50} for juvenile Burbot (2.4 g) was 1.2×10^5 CFU/fish, sixty times below the injection dose of this challenge (7.2×10^6 CFU/fish). With the additional thermal stress it was hypothesized that Burbot (30+ g) would also be susceptible to bacteria known to be pathogenic to earlier life stages of Burbot. It

appears that while the increase in temperature did decrease growth, and potentialy competitive ability, it did not decrease disease tolerance as suggested by Wedemeyer and McLeay (1981). The bacterial challenge coupled with the data from the serum lysozyme analysis suggests that no major differences in disease susceptibility or immune response exist between fish in the treatment and control groups. The results are contrary to our preliminary hypothesis of daily high temperatures having negative effects on both disease susceptibility and immune response. A possible factor contributing to the lack of differences between the groups is the duration of the stress event was not long enough or severe enough to elicit the hypothesized response. Alternatively, Burbot were likely large enough to have a more developed immune response, and thus susceptibility to the dose administered was low.

The thermal maxima trial showed the variability of different fish to withstand temperatures as high as 31.5°C. Results were generally similar to previous studies that attempted to define the thermal endpoint for Burbot (Hofmann and Fisher 2002). The method by which we conducted this trial provides better resolution of the upper lethal temperatures of juvenile Burbot. In previous studies, fish were removed following 10 minutes of stress (Hofmann and Fisher 2002). The present study also used daily variability similar to what would be expected in natural systems instead of slowly increasing temperatures until the fish become stressed. Some fish from the temperature treatment were not exhibiting equilibrium problems, or rapid ventilatory movements as signs of thermal stress (Baroudy and Elliott 1994; Elliott et al. 1994) at temperatures of 30°C, but most fish were actively swimming at temperatures above 28°C. The upper lethal temperature is one which would rarely be encountered for Burbot given their distribution or preferred microhabitats within streams and lakes. Our results show that individual fish are quite tolerant of high temperatures. This suggests that Burbot may acclimate to higher water temperatures as a result of climate uncertainty; however, the need for temperatures ≤ 5°C during spawning and egg incubation suggests that reproduction could be limiting.

The current study suggests that juvenile Burbot are able to survive and withstand summer stressors caused by high diel temperatures, but water temperatures already observed in natural systems induced a stress response in our trials. When compared to field sites where Burbot survival has been a concern, the thermograph did not exactly mimic the thermal units of the 2015 summer, which had an unusually warm June (NOAA 2015), and was a low water

year for the Northern Rocky Mountains (NRCS 2015). The experiment did reach higher daily temperatures than in Deep Creek on most days, but nightly temperatures were also lower than Deep Creek. Both sites did reach 27°C over the summer (June) with the MacArthur location reaching 28.5°C on June 28 and heating to over 25°C for a few days in August. To better mimic natural locations with high diel temperatures future studies could provide less nightly thermal refuge, and better mimic the natural thermograph. Additionally, there are likely areas of hyporheic exchange where fish can find thermal refuge even when temperatures reach peak levels for the day or season that would be hard to mimic in a laboratory setting. Gene expression of *hsp70* and hsp70 induction was significantly higher at both day 15 and day 30 in our study, suggesting a compensatory response to thermal stress. Now that *hsp70* and reference genes have been optimized from Atlantic Cod for Burbot, non-lethal tissues including fin rays and red blood cells may be useful for future use in gene expression studies of Burbot.

There are multiple costs associated with surviving increased temperatures for Burbot. There is lower growth seemingly because of a lower appetite and associated feeding behavior. Sublethal temperatures may have significant physiological effects including the increase in *hsp70* gene expression and hsp70 induction. The hsp70 mRNA levels in tissue provide a sensitive indicator of thermal stress in Burbot, as is the case with other fish (Currie et al. 2001; Lund et al. 2003; Aursnes et al. 2011). The use of Atlantic Cod primers for both target and reference genes appear to be appropriate and useful. Water temperatures that induce a stress response have already occurred over portions of the distribution of Burbot. In the future it is likely that aquatic thermal stress will increase for thermally sensitive species, such as Burbot, as a result of global climate change. The current study provides methodological and physiological information for continued monitoring of Burbot in regards to thermal stress.

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Table 3.1. Summary statistics for each reference gene, *BestKeeper* results of repeated pair-wise correlation analysis of gene specific stability for each reference gene, and gene specific *BestKeeper* index vs. each tissue type comparing expression stability. The shaded entries indicate tissue with high variability (standard deviation) and that all tissues have similar stability (p-value).

	rplp1				*ubiq*			
	Gill	Liver	Muscle	BestKeeper	Gill	Liver	Muscle	BestKeeper
n	59	71	59	72	59	71	59	72
geo Mean (C_p)	22.060	22.600	22.012	22.278	17.200	17.991	16.271	17.252
ar Mean (C_p)	22.078	22.626	22.053	22.287	17.213	18.015	16.390	17.269
min (C_p)	20.377	19.831	19.324	21.022	15.353	14.466	12.917	15.302
max (C_p)	24.109	26.153	24.713	23.658	19.561	21.549	19.335	18.744
std dev (± C_p)	0.751	0.865	1.197	0.556	0.554	0.648	1.859	0.630
CV (% C_p)	3.401	3.823	5.428	2.494	3.217	3.596	11.344	3.650
Repeated pair-wise correlation analysis								
vs.	Gill	Liver	-	-	Gill	Liver	-	-
Liver	0.252	-	-	-	0.161	-	-	-
p-value	0.0562	-	-	-	0.2276	-	-	-
Muscle	-0.377	-0.141	-	-	-0.159	-0.219	-	-
p-value	0.0076	0.2847	-	-	0.2769	0.0965	-	-
BestKeeper vs.								
Pearson cor (r)	0.49	0.629	0.566	-	0.295	0.253	0.846	-
(r^2)	0.24	0.396	0.32	-	0.087	0.064	0.716	-
p-value	0.0010	0.0010	0.0010	-	0.0234	0.0335	0.0010	-

Table 3.2. Summary of *BestKeeper* comparisons of all tissues and both reference genes, the result for *rplp1* gill is highlighted as it might be considered less stable than the other gene/tissue combinations, with muscle being the most stable.

Repeated pair-wise correlation analysis						
	rplp1			*ubiq*		
vs.	Gill	Liver	Muscle	Gill	Liver	Muscle
Liver	0.252	-	-	-	-	-
p-value	0.056	-	-	-	-	-
Muscle	-0.377	-0.141	-	-	-	-
p-value	0.008	0.285	-	-	-	-
Gill (*ubiq*)	0.579	0.119	-0.23	-	-	-
p-value	0.001	0.372	0.112	-	-	-
Liver (*ubiq*)	0.192	0.665	-51.981	0.161	-	-
p-value	0.150	0.000	0.000	0.228	-	-
Muscle (*ubiq*)	-0.488	-0.25	0.914	-0.159	-0.219	-
p-value	0.001	0.056	0.001	0.277	0.096	-
BestKeeper vs.						
Pearson cor (r)	0.172	0.332	0.772	0.314	0.343	0.743
(r^2)	0.03	0.11	0.596	0.099	0.118	0.552
p-value	0.192	0.005	0.001	0.015	0.003	0.001

Table 3.3. Summary of repeated measures ANOVA results for the gene expression and protein quantification analyses including results for significance tests of sphericity and fixed effects.

Analysis	Tissue	Test of Sphericity	Significance Tests		
			Treatment	Time	Trt × Time
RT-qPCR	Gill	0.811	< 0.001	< 0.001	< 0.001
	Liver	0.066	< 0.001	0.008	0.005
	Muscle	0.803	< 0.001	< 0.001	< 0.001
	All	0.110	< 0.001	0.002	0.001
Western Blot	Liver	0.02	0.008	0.061	0.089
	Muscle	0.358	0.037	0.075	0.134
	Both	0.358	0.038	0.075	0.134

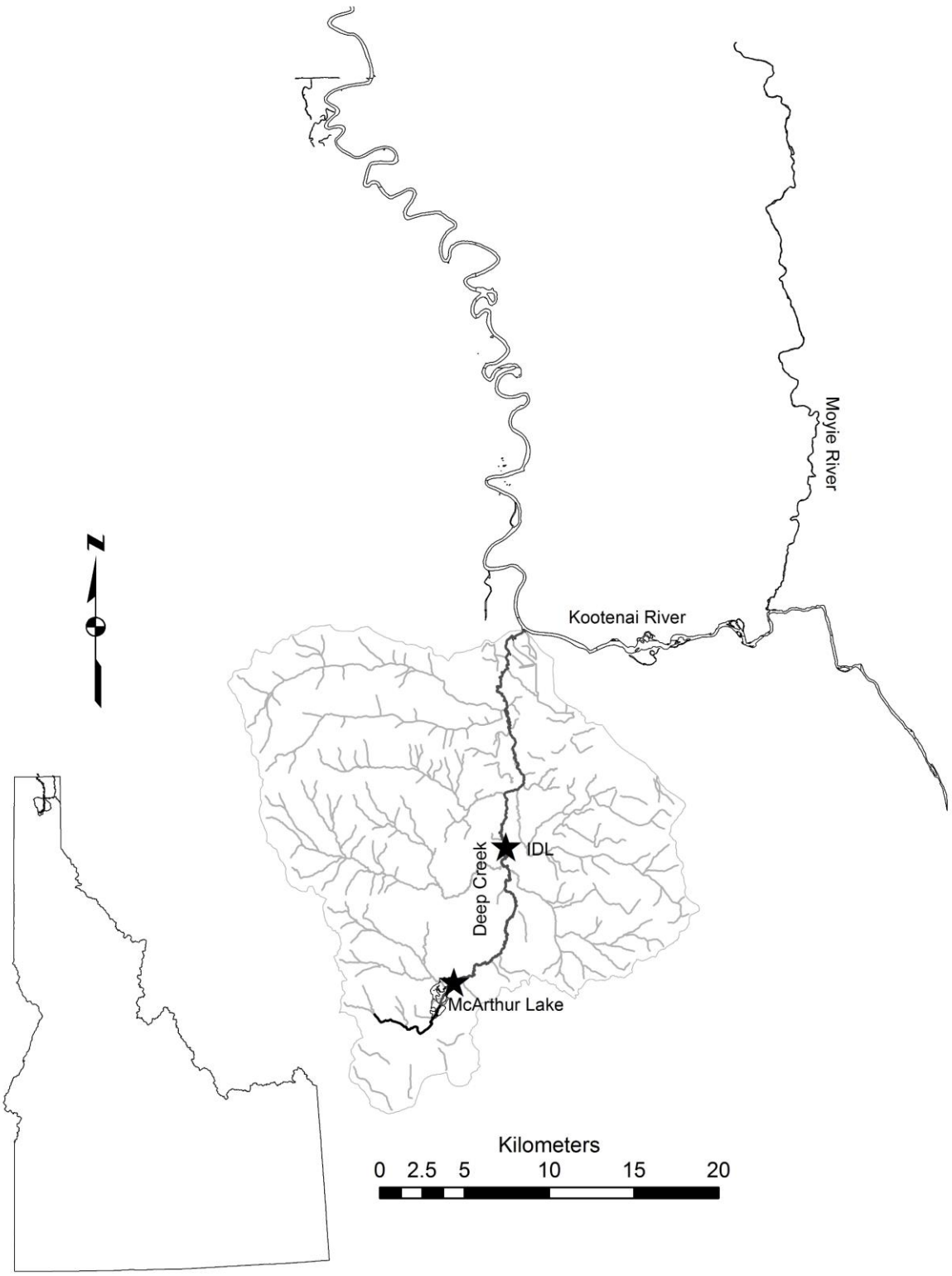

Figure 3.1. Map showing the Kootenai River drainage in northern Idaho including Deep Creek and temperature logging locations at IDL and McArthur Lake. Map courtesy of Zach Beard (University of Idaho).

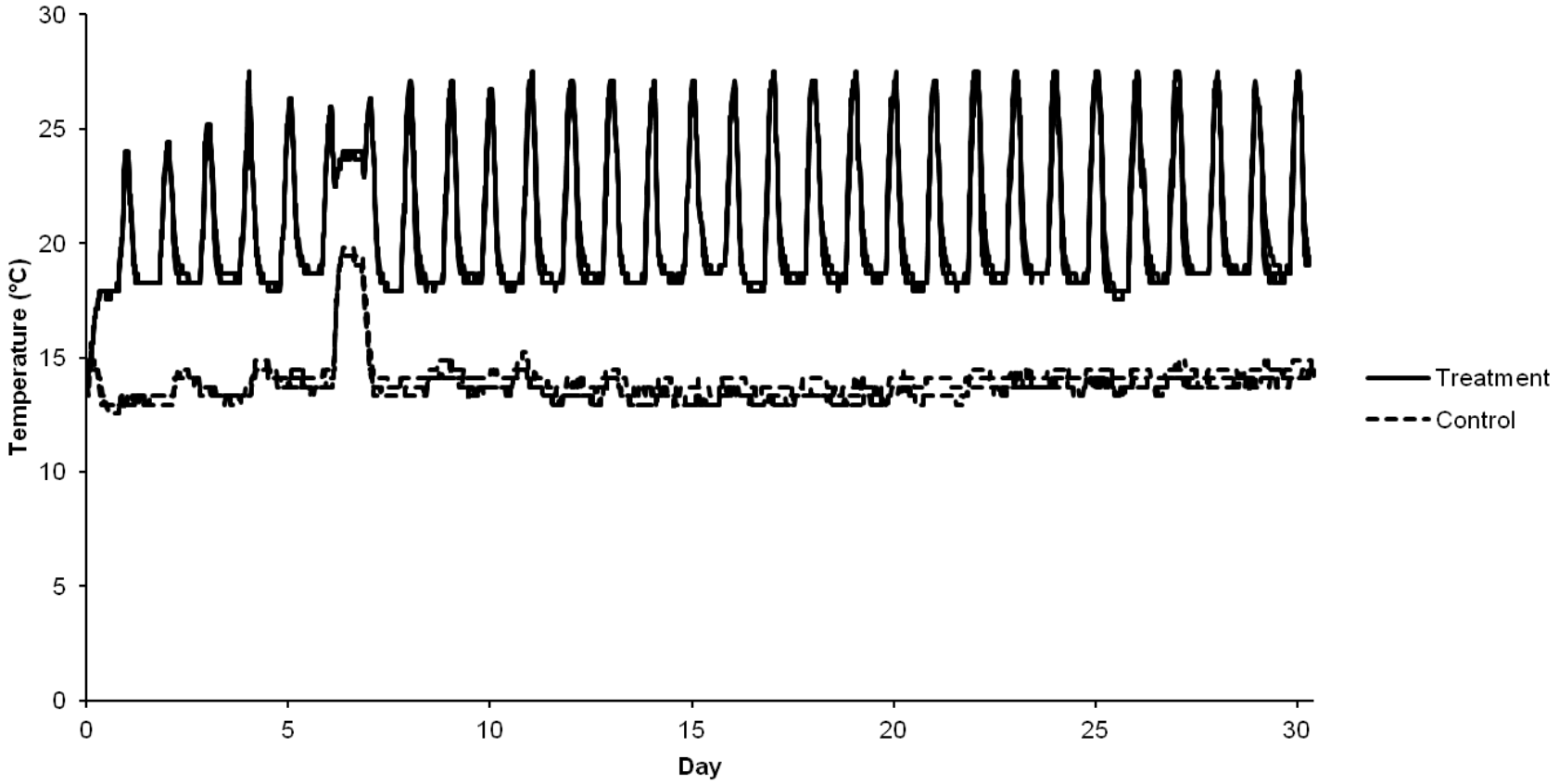

Figure 3.2. Temperature profiles for the 30 day experiment, note the temperature irregularity on day 6.

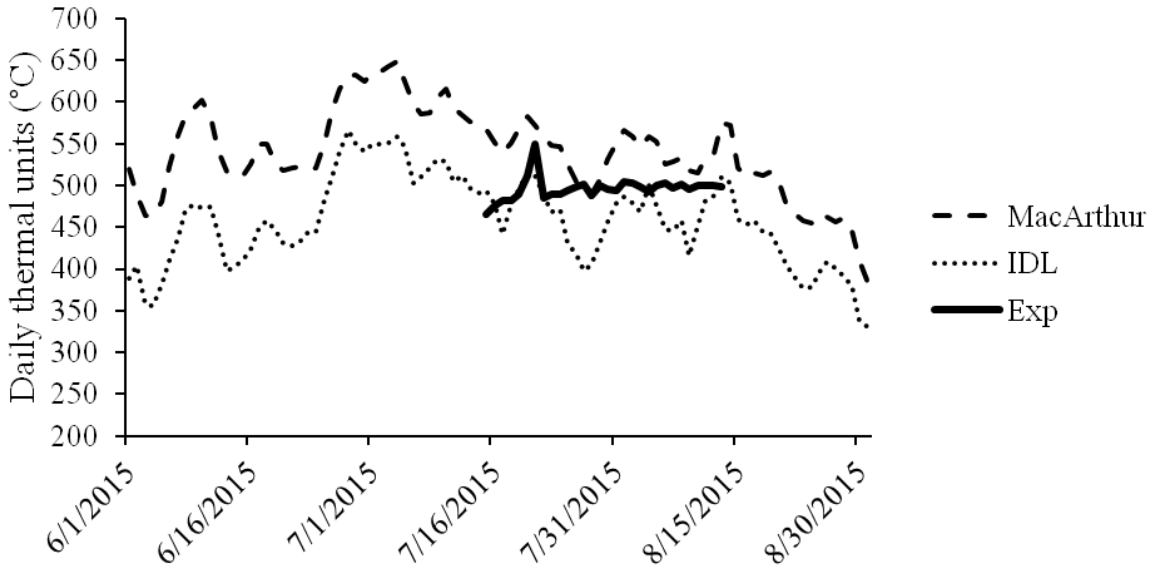

Figure 3.3. Daily thermal units for the experiment and two Deep Creek sites over the 2015 summer show similar daily thermographs for the field sites and the experiment.

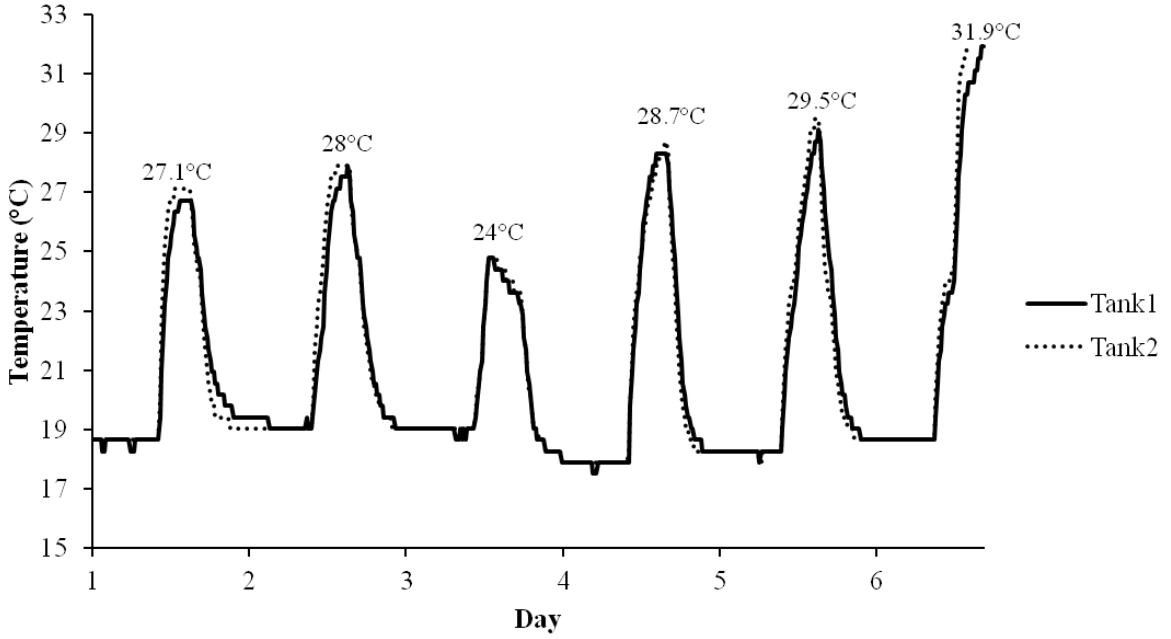

Figure 3.4. Temperatures for the 6 day extreme temperature trial. Day 3 is when the water was turned off for pipe repair and day 6 was the final day of the trial with thermal maxima attained for Burbot.

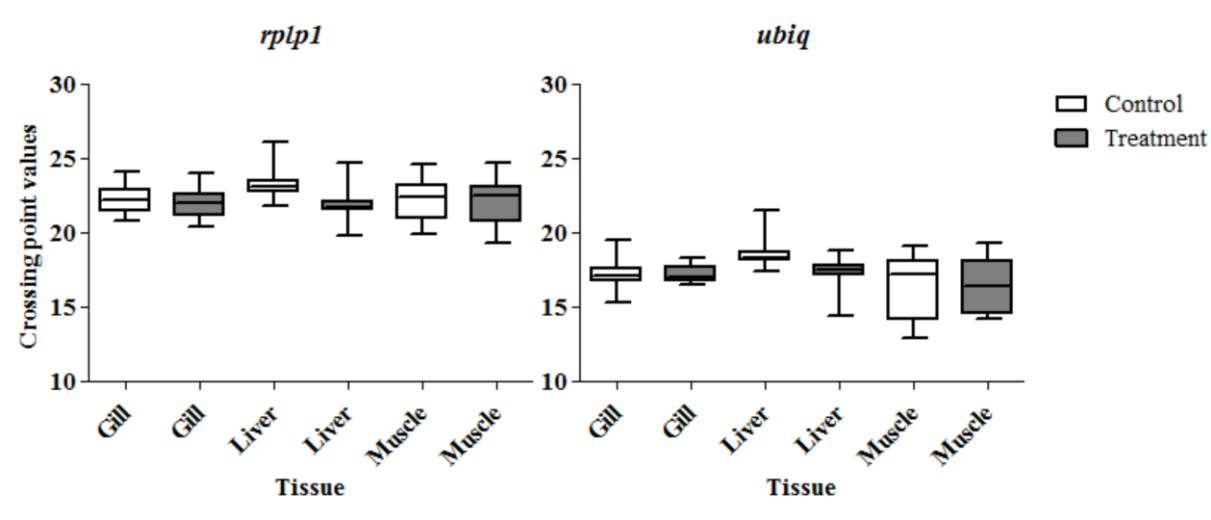

Figure 3.5. Crossing point values for all tissue and treatment combinations of reference genes *rplp1* and *ubiq* showing stable expression across tissue and treatment combinations.

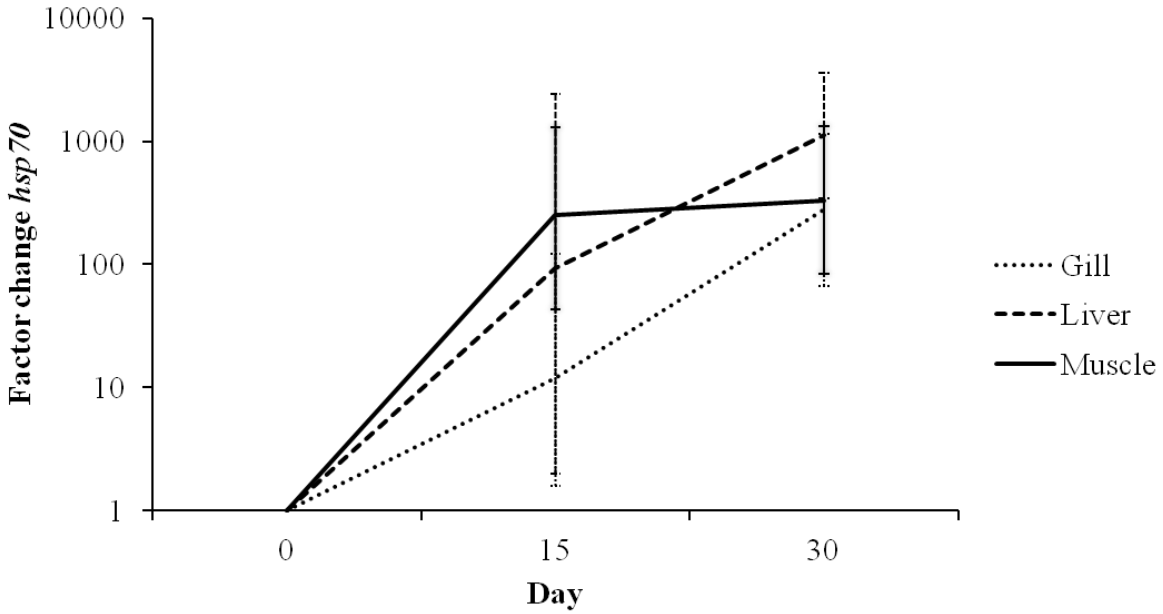

Figure 3.6. Factor change *hsp70* gene expression (± 95% C.I.) from REST analysis for all treatment tissues normalized against controls (set at 1) for both reference genes at day 15 and day 30. The graph shows increasing gene expression for gill and liver tissue from day 15 to day 30 and lower variability for all tissues at day 30 than day 15.

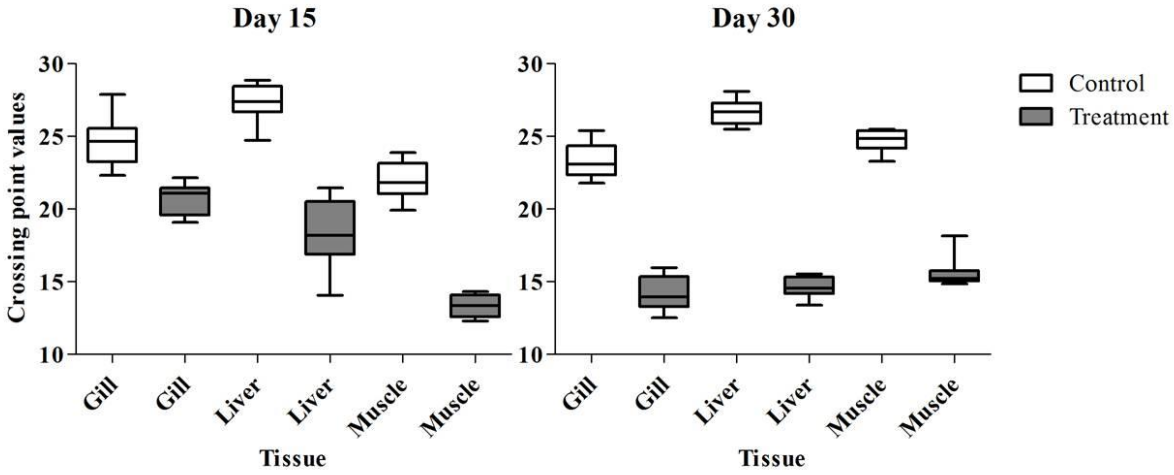

Figure 3.7. *hsp70* crossing point values for all tissue and treatment combinations of *hsp70* for day 15 and day 30. There is more separation between treatment and controls and less variability at day 30 than at day 15.

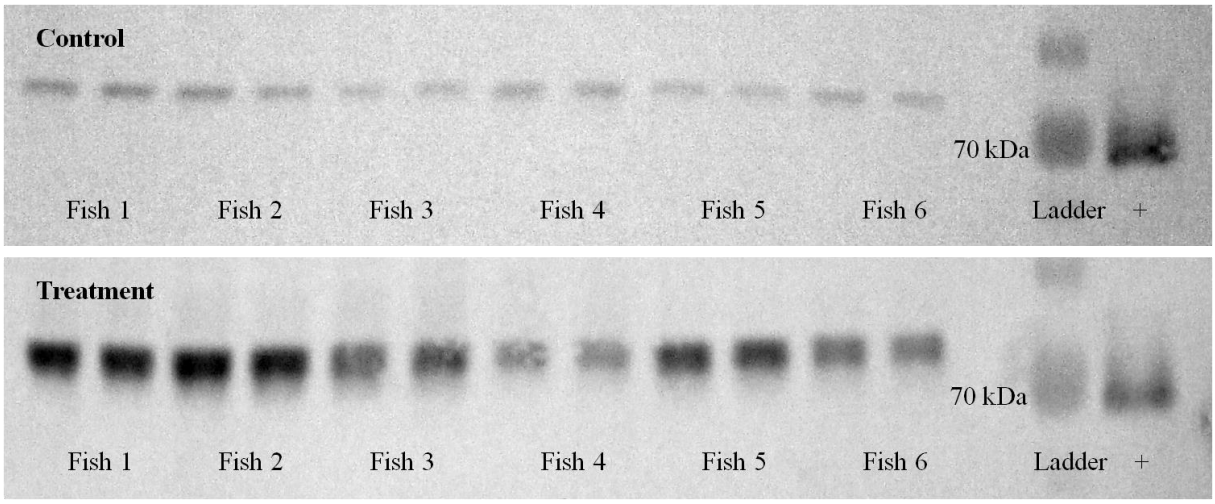

Figure 3.8. Western blot gel image shows the difference in band intensity between contol and treatment gels. Samples are run in duplicate wells with one empty well between fish 6 and the ladder and positive control bands. Image J software was used to analyze band density for each sample and allow for comparison to positive control band for each gel to compare to a known concentration of hsp70.

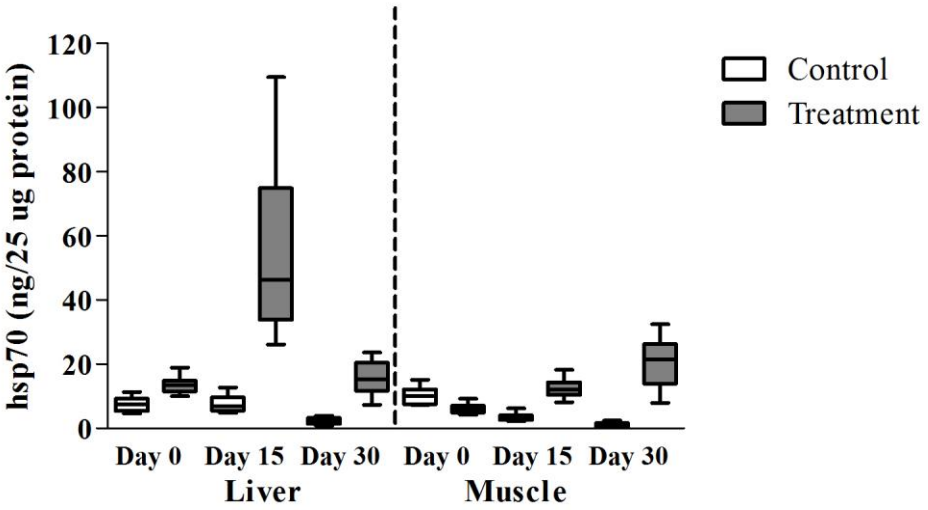

Figure 3.9. Results of the Western Blots showing hsp70 concentration for each tissue and treatment combination.

General Conclusions

The current studys identified two bacterial species, isolated during stress events and most closely related to *Aeromonas veroii*, as potential stress-mediated bacterial pathogens of Burbot. The pathogens are common Aeromonads found in water and associated with culture of other species of fish. There was differential mortality caused by the two *Aeromonas* sp. with one isolate in particular being very pathogenic to juvenile Burbot. The results suggest Burbot are among many species susceptible to *Aeromonas veronii* infections, similar to fish from Bangladesh (Rahman et al. 2002), snakehead *Ophiocephalus argus* (Zheng et al. 2012), in addition to Common Carp from Poland (Kozińska 2004), and the isolates identified as pathogenic to Burbot are potentially unidentified species. The inability to induce mortality with immersion treatment suggests other factors contributed to mortality of Burbot during the epizootics, similar to Mellergaard and Dalsgaard (1987), where our screening isolates were initially obtained.

The study also increased the knowledge of thermal tolerances and resiliency of Burbot and is the first study to outline the physiological response of Burbot to high temperatures. Our observed thermal maxima are similar to what Hofmann and Fisher (2002) determined to be critical thermal maxima for juvenile Burbot (around 31.5°C). The results outlined the gene expression of the critical thermal stress protein *hsp70* and this is one of few studies to use reference genes from Atlantic Cod with success in Burbot, another being Olsvik et al. (2013). We found gene expression and hsp70 production was increased in Burbot from the thermal treatments, indicating sub-lethal stress. We also found many growth parameters indicated inferior performance of Burbot in the thermal treatments compared to the controls. The proximate composition of Burbot exposed to high daily temperatures was similar to bull trout (Selong et al. 2001), and control fish, we think because of the ability of Burbot to down-regulate liver and muscle enzymes (Hardewig et al. 2004). This study also suggests that following thermal stress Burbot are immuno-competent enough to withstand a challenge from a known pathogenic isolate to smaller Burbot. Both the serum lysozyme and challenge results suggest that the immune response and competency following thermal challenge are not different than control fish.

The key findings of this research are that common bacterial species are very pathogenic to Burbot. The pathogenicity of these bacteria to Burbot is important for continued

population rehabilitation in the Kootenai River. Because the bacteria are common any captive rearing effort for Burbot should be aware of and try to mitigate any effects of these pathogens to Burbot. Burbot also show a robust response to repeated exposure to high water temperatures. In the event of changing climatic conditions across the current distribution of Burbot this is good because Burbot may persist in locations thought to be less than ideal based on temperature. Temperatures are expected to increase more at latitudes considered temperate and farther north or south. The changing of temperatures across the current distribution of Burbot will occur, and Burbot appear to have a resiliency to warm temperatures once hatched. As temperatures increase locations considered ideal for recruitment may shrink, or weather stochasicity may only allow for intermittent recruitment.

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Appendix 1. IACUC protocol 2013-97 approval.

University of Idaho
Institutional Animal Care and Use Committee

Date: Tuesday, September 15, 2015
To: Kenneth D. Cain
From: University of Idaho
Institutional Animal Care and Use Committee
Re: Protocol 2013-97
Development of conservation aquaculture techniques for Burbot: continued maintenance of broodstock and progeny

Your requested renewal of the animal care and use protocol shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Tuesday, September 15, 2015.

This protocol was originally submitted for review on: Tuesday, October 15, 2013
The original approval date for this protocol is: Friday, January 31, 2014
This approval will remain in effect until: Thursday, September 15, 2016
The protocol may be continued by annual updates until: Tuesday, January 31, 2017

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Barrie Robison, IACUC Chair

Appendix 2. IACUC protocol 2014-88 approval.

University of Idaho
Institutional Animal Care and Use Committee

Date: Tuesday, September 15, 2015
To: Kenneth D. Cain
From: University of Idaho
Institutional Animal Care and Use Committee
Re: Protocol 2014-88
Establishing Stress Mediated Disease Susceptibility Risks for Burbot

Your requested renewal of the animal care and use protocol shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Tuesday, September 15, 2015.

This protocol was originally submitted for review on: Wednesday, September 24, 2014
The original approval date for this protocol is: Wednesday, November 12, 2014
This approval will remain in effect until: Thursday, September 15, 2016
The protocol may be continued by annual updates until: Sunday, November 12, 2017

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Barrie Robison, IACUC Chair