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Effects of a simulated Klamath River summer temperature profile on juvenile Chinook salmon (*Oncorhynchus tshawytscha*) immune function.

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Summary:

Juvenile Klamath River Chinook salmon were reared for 21 days under two fluctuating temperature profiles (mean daily temperature (MDT) of 18° and 23° C) reflective of the lower Klamath R. during smolt migration. Immune function (*Vibrio (Listonella) anguillarum* challenge of fish vaccinated against the bacteria, plasma protein analysis, plasma complement and lysozyme activity, leukocyte profile and phagocyte activity, and spleen cell cytokine gene expression) and physiological measurements (growth, plasma cortisol, and gill Na⁺-K⁺-ATPase activity) were monitored over the experiment. As evident by low cortisol levels and positive growth, salmon adapted to the 23° MDT laboratory conditions. No overt effect of elevated temperature on immune function or fitness was detected in the salmon. While cytokine gene expression was consistently lower in 23°MDT fish, it was not associated with impaired performance in the *V. anguillarum* challenges. Survival after challenge was variable among the temperature groups while complement activity, plasma proteins, and both immune cell profile and function were similar. Based on these results, disease occurrence in the late spring and summer out-migration period is likely to be influenced by pathogen replication rate at elevated temperatures and infectious dose, rather than host immunosuppression. Further work is needed to identify physiological thresholds in both elevated water temperature and exposure duration that negatively influence fitness.

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Introduction

Juvenile Fall-run Chinook salmon (*Oncorhynchus tshawytscha*), are present in the lower Klamath River from their emergence as fry in January through their smolt emigration between May and August. Movement of salmon into the Klamath estuary has been reported to range from late June through August (Wallace and Collins 1997). Similarly, peak emigration for natural juvenile Chinook past Big Bar (rkm 80) occurred in late June 1996 (USFWS 1999). During late June to August, juveniles encounter mean water temperatures of 20 – 22.5°C with summer maxima of 26.6 °C (Bartholow 2005). Iron Gate Hatchery Chinook salmon smolts are released at the limit of anadromous fish range (rkm 307) and are reported to have median travel times of 30 -34 days (California Department of Fish and Game 2003). Sutton et al. (2007) describe the use of thermal refugia by juvenile Klamath R. salmon. They report that fish would utilize Beaver creek refugia when the river temperature reached 22 - 23°C. Given the migration timing and water temperature profile, juvenile Klamath R. Chinook salmon must rear and migrate in water temperatures in excess of 21°C for as long as 3 to 6 weeks. The incidence of parasitic disease can be high for juvenile Klamath salmon in the spring – summer period (Nichols et al. 2009, True et al. 2010). Temperature is a physiological stressor for fish and can induce immunosuppression. When challenged with a pathogen, this stressor may tip the balance between a protective immune response and succumbing to disease. Our goal in this study was to determine if elevated water temperatures induce immunosuppression and therefore increase disease incidence in juvenile Klamath River Chinook salmon.

An acute lethal water temperature threshold for juvenile Chinook salmon has been estimated at approximately 25°C (Brett 1952, Orsi 1971). This data was generated using the upper incipient lethal temperature (UILT) method with an acclimation temperature of 20 - 21°C (Fry et al. 1942). Baker et al. (1995) used coded wire tag recovery data in the Sacramento – San Joaquin delta to estimate an UILT of 23°C for juvenile Chinook. It is unclear whether this observation was a direct or indirect (i.e. increase predation) effect of elevated water temperatures. In contrast to the UILT data, Marine and Cech (2004) report that juvenile Fall Chinook salmon could survive and grow at 21 - 24°C (mean 22.3°C) when offered 60 – 80% satiation rations. Elevated water temperature will also induce sub-lethal physiological responses that could reduce smolt survival. Elevated temperature has been reported to impair smoltification changes such as increased gill Na-K-ATPase activity (Zaugg 1981, Duston et al. 1991). U.S. EPA Region 10 Guidance for Pacific NW State and Tribal Temperature Water Quality Standards (2003) describes constant water temperatures of 18 - 20°C as posing a “high” disease risk to juvenile salmonids. Therefore maximum 7 day average of daily maximums (7DADM) for migrating juvenile salmonids was set at 18 °C. Based on the aforementioned data, juvenile salmon in the Klamath R. are exposed to water temperatures near or at their reported thermal limits.

In the last several decades, temperature studies have used diel fluctuation profiles, representative of actual stream conditions, in comparison to chronic single temperature exposure (Dickerson and Vinyard 1999, Geist et al. 2010, Thomas et al. 1986). Diel fluctuation provide fish a daily minima respite as well as a limited daily maxima. These

studies report higher survival in fluctuating regimes incorporating UILT maxima compared to similar experimental groups held a constant but similar mean daily temperature.

Fish have both specific (immunoglobulin and cytotoxic T lymphocytes) and innate immune mechanisms to resist pathogen infection. Cellular components of the innate system include phagocytes such as macrophages and neutrophils while humoral elements include lysozyme, complement, interferon, C-reactive protein, transferrin and multiple lectins (Yano 1996). Cytokines are immune function regulatory proteins that can have pro-inflammatory, antiviral, or regulatory activities in fish (Secombes et al. 2009). Infection and stress influence cytokine gene expression in fish (Raida et al. 2008, Castillo et al. 2009). Lysozyme is produced primarily by macrophages and has bactericidal properties through its hydrolysis of bacterial peptidoglycans, opsonin function for phagocytes, and ability to activate the complement system (Paulsen et al. 2003). Mock and Peters (1990) report significant declines of blood lysozyme levels in chronically stressed trout. Fish have complement protein components similar to higher vertebrates that are essential to microbial killing via innate and specific pathways (Holland and Lambris 2002). There are 2 convergent pathways of complement activation: immunoglobulin-dependent requiring both Ca^{2+} and Mg^{2+} (classical) and immunoglobulin-independent requiring only Mg^{2+} (alternative). The alternative complement activity is measured as ACH50 units in a hemolytic assay (Collazos et al. 1994).

The specific immune response in this study was evaluated through a vaccination and challenge protocol. Fish vaccination is routinely performed via an immersion or injection technique however immersion is typically chosen for large numbers of smaller fish (Johnson et al. 1982). Intra-peritoneal injection of known quantities of bacteria has been reported to be the most reliable challenge method in fish immune system studies (Nordmo et al. 1997). Both innate and specific immune functions are involved in resistance to bacterial infection. *Vibrio (Listonella) anguillarum* was chosen as a challenge pathogen given its significance to salmon mortality in saltwater environments, unlikely exposure of our freshwater hatchery salmon to this pathogen (novel antigen), availability of commercial immersion vaccine, and phenotypic characteristics in culture that allow for rapid identification (Actis et al. 1999). Survival after bacterial challenge was also chosen as an overall performance measure as single immune function changes may not correspond to host immune performance. As an example, phagocyte activity does not always correlate to disease resistance. Aoshima et al. (2005) report heightened phagocytosis, reactive oxygen species production, and bacteriocidal capacity in rainbow trout during temperature stress, however, bacterial challenge still resulted in high mortality.

Qualitative evaluation of leukocyte profile and function, from both lymphoid organs and peripheral blood, can provide information on the fish's immune status. Differential leukocyte counts can demonstrate either granulocyte increase associated with infectious disease or lymphopenia observed in fish exposed to pollutants (Blaxhall and Daisley 1973, Dixon and Pick 1985, McLeay 1975). Respiratory burst activity, the

production of reactive oxygen intermediates such as H_2O_2 , O_2^- , and OH^- within the phagocyte, is an important feature of microbial killing by phagocytes (Chettri et al. 2010). Similarly, the ability of phagocytic cells to engulf foreign targets, such as opsonized particles, is another key measurement of their involvement in microbial defense (Solem et al. 1995). Chinook salmon plasma is reported to contain a 65 kDa albumin that has anionic migration in agarose electrophoresis, binds the fatty acid palmitate and is at a concentration of 15 mg/mL (Metcalf et al. 1998). Similarly Xu and Ding (2005) purified and describe an albumin protein from Atlantic salmon plasma. Globular plasma proteins such as immunoglobulin, complement components, alpha-2 macroglobulin, transferrin, C-reactive protein, ceruloplasmin, and fibrinogen function in immune response to infection and injury. An Albumin : Globulin ratio below 1.0 can indicate chronic infection or liver (Silverman and Christenson 1994). Temperature can also influence the production of globular proteins in fish. Meisner and Hickman (1962) report that the A/G ratio of rainbow trout sera was greater in fish reared at 8°C than 16°C. Foott et al. (2004) did not observe a difference in either plasma protein levels or hemolytic activity (alternative complement) of juvenile Klamath R. salmon reared at 16°C compared to 20°C for one week. Whereas plasma lysozyme and kidney phagocyte (MTT bactericidal assay) activity was markedly elevated in the 20°C fish in this study.

Snieszko (1974) discussed the interaction of infectious disease with virulent pathogens and stressful environmental conditions. Virulence of some fish bacterial and parasitic diseases has a positive relationship with water temperature (Groberg et al. 1978, Holt et al. 1975, Udey et al. 1975). Elevated water temperatures will tend to favor microbial replication rates over host defenses. A fish's immunodefense against infectious disease is influenced by the duration and magnitude of its stress response (Sink and Strange 2004, Houghton and Matthews 1990, Small et al. 2008, Pickering et al. 1989). Maule et al. (1989) described the dynamics of the stress response and immune system in juvenile Chinook. The authors report a reduction, in both antibody-producing cell (APC) response and survival following bacterial challenge, in fish 4h after an acute stressor. At 24h post-stress, both plasma cortisol and APC response was similar to controls and challenge survival was enhanced. Tripp et al. (1987) report that suppressed lymphokine production is associated with this stress-mediated APC impairment. Later work by this group showed how leukocyte numbers in different organs and peripheral blood varied after acute stress (Maule and Schreck 1990). Ruis and Bayne (1997) determined that acute stress can stimulate trout opsin-mediated phagocytosis. They postulate acute phase proteins, cortisol, and catecholamines were associated with this response. Chronic stress and the associated elevated blood cortisol induced lymphopenia and an increase of both immature and mature neutrophils in carp (Wojtaszek 2002). The mechanism for lymphopenia is through specific leukocyte mortality. Verburg-van Kemenade et al. (1999) and Weyts et al. (1998) report that cortisol stimulates carp lymphocyte apoptosis while inhibiting a similar apoptotic responses in carp neutrophils. The immune system response to environmental conditions is dynamic and difficult to characterize as a whole until significant changes occur to the host.

In this report we address 3 questions:

- 1) Will a high summer temperature profile, representative of the lower Klamath River, result in reduced innate or specific immune functions in juvenile Klamath River Chinook salmon ?
- 2) Is there a duration threshold for such immune function changes over a 3 week period?
- 3) Does a chronic stress response, as indicated by elevated plasma cortisol, correspond to any immune function changes?

This experiment measured the survival of immunized smolts reared under 2 temperature profiles and later challenged by bacteria. Physiological parameters such as plasma cortisol, blood cell counts, enzyme activity and cytokine gene expression were compared between fish subjected to the described immunization and infection trial under two temperature regimes. We hypothesize that immunosuppressed fish would succumb to bacterial infection whereas fish with a normal functioning immune system would survive due to the immune protection induced by the vaccine.

Methods:

Temperature system - Experimental (23° C mean daily temperature, **MDT**) and control (18° C MDT) salmon were each reared in separate systems that consisted of four replicate, 352L circular tanks that received 7.6 L/min of ozone-treated Coleman National Fish Hatchery water. Each system was supplied water from a temperature blending valve regulated by a Fuji Electric Systems Proportional Integral Derivative digital controller (model PXG, Temperature Blending Unit designed and built by Mike Hosford, Aquaculture Solutions, Klamath Falls, OR, see photo on report cover). Each controller had access to ambient hatchery water, chilled water (14°C) and heated water (28° C) at equal pressure (25psi). Hot and cold temperatures were achieved by passing water through a commercial pool-heater or three in-series chillers (3hp, 4hp, and 10hp) with each temperature system fed into separate 227,400 L thermal reservoirs. Water was pumped from the reservoir and supplied to each controller. Water not used by the controller was re-circulated back into the hot or cold system. Ambient water was also supplied to the controllers by a Variable Frequency Drive (VFD) pump at equal pressure. Appropriate temperatures were achieved by an in-line sensor constantly relaying the blended water temperature to the controller. The controller constantly calculated the relationship of the valve position to the blended water temperature and adjusted the valve to achieve the set point temperatures. Programming temperature profiles into each controller required only two parameters: required temperature and duration (in hours). Temperature profiles required a minimum temperature, a maximum temperature, and the duration times to hold temperatures (soak) or to reach highs/lows (ramp).

Table 1. Settings for 18CMDT (control) and 23MDT (heated) controllers.

Control System	Heated System	Time/Action
16°C	22°C	8 hour soak
19°C	25°C	4 hour ramp
19°C	25°C	5 hour soak
16°C	22°C	7 hour ramp

Fish culture - Juvenile Chinook salmon (1500 fish at an average of 1.3g, 50 mm FL), Klamath River origin from the California Department of Fish and Game's Iron Gate Hatchery, were transported to the wet lab on April 23, 2011. Groups of 380 salmon were reared in four 352L circular tanks at ambient temperatures (12 - 14°C) and fed 1.0mm Silvercup salmon diet at 4% bodyweight / d. Growth rate over a 2 month period averaged 0.55mm / d. To establish protective immunity to *Vibrio anguillarum*, nine hundred and sixty salmon were netted from the population tanks, vaccinated, and distributed as groups of 120 fish to 352L circular tanks. On June 21, vaccination occurred in groups of 60, by a 40 s immersion in *Vibrio anguillarum* vaccine (10x dilution in tank water of killed bacterin custom prepared by AquaLife Veterinary Services, Kirkland, WA, lot CALI04021810V_a12/13V_o6/7012). Each tank was subsequently loaded with 120 vaccinated salmon. Due to space and water treatment limitations, unvaccinated fish were not part of the study. Between June 21 and June 24, temperature was raised for all fish from an ambient 15.1 °C to a constant 18°C. In the experimental tanks (23°MDT), temperature was then raised to a constant 19°C for 3d and then to 20°C for one day prior to the beginning of diurnal profiles. Control fish (18°MDT) remained at a constant 18°C until initiation of diurnal profiles on June 29. An error with temperature ramping occurred on the morning of June 29 approximately 45min prior to the collection of time zero samples in the 23°MDT group and 2 h prior to 18°MDT group sampling (Figure 1). Temperature increased 3.3 to 3.6°C within an hour to the groups' maximum daily temperature instead of slowly ramping to maximum temperature in 6 hours. The following day another attempt to match our planned profile was made at 6 am but was canceled in 2 h as ramping did not occur in the controller program. Maximum temperatures were initiated at 10 am that same day and the diurnal pattern was maintained for the rest of the 3 week experiment (Figure 1). Salmon were fed at 2% bodyweight / d, to approximate the feeding rate of juvenile salmon in coastal waters, with a belt feeder during the 3 week experiment (Benkwitt et al. 2009). Hourly temperature measurements were recorded with Onset temperature probes and dissolved oxygen was measured twice per week with a HACH LDO oxygen meter.

Sampling – One 18° and 23°MDT tank was sampled weekly (selected by random number generator and referred to as “tank population” (in comparison to 24h post-*Vibrio* challenge fish) beginning at time zero (June 29, 8 days post vaccination). The first 8 fish netted from a tank were rapidly euthanized in an overdose of benzocaine, bled from the caudal vessels into heparin-coated natelson tubes and expressed into microcentrifuge tubes, plasma collected by centrifugation for cortisol (5µL) and bacteriocidal activity (10µL). Weight (0.001 g) and fork length (mm) was measured from the next 20 fish and condition factor calculated as $KFL = (\text{weight} / \text{fork length}^3) \times 10^5$

(Anderson and Gutreuter 1983). Plasma and selected tissues were collected from 44 fish per group. Tissue samples included: blood for differential leukocyte counts, gill for Na-K-ATPase assay, anterior kidney for NBT and phagocytic assays, and spleen for cytokine gene expression. The remaining fish were used for the *Vibrio anguillarum* challenges.

Bacterial challenge – Four challenges were performed on a weekly basis with the “time zero” challenge occurring 8 days post-vaccination on 29 June (start of diurnal temperature profiles). A challenge suspension of *Vibrio (Listonella) anguillarum* (Va32 isolate, Fish Pathology laboratory, University of California, Davis) was prepared from a 18 h Brain Heart Infusion broth (BHIB) culture that was centrifuged (1400 x g x 10 min, 10°C), re-suspended to original volume in BHIB, the suspension’s concentration estimated by absorbance (OD_{600nm} of 0.8 – 0.9 was equivalent to 10⁹ cfu/mL), a plate count performed, and 10⁻⁴ dilution prepared in BHIB. Previous work had shown that the LD₅₀ dosage for juvenile Chinook to be between 10⁴ and 10⁵ cells/ mL. For each challenge, duplicate 15 fish groups from the 18° and 23°MDT treatments were lightly anesthetized with MS222, given a 0.1 mL intra-peritoneal injection of a 10⁵ *V. anguillarum* / mL and held for 7 d in 18°C flow-through 40L aquaria. One additional aquaria of 10 – 12 challenged fish per group was sampled at 24 h post-injection (24 hpi) for plasma protein and lysozyme activity as well as spleen tissue (cytokine gene expression). Mortalities were monitored twice daily and kidney tissue from the first 5 “fresh” mortalities inoculated onto BHI agar. *V. anguillarum* isolation was determined by the following criteria: gram-negative motile rod, cytochrome oxidase positive, sensitivity to 0.1% vibriostatic agent O/ 129 disks (2, 4-diamino-6,7-diisopropylpteridine phosphate).

Gill ATPase activity - Gill Na⁺, K⁺-Adenosine Triphosphatase activity (ATPase) was assayed by the method of McCormick (1993). Briefly, gill lamellae were dissected and frozen in sucrose-EDTA-Imidazole (SEI) buffer on dry ice. The sample was later homogenized, centrifuged and the pellet sonicated prior to the assay. ATPase activity was determined by the decrease in optical density (340 nm) over time as NADH is converted to NAD⁺. This activity was reported as μmol ADP/mg protein/hour as there is a 1:1 relationship between NAD produced and ADP generated in the reaction. Data analysis was performed by ANOVA and Tukey’s multiple comparison test.

Differential blood leukocyte count- Blood was diluted 20x in Leibovitz (L-15) 1% Chinook plasma (0.2μm filtered), centrifuged onto glass slides (Statspin Cytofuge, Statspin Inc. Norwood MA), fixed in absolute methanol, and stained with Leishman – Giemsa. A differential leukocyte count performed (number of lymphocytes, thrombocytes, monocytes / macrophage, and neutrophils observed in the first 100 leukocytes (Yasutake and Wales 1993).

Total protein, albumin, lysozyme activity- Upon thawing, lysozyme activity of the plasma (mOD / min / mL) was assayed from single 10 μL samples by a microplate turbidimetric method (Ellis 1990). Briefly, sample and hen egg white lysozyme standards (0,5,10,15 μg/mL in 0.04M phosphate buffer, pH 6.2) were added to a 96 well

ELISA plate followed by 200 μ L of a 0.25 mg / mL suspension of freeze-dried *Micrococcus lysodeikticus* in 0.02M acetate buffer (pH 5.5). The decrease in absorption (450nm, 25°C) was immediately measured in a microplate reader at 30 s intervals for 10 min. and the maximum velocity of 15 consecutive measurements recorded (mOD / min). Subsequently, duplicate plasma samples from the same fish (2 μ L albumin and 4 μ L protein) were assayed on 96 well microplates (NUNC ELISA) using Pointe Scientific chemistry kits (Pointe Scientific Inc, Canton MI 48188). The albumin kit used bromocresol green and protein was assayed by the Biuret color reaction. The Albumin: globulin ratio (A/G) was calculated as (Albumin (mg/mL) / [protein (mg/mL) – albumin]). Bovine serum albumin was used as the standard in both assays.

Plasma cortisol – Plasma was collected from the first 8 fish captured on each sample day and immediately euthanized by an overdose in benzocaine. A 5 microliter aliquot was frozen on dry ice and stored at -70°C until assayed with an EIA kit (Cortisol EIA #402710, Neogen Corp., 628 Winchester Road, Lexington Kentucky 40505). Duplicate samples of a 100x dilution were tested from each fish and reported as ng Cortisol/ mL plasma.

Alternative complement pathway activity – The activity of the alternative pathway (ACH50) was determined with rabbit erythrocytes (RRBC) in the presence of the Ca²⁺ blocker ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma #E-0396) and magnesium using the method of Alcorn et al. (2002). Briefly, GVB-EGTA-Mg buffer was produced by adding 1 part 0.1 M EGTA and 0.1M MgCl₂ to 9 parts gelatin veronal buffer (GVB, Sigma# G6514; 0.15 mM CaCl₂, 141 mM NaCl, 0.5 mM MgCl₂, 0.1% gelatin, 1.8 mM sodium barbital and 3.1 mM barbituric acid, pH 7.4.). The presence of 0.15mM calcium in the GVB was considered inconsequential to the ACH pathway as the buffer also contained 0.01M EGTA. Rabbit RBC (RRBC, Innovation Research, Fisher Scientific) was washed 3x in GVB-EGTA-Mg buffer (5,000xg, 10min, 4°C) and the pellet re-suspended to approximately 1% solution in the buffer. The RRBC concentration was standardized as follows: 1.5mL of distilled water (4°C) was added to 0.5mL of the RRBC suspension mixed for 1 min, 3mL GVB-EGTA-Mg buffer added, and the OD₅₄₀ determined by spectrophotometry. The original RRBC suspension was adjusted so that the OD₅₄₀ was 0.370.

Each 25 μ L plasma sample was defrosted and immediately diluted with 745 μ L cold GVB-EGTA-Mg buffer (20x dilution) and in 2x serial dilutions (40,80,160x). Duplicate wells were filled with 100 μ L of each dilution followed by 100 μ L of 1%RRBC. GVB-EGTA-Mg buffer was added to one 20x well to serve as a plasma background control. The reaction occurred for 60 min at 15°C and the plates were then centrifuged at 1000 x g, 10 min, 4°C. Two hundred microliters of the supernatant was transferred to another ELISA plate and the absorbance measured at 540nm.

On each plate, duplicate wells contained 200 μ L 1%RRBC, 200 μ L GVB-EGTA-Mg buffer, 100% hemolysis (1%RRBC 100 μ L + 100 μ L 0.1% saponin in distilled water), and 100% blank (100 μ L 0.1% saponin+ 100 μ L GVB-EGTA-Mg buffer).

Background (OD_{540} 1%RRBC subtracted by OD_{540} GVB-EGTA-Mg buffer) was subtracted from each sample and the 100% hemolysis value was calculated as OD_{540} 100% hemolysis subtracted by OD_{540} 100% blank.

The ACH50 value was calculated according to Yano (1992). The value Y (percent hemolysis sample OD_{540} / 100% hemolysis OD_{540}) was calculated for each sample dilution and used in the equation $\log(y / 1-y)$. A linear regression was performed with \log (inverse dilution) to derive the y-intercept which is equivalent to the \log (ACH50). In a log-log graph, 50% hemolysis is equal to $\log(y/1-y) = 0$.

Anterior kidney cell oxidative burst and phagocytosis - The anterior kidney (cephalic horn region) was aseptically removed and placed into 1.5mL of 4°C L-15-1 medium containing 1% filter-sterilized salmon sera. A single cell suspension was created by trituration with a 21G needle attached to a 1cc syringe. Supernatant containing a mixed population of cell types was removed for the 2 assays following a 3 min. settlement period on ice. Because of blood collection prior to kidney dissection, fewer than 5% of the kidney cell suspension contained erythrocytes. Over 95% of the cells were deemed viable when a subsample was mixed with 0.2% trypan blue was viewed microscopically within a hemocytometer.

Oxidative burst response to stimulation and phagocytosis of opsonized beads by anterior kidney cells was assessed by the modified method of Anderson (1992). Briefly, one hundred microliters of cell suspension was mixed with 100 μ L of Nitroblue tetrazolum (NBT) solution (2 mg/mL NBT, 1 μ g /mL Phorbol myristate, in L-15-1), incubated in moist chamber at 15°C for 30 min, washed gently with PBS, fixed in absolute methanol for 5 min, and counter stained with 2 % safranin red stain. One hundred cells were microscopically evaluated for cytoplasmic blue stain to determine percent NBT positive cells.

Latex beads (3.0 μ m, Polysciences, Inc), opsonized with salmon plasma, were mixed with kidney cell suspension. A 20 μ L aliquot of stock latex beads (approximately 10^5 / mL of beads washed once with L-15 by centrifugation) was mixed with 2 mL of a 10% salmon plasma solution (diluted 10x with L-15) for 1 h at 25°C. One hundred microliter of the opsonized beads was continuously mixed for 1h at 20°C in 2mL tubes containing 100 μ L of the kidney cell suspension. A cytopsin slide was then prepared as described for the peripheral blood samples, fixed in absolute methonal, air dried, stained with Leishman Giemsa, air dried, and rinsed in absolute xylene for 15 min to dissolve beads external to cell membranes. The number of cells ingesting 1 or more beads divided by the total cells examined (100 – 135 cells) is referred to as the phagocytic index. Reagents were purchased from Sigma chemical.

Spleen cytokine gene expression - Entire spleens (<5 mg) were dissected and placed into 200 μ L of 10°C RNAlater (Applied Biosystems) and after 2 h stored at -70°C until later processed. RNA was extracted with RNeasy kit (Invitrogen) by the manufacturer's protocol with RNA concentration measured on a Nanodrop spectrophotometer (yields of 15 – 304 ng / mL RNA). An Invitrogen superscript III kit

and Eppendorf Mastercycler thermocycler was used to produce cDNA . Quantitative PCR was used to amplify and compare of the expression of 8 cytokine genes (IL-1 β , IL-2, IL-6, IL-8, IL-10 IFN, TNF α and TGF β). Primers for amplification of IL-1 β , IFN γ , TNF α , and IL-8 were designed from sequences from Chinook salmon in GenBank. Primers for amplification of IL-2, IL-6, IL-10, and TGF β transcripts were designed based on rainbow trout sequences in GenBank. Serial dilutions of a standard cDNA preparation were used to assess PCR efficiency. Primer concentrations and reaction efficiency and GenBank accession numbers are listed in Table 2. The dissociation curve of the PCR products were analyzed to verify a single peak and products were separated by gel electrophoresis to corroborate the presence of only one product and to evaluate product size. Quantitative PCR was performed in 10 μ l reactions containing 5 μ l Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA) 1 μ l of forward and reverse primer and 2 μ l cDNA and 2 μ l of cDNA (10x dilution). A β actin PCR product was synthesized and included in each assay as an endogenous control and to assess inter-assay variation. MicroAmp Fast Optical 96 well reaction plates were used. Each sample was run in duplicate wells through 40 cycles on an ABI 7500 Fast Real-time PCR system on the Standard 7500 setting with an added dissociation step and ROX used as passive reference. After 50 $^{\circ}$ C for 2 min, amplification proceeded for 40 cycles at: 95 $^{\circ}$ C for 10 min, 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C 1 min, then the dissociation curve consisted of 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 1 min followed by 95 $^{\circ}$ C for 15 s. Only samples for which both wells fluoresced were considered positive. If the standard deviation of the duplicate wells for an individual sample was >1, the sample was re-run. If the dissociation curve of the amplified product was inconsistent with the dissociation curve of a control amplicon (as can occur when there is DNA contamination) then the sample was excluded from the analysis.

Table 2. Primer sequences for Chinook salmon cytokine expression analysis, primer concentration, reaction efficiency, size of amplification product and GenBank accession number.

Gene	Primer sequences (5'-3')	Primer concentration and efficiency	Product (bp)	GenBank #
β actin	(F) GGA CTTTGAGCAGGAGATGG	4 μ M, 99.4%	186	Chinook salmon FJ546418
	(R) ATGATGGAGTTGTAGGTGGTCT			
IL-1 β	(F) ACCGAGTTCAAGGACAAGGA	6 μ M, 99.9%	181	Chinook salmon DQ778946
	(R) CATTCA TCAGGACCCAGCAC			
IL-2	(F) TTTCTTTTTGACGCTTTTTCTCA	4 μ M, 99.6%	204	Rainbow trout NM_001164065
	(R) CGAGGCATTCTACTTTCACAGT			
IL-6	(F) CAGTTTGTGGAGGAGTTTCAGA	2 μ M, 99.3%	118	Rainbow trout NM_001124657
	(R) TGTTGTAGTTTGAGGTGGAGCA			
IL-8	(F) GAGCATCAGAATGTCAGCCAG	6 μ M, 97.5%	77	Chinook salmon DQ778948
	(R) CTCTCAGACTCATCCCCTCAG			
IL-10	(F) CTACGAGGCTAATGACGAGC	6 μ M, 99.5%	100	Rainbow trout AB118099
	(R) GATGCTGTCCATAGCGTGAC			
IFN γ	(F) CAACATAGACAAACTGAAAGTCCA	4 μ M, 99.4%	129	Chinook salmon GT897806
	(R) ACATCCAGAACCACACTCATCA			
TGF β	(F) AGATAAATCGGAGAGTTGCTGTG	2 μ M, 99.9%	275	Rainbow trout X99303
	(R) CCTGCTCCACCTTGTTGT			
TNF α	(F) ACCAAGAGCCAAGAGTTTGAAC	2 μ M, 98.0%	154	Chinook salmon DQ778945
	(R) CCACACAGCCTCCATAGCCA			

The expression level of 4 cytokine genes (IL-1 β , IL-6, TNF α and TGF β) was analyzed by the comparative Ct method ($2^{-\Delta\Delta C_t}$) described by Schmittgen and Livak (2008). Four genes (IL-2, IL-8, IL-10, and IFN) had little to no amplification. Cytokine gene C_t data was normalized with respect to the housekeeping gene (β -actin) C_t of the sample. Fold difference of a 23 $^{\circ}$ MDT group compared to a cohort 18 $^{\circ}$ MDT group was calculated:

- 1) $\Delta = (\text{mean } C_t \text{ 23MDT cytokine} - \text{mean } C_t \text{ beta-actin of 23}^{\circ}\text{MDT}) - \text{mean } C_t \text{ 18MDT cytokine} - \text{mean } C_t \text{ beta-actin of 18}^{\circ}\text{MDT}$
- 2) $X = 2^{-\Delta}$
- 3) Fold change = $-1/X$

We considered a fold change ≥ 2.0 to demonstrate a significant response difference (Dios et al. 2010).

Statistical analysis - Analysis was performed with SigmaStat 3.1 software on raw data. Percentage data such as NBT and phagocyte activity was arcsine square

root transformed prior to analysis (Gotelli and Ellison 2004). Normality was tested by the Kolmogorov – Smirnov method at the P= 0.05 level. One-way ANOVA or T-test (data with normal distribution, reported with F or t value) or Kruskal-Wallis ANOVA or Mann-Whitney U test on ranks (non-parametric analysis) with subsequent multiple comparison procedures (Holm-Sidak or Dunns method respectively, $\alpha \leq 0.05$) was used to compare groups.

Results:

Both temperature groups experienced an 8d acclimation period that bifurcated at 18°C on day 4 (Fig. 1). The experimental 23°MDT group increased to 19°C for 4d and 20°C for 1d before the initiation of diurnal profiles. After the initial temperature control problem on June 29 and 30, daily profiles were consistently met throughout the 3 week study (Fig. 2). Dissolved oxygen concentrations were above 8 mg / L and obvious accumulation of feed was not observed in any tanks.

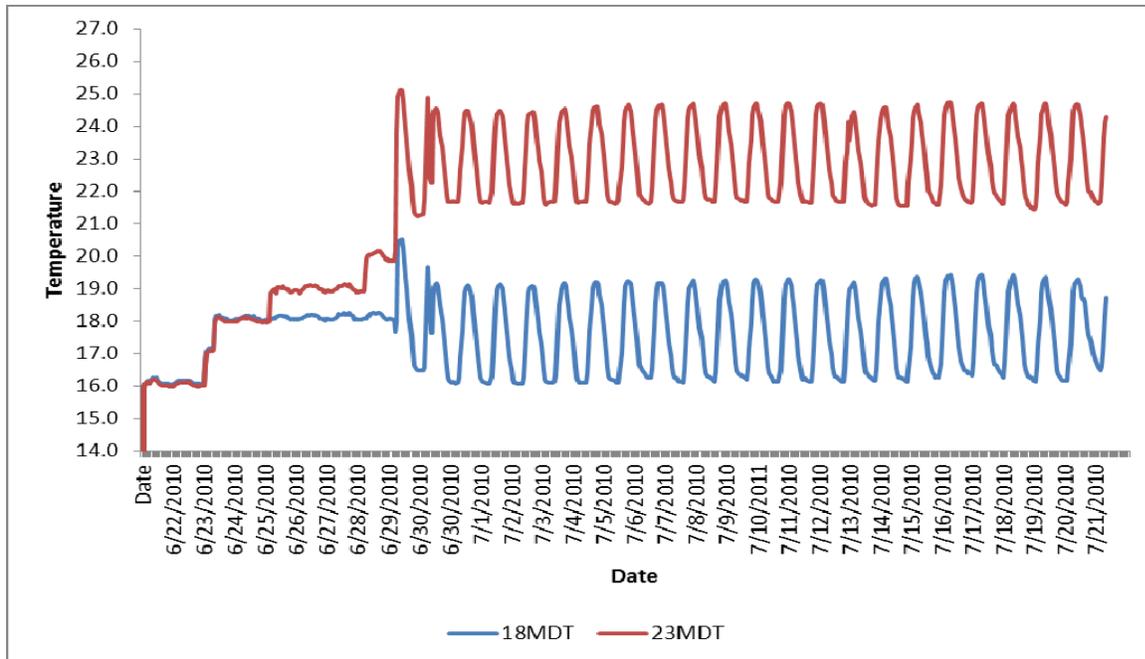


Figure 1. Daily profile of experimental 23° MDT and control 18°MDT groups during 8d acclimation and 3 week study period.

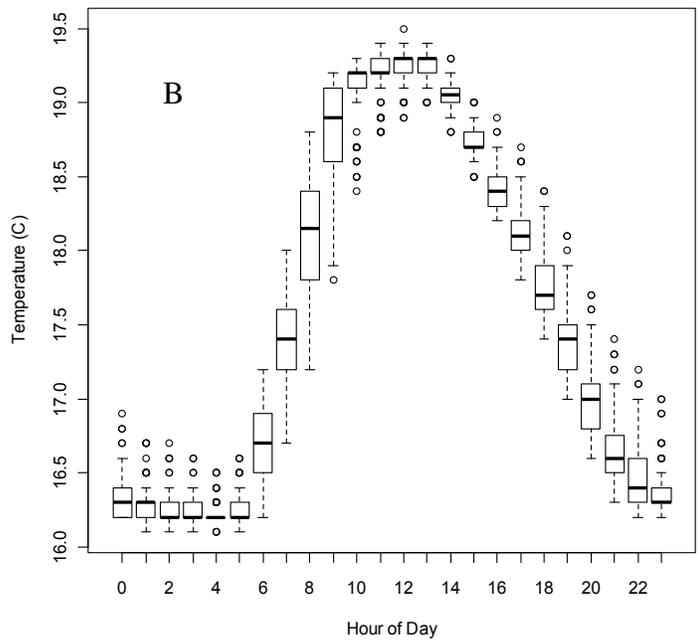
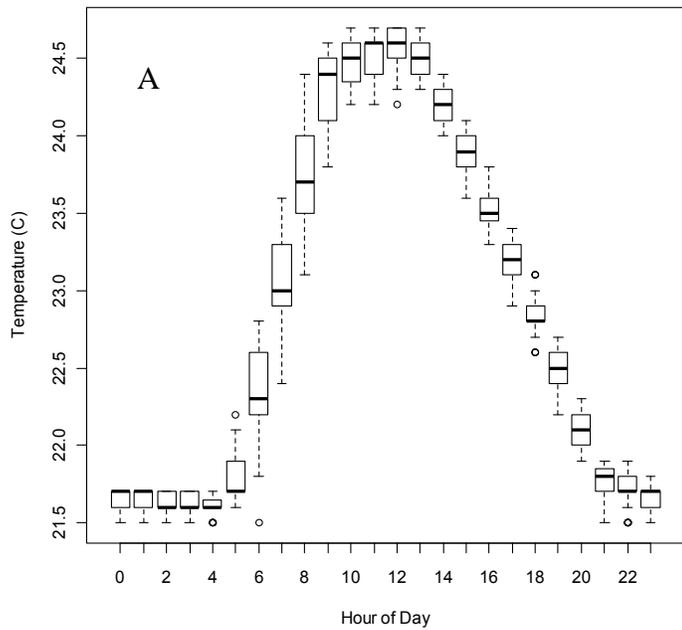


Figure 2. Daily profile of the experimental 23°MDT (A) and control 18° (B) groups. Data reported as quantiles (10, 25, 50, 75, and 90th percentile) of hourly temperature measurements over the 22 d experiment.

Growth and smolt development - Both length and weight of the 2 groups was similar at the beginning of the experiment and remained relatively level until the 3rd week (Table 3, Fig.3). As indicated by length and condition factor, 18° MDT salmon were significantly longer for a given weight than the 23°MDT fish. The mean growth rate over the 22d experiment was 0.36 mm/d for the 18°MDT fish compared with 0.22mm /d for the 23°MDTgroup. In contrast, mean weight gain was similar over the same period (1.37x for 23°MDT and 1.40x for 18°MDT fish). The 2%BW/d feed rate of commercial fish food was sufficient for positive growth at both temperature regimes. There were no mortalities during the study. Gill Na-K-ATPase activity tended to be higher in 23°MDT salmon until week 2 when this trend was reversed (Fig. 4). Activities ranged from 2.40 to 13.34 $\mu\text{mol ADP/mg protein/h}$. This is a range we consider normal for juvenile Chinook in our laboratory. Mean activity ranged from 5 to 8 $\mu\text{mol ADP/mg protein/h}$ throughout the study which is indicative of early smolt development. Elevated temperature reduced Na-K-ATPase activity over time.

Table 3. Fork length (mm), weight (g), and condition factor ($\text{wt/FL}^3 \times 10^5$) of salmon sampled from the 23° and 18° MDT groups at time zero (T0) – 3 weeks following start of diurnal temperature profiles. Data reported as mean (standard deviation). Significant difference between temperature groups at a given sample time is indicated by (*).

	Time0	1week	2week	3week
<u>Fork length</u>				
23mdt	82 (5)	81 (4)	84 (4)	87 (4)
18mdt	82 (5)	82 (4)	82 (7)	90 (4) *
<u>Weight</u>				
23mdt	5.9 (1.1)	6.0 (1.1)	6.8 (1.1)	8.1 (1.2)
18mdt	6.0 (1.2)	6.3 (1.0)	6.5 (1.4)	8.4 (1.1)
<u>Condition factor</u>				
23mdt	1.074 (0.081)	1.135 (0.094)	1.127 (0.092)	1.218 (0.100)
18mdt	1.089 (0.084)	1.126 (0.104)	1.163 (0.096)	1.146 (0.063)*

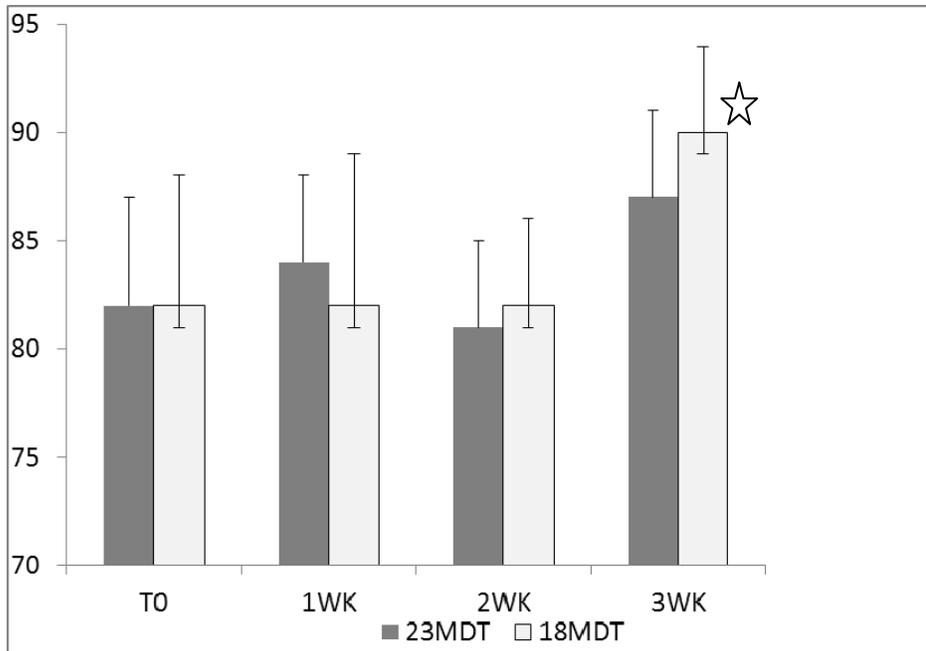


Figure 3. Mean fork length (mm) of salmon sampled from the 23° and 18°C MDT groups at time zero (T0) – 3 weeks (3wk) following start of diurnal temperature regimes. Significant difference (T-test, $P \leq 0.05$) between groups is indicated by star.

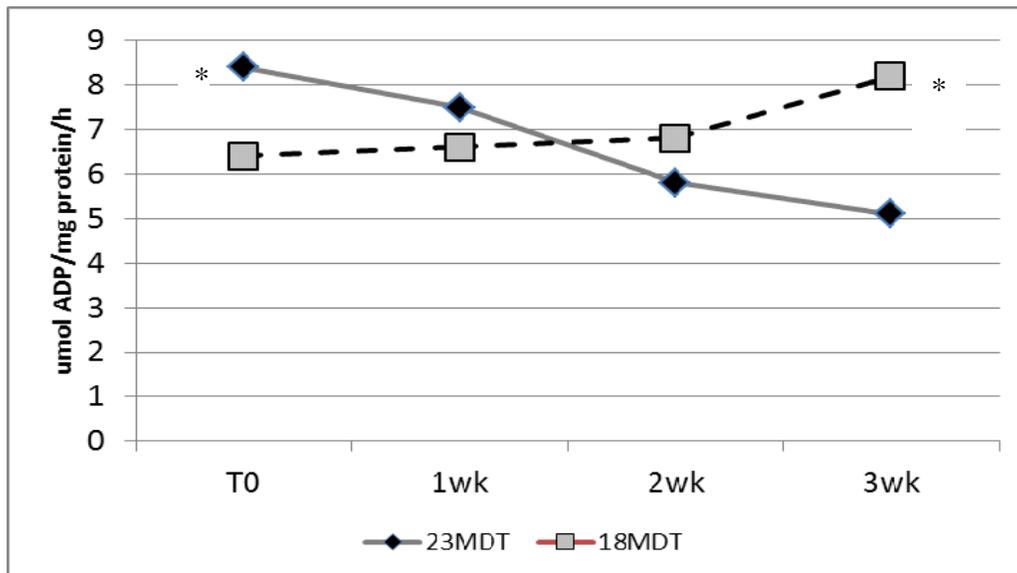


Figure 4. Mean Na-K-ATPase activities for salmon sampled from the 23° and 18°C MDT groups at time zero (T0) – 3 weeks (3wk) following start of diurnal temperature regimes. Significant difference (T-test, $P \leq 0.05$) between groups is indicated by asterisk.

Plasma cortisol - Cortisol was elevated in both 18° and 23° MDT salmon sampled at time zero (Fig. 5). The rapid temperature increase on 29 June immediately prior to the time zero sample is the likely cause of the high plasma cortisol levels. Cortisol remained elevated above basal levels in the 23°MDT group at 1 week. Salmon in 23°MDT group had significantly higher cortisol values than the 18°C fish at both time zero and 1 week (T-test, $P < 0.05$). Plasma cortisol was at basal levels (mean 7 ng/mL) for both groups in the final 2 weekly samples.

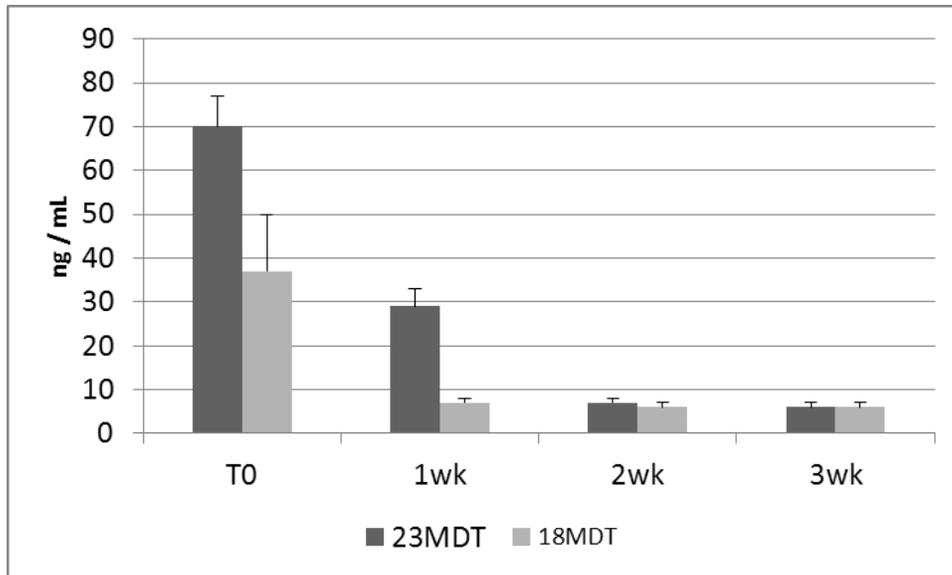


Figure 5. Mean plasma cortisol concentration for salmon sampled from the 23° and 18°C MDT groups at time zero (T0) – 3 weeks (3wk) following start of diurnal temperature profiles. Bars indicate standard error of the mean.

Vibrio anguillarum challenge - No obvious trend in challenge mortality was observed between the temperature groups (Fig. 6). Cumulative percent mortality was similar for the time 0 and week 1 challenges. Salmon in the 23°MDT group had significantly greater mortality in week 2. This trend shifted the following week with 18°MDT salmon having the highest mortality. The acute nature of the challenge was demonstrated by peak mortality occurring on day 2 post-injection with no mortality after 3 d post-injection. Mortalities had typical clinical signs of septicemia (internal and external hemorrhage) and *Vibrio anguillarum* was the only bacteria isolated from mortalities

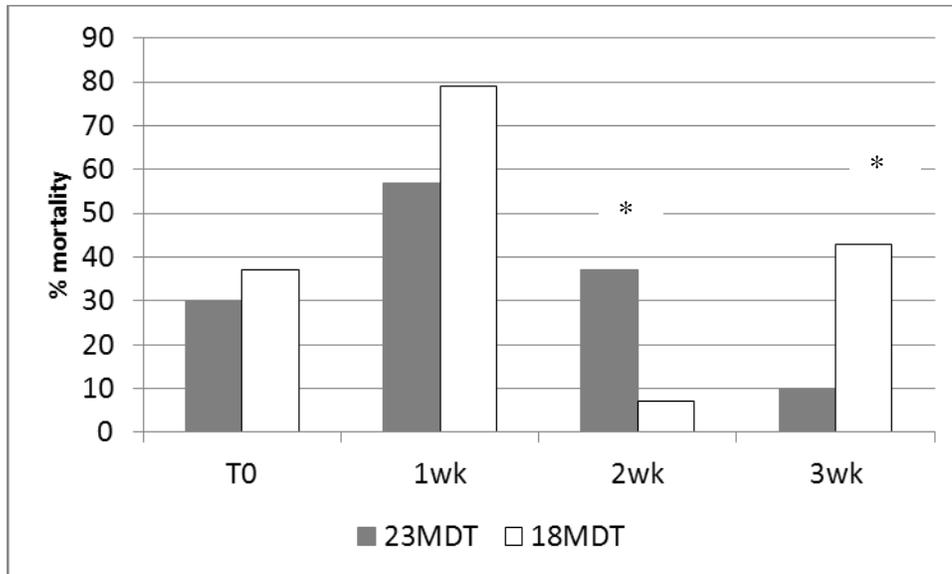


Figure 6. Ten day cumulative mortality (%) of salmon in the 23° and 18°C MDT groups challenged with $1.5 - 5.1 \times 10^4$ cfu *Vibrio anguillarum* by intra-peritoneal injection. Salmon were challenged at time zero (T0) and at weeks 1 – 3 after diurnal temperature exposure. Significant difference (Fishers exact test, $P \leq 0.05$) between groups is indicated by asterisk.

We attempted to determine specific plasma immunoglobulin titer to *V. anguillarum* using a monoclonal antibody to salmonid immunoglobulin (Aquatic Diagnostics Ltd., Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA) by the ELISA method of Skinner et. al (2010) and in an agglutination microplate assay (20x dilution sera with killed *V. anguillarum* vaccine pellet). No positive reaction occurred with either method. In a pilot study involving intra-peritoneal injection of vaccine into juvenile salmon, the ELISA method did produce positive titers.

Anterior kidney cell phagocytes - The mixed cell type suspensions from the anterior kidney of 23°MDT salmon did not demonstrated a consistent difference from the 18° MDT group in either oxidative burst response or phagocytosis. The oxidative burst response, (nitroblue tetrazolium staining) of activated phagocytes, was observed in 0% - 38% of the mixed kidney cell suspensions stimulated with phorbol myristate. Mean proportions ranged from 6 – 16 % of the cell population (Table 4). Initially, 18°MDT fish samples had higher proportion of NBT positive cells than 23°MDT cohorts but this trend reversed at week 2 (T-test, $P < 0.05$). The assay results were judged to be highly variable with coefficient of variation ranging from 34 to 68%.

Similarly, the phagocytic activity of the same kidney suspension showed a large degree of variation (coefficient of variation ranging from 34 to 54%). A higher percentage of 23°MDT fish kidney cells contained latex beads than 18° cohorts during week 1 (Table 4). In error, time zero samples were conducted on glass adherent cells (1h attachment followed by a wash) instead of a mixed kidney cell population. Percent phagocytosis in time zero samples were similar between temperature groups (mean 60% and 57%) and

higher in this macrophage rich cell population than the mixed kidney cells of later weekly samples. Sampling bias, during microscopic examination of a non-normal distribution of phagocytes, is a likely influence on the observed variability of both cell assays.

Table 4. Percent of anterior kidney cells demonstrating phagocyte oxidative burst (nitroblue tetrazolium (NBT) cytoplasmic staining) and phagocytosis of opsonized beads (Phagocytic activity) in salmon sampled from the 23° and 18°C MDT groups at time zero (T0) – 3 weeks (3wk) following start of diurnal temperature regimes. Data reported as mean (standard error) with significant difference (T-test, $P \leq 0.05$) between groups indicated by asterisk.

	Time0	1week	2week	3 week
NBT positive				
23°MDT	6 (1)	16 (2)	13 (2) *	12 (2)
18°MDT	14 (2) *	14 (1)	8 (1)	14 (3)
Phagocytic activity				
23°MDT	N/A	22 (4) *	22 (4)	40 (3)
18°MDT		12 (2)	28 (4)	34 (2)

N/A Glass adherent cells were assayed unlike the mixed anterior kidney cell suspension used in week1 – 3. Mean (SE) phagocytic activity for time zero 23°MDT was 60% (3%) and 57% (5%) for 18°MDT fish.

Differential leukocyte count of peripheral blood – Over the first 2 weeks of the experiment, lymphocyte numbers declined in both temperature groups and then moderately increased in week 3 (Fig. 7, Table 5). There was no significant difference in lymphocyte counts among the temperature groups at any specific sample date. Higher numbers of neutrophils were observed in the 23°C MDT group at time zero and week 3. This granulocyte increase resulted in a lower Lymphocyte : Granulocyte (L:G) ratio at these sample times (T-test, $P = 0.037$ for T0 and $P < 0.01$ for week 3). The time zero L:G difference is likely a stress response to the temperature ramping error while the week 3 data may represent a shift in the peripheral blood profile. Differential leukocyte count data from the 24 hpi challenge salmon was limited by both low sample number (mortality) and a high prevalence of “smudge” cells in the blood smears. It is possible that cell fragility increased due to inflammatory response. Generally, neutrophil and monocyte counts were elevated in the challenged salmon compared to the tank populations (Table 5).

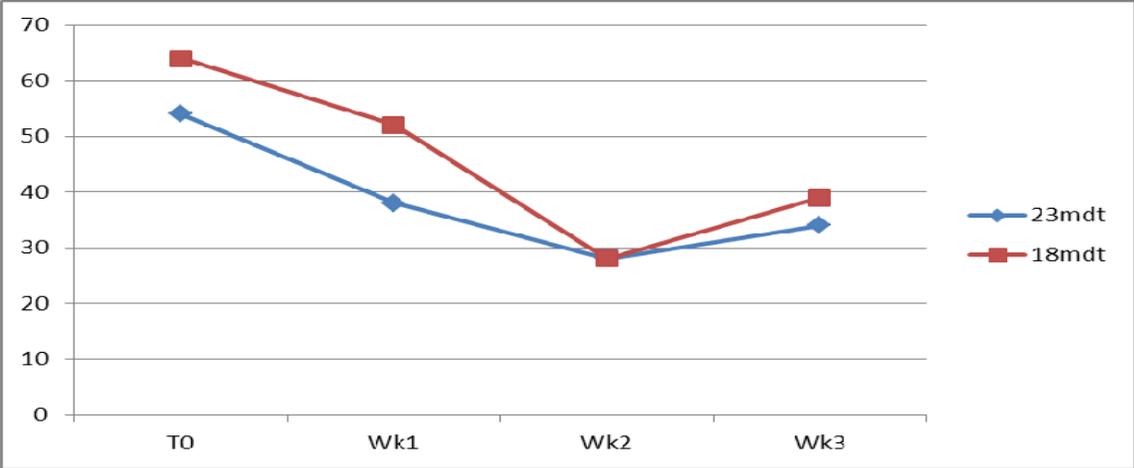


Figure 7. Mean percentage of blood lymphocytes in 18° and 23°C MDT salmon sampled weekly (Wk1-3) after initiation of diurnal temperatures profile (T0).

Table 5. Differential leukocyte counts of 23° and 18°C MDT salmon sampled at time zero (T0) and at weeks 1 – 3 (wk1-3) after elevated temperature profiles. Samples came from the tank population as well as cohorts challenged on the same date with $1.5 - 5.1 \times 10^4$ cfu *Vibrio anguillarum* by intra-peritoneal injection (IP). Data reported as mean (SEM), sample number (no.), and lymphocyte:granulocyte ratio (L/G). Bold L/G values are significantly different from cohorts.

		no.	Lymphocyte	Thrombocyte	Neutrophil	Monocyte	L / G
T0	23MDT	6	53.8 (5.1)	41.8 (4.8)	1.8 (0.3)	0.9 (0.1)	35 (8)
	18MDT	10	64.1 (4.3)	33.2 (4.1)	0.7 (0.2)	1.2 (0.4)	76 (11)
	23MDT-1P	10	39.3 (5.8)	55.6 (6.2)	1.5 (0.8)	2.1 (0.5)	61 (11)
	18MDT-IP	7	45.9 (5.5)	39.6 (6.2)	4.8 (1.4)	5.0 (0.9)	24 (13)
WK 1	23MDT	12	38.4 (1.6)	58.2 (1.7)	0.8 (0.1)	1.6 (0.5)	57 (8)
	18MDT	10	51.9 (1.8)	44.6 (1.6)	1.5 (0.4)	0.5 (0.2)	58 (12)
	23MDT-1P	1	7	93	0	0	na
	18MDT-IP	2	33.7 (11.1)	46.3 (19.5)	4.5 (4.0)	15.5 (4.4)	25 (20)
WK 2	23MDT	11	27.7 (2.8)	70.7 (2.7)	0.5 (0.2)	0.3 (0.1)	63 (9)
	18MDT	11	28.1 (4.5)	69.0 (4.3)	1.2 (0.5)	0	64 (11)
	23MDT-1P	8	20.0 (2.4)	72.1 (3.9)	2.9 (1.0)	2.0 (0.6)	32 (15)
	18MDT-IP	2	20.1 (0.1)	69.7 (5.4)	3.6 (2.1)	2.9 (1.4)	8 (5)
WK 3	23MDT	10	33.6 (2.9)	61.9 (3.2)	1.4 (0.3)	1.7 (0.4)	36 (9)
	18MDT	12	39.4 (3.5)	59.1 (3.4)	0.2 (0.1)	0.7 (0.2)	82 (7)
	23MDT-1P	8	38.2 (5.0)	44.5 (5.0)	6.3 (1.8)	4.6 (1.2)	21 (12)
	18MDT-IP	6	23.2 (6.5)	40.4 (10.7)	7.3 (4.0)	21.7 (8.8)	16 (6)

Total protein, albumin, lysozyme activity- Plasma protein concentration ranged from 8 – 39 mg / mL with the group means of 22 – 33 mg/mL (Table 6, Fig. 8). Significant differences (T-test, $P < 0.05$) were observed between the time zero *Vibrio* challenge 24hpi fish of both temperature groups and week 2 tank and 24 hpi challenge fish (Fig. 8). While not statistically significant, 23°MDT salmon tended to have higher plasma protein levels than 18°MDT cohorts except in week 2. Protein concentrations of 18 °MDT tank populations were significantly higher in week 1 and 2 compared to time zero and week 3 (ANOVA, $P < 0.01$). No such trend was detected for the 23°MDT salmon.

Table 6. Plasma chemistry data (mean, standard error) for juvenile Chinook reared for 21 d under diurnal temperature profiles of 18°C and 23°C MDT. Salmon were also sampled at time zero (T0) and at weeks 1 – 3 (1-3WK) after elevated temperature profiles and 24h after intra-peritoneal (IP) challenge with *Vibrio anguillarum*. Data includes protein and albumin (mg calculated globulin (Protein – albumin), Albumin / Globulin ratio (A/G), lysozyme activity (mOD/min/mL), no lysozyme activity (0Activity LZ = number lysozyme samples with no activity / total sample (%), alternative complement activity (ACH50), and cortisol concentration (ng/mL).

Group	Protein	Albumin	Globulin	A/G	Lysozyme	0 Activity LZ	ACH50	Cortisol
23MDT T0	28.1 (1.6)	8.9 (0.5)	19.2 (1.3)	0.48 (0.03)	653 (156)	1 / 8 (13%)	20.6 (4.3)	70.2 (6.5)
no.	8	8	8	8	7		12	8
18MDT T0	25.2 (1.2)	7.2 (0.5)	18.0 (1.0)	0.41 (0.04)	517 (117)	1 / 7 (14%)	19.3 (4.5)	37.1 (13.2)
no.	8	8	8	8	6		12	8
23MDT T0-IP	27.3 (1.0)	8.7 (0.4)	18.5 (0.8)	0.48 (0.03)	682 (222)	3 / 10 (30%)	nd	nd
no.	10	10	10	10	7			
18MDT T0-IP	22.3 (1.8)	6.1 (0.5)	16.2 (1.4)	0.38 (0.02)	968 (154)	2 / 6 (33%)	nd	nd
no.	8	8	8	8	4			
23MDT 1WK	32.1 (1.2)	8.0 (0.4)	24.1 (1.0)	0.33 (0.01)	717 (98)	1 / 13(8%)	15.4 (1.7)	29.1 (3.7)
no.	7	7	7	7	12		12	8
18MDT 1WK	32.9 (0.7)	8.7 (0.4)	24.2 (0.4)	0.36 (0.01)	555 (89)	6 / 10 (60%)	13.3 (2.5)	7.2 (1.2)
no.	10	10	10	10	4		12	8
23MDT 1WK-IP	16.9	1.8	15.1	0.12	nd	nd	nd	nd
no.	1	1	1	1				
18MDT 1WK-1P	22.2 (3.3)	7.0 (0.8)	15.2 (2.8)	0.42 (0.03)	664 (132)	1 / 7 (14%)	nd	nd
no.	8	8	8	7	6			
23MDT 2WK	28.6 (0.6)	4.6 (0.1)	24.0 (0.5)	0.19 (0.00)	748 (91)	6 / 19 (32%)	19.0 (2.4)	7.2 (1.1)
no.	20	20	20	20	13		12	8
18MDT 2WK	31.1 (0.8)	5.1 (0.1)	26.0 (0.8)	0.20 (0.00)	610 (144)	5 / 12 (42%)	21.6 (4.6)	5.8 (0.8)
no.	18	16	16	16	7		12	8
23MDT 2WK-IP	24.9 (1.5)	6.1 (0.3)	18.8 (1.4)	0.34 (0.03)	1014 (344)	6 / 10 (60%)	nd	nd
no.	10	10	10	10	4			
18MDT 2WK-IP	30.5 (1.8)	7.1 (0.4)	23.3 (1.4)	0.31 (0.01)	619 (218)	7 / 10 (70%)	nd	nd
no.	10	10	10	10	3			
23MDT 3WK	28.5 (0.8)	6.5 (0.2)	21.9 (0.7)	0.30 (0.01)	702 (185)	14 / 19 (74%)	26.5 (3.8)	6.2 (0.8)
no.	20	20	20	20	5		12	8
18MDT 3WK	26.2 (0.9)	6.9 (0.3)	19.4 (0.6)	0.36 (0.01)	677 (458)	11 / 13 (85%)	22.1 (2.2)	6.3 (0.6)
no.	20	20	20	20	2		12	8
23MDT 3WK-IP	27.3 (1.5)	7.8 (0.8)	19.5 (2.9)	0.41 (0.06)	757 (105)	3 / 8 (38%)	nd	nd
no.	5	5	5	5	5			
18MDT 3WK-IP	21.6 (3.2)	6.0 (0.6)	15.6 (2.6)	0.39 (0.03)	801 (109)	0 / 4 (0%)	nd	nd

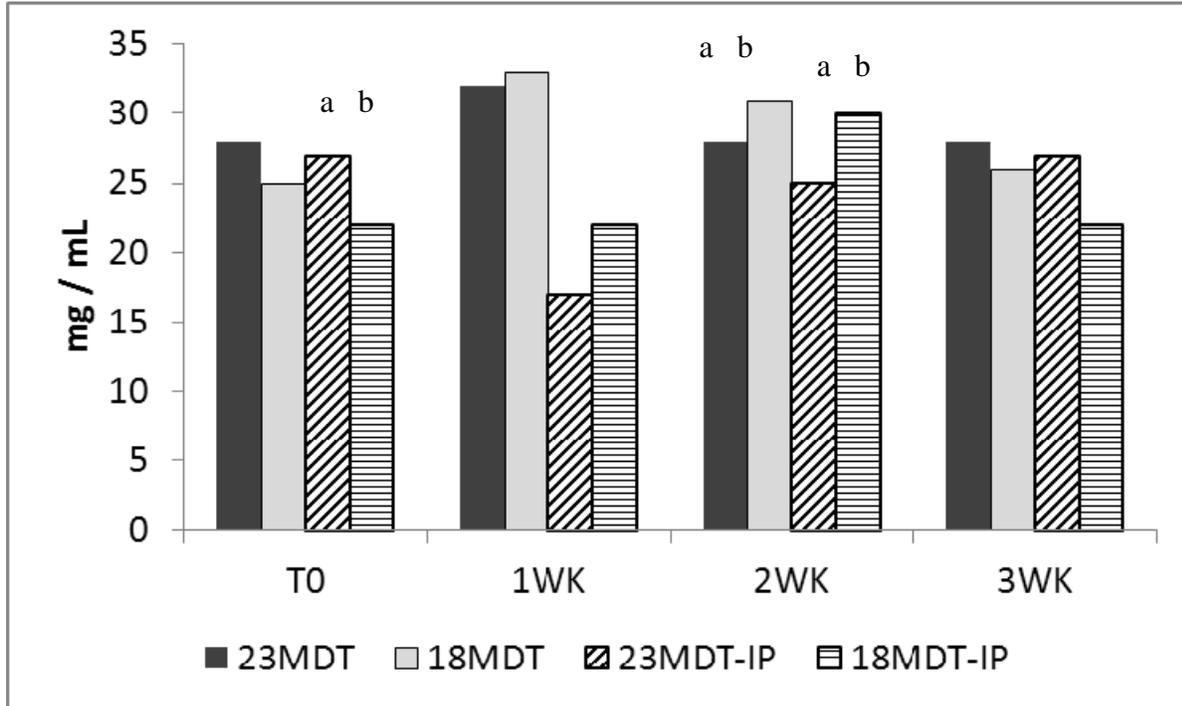


Figure 8. Mean protein concentration (mg/mL) of plasma samples from 23° and 18°C MDT salmon sampled at time zero (T0) and at weeks 1 – 3 (1-3WK) after elevated temperature profiles. Samples came from the tank population as well as cohorts challenged on the same date with $1.5 - 5.1 \times 10^4$ cfu *Vibrio anguillarum* by intra-peritoneal (IP) injection. The 1week 23°MDT-IP data is from a single fish. Significant difference between 2 sample groups on a given week indicated by different letters (t-test, $P < 0.05$).

Plasma albumin concentration ranged from 4 – 12 mg/mL with mean values of 6 – 9 mg/mL (Table 6). Mean albumin represented 16 – 33% of the mean total protein content with trends in group means matching those of total protein (graphical data not shown). Significant differences between temperature groups occurred in both the tank and 24hpi salmon at time zero and week 2 (T-test, $P < 0.05$). The 23°MDT salmon had higher albumin levels on time zero and lower values in week 2 than 18 °MDT.

Calculated globulin concentrations ranged from 10 – 31 mg/mL with mean values of 15 – 26 mg/mL (Table 6). Plasma globulin from challenged fish (24hpi) was either similar or lower than the tank population sampled the day prior. Significant differences (T-test, $P < 0.05$) were observed between 23°MDT and 18°MDT salmon in week 2 (23°MDT < 18°MDT) and week 3 (23°MDT > 18°MDT).

The calculated albumin : globulin ratio (A/G) ranged from 0.16 to 0.65 with mean values of 0.19 – 0.48 (Table 6, Fig. 9). Week 2 salmon in both groups had the lowest ratios

(0.19 and 0.20) due to their lower albumin levels. Significant differences (T-test, $P < 0.05$) were observed between 23°MDT and 18°MDT salmon in time zero 24hpi groups (23°MDT > 18°MDT) and week 3 tank population (23°MDT < 18°MDT).

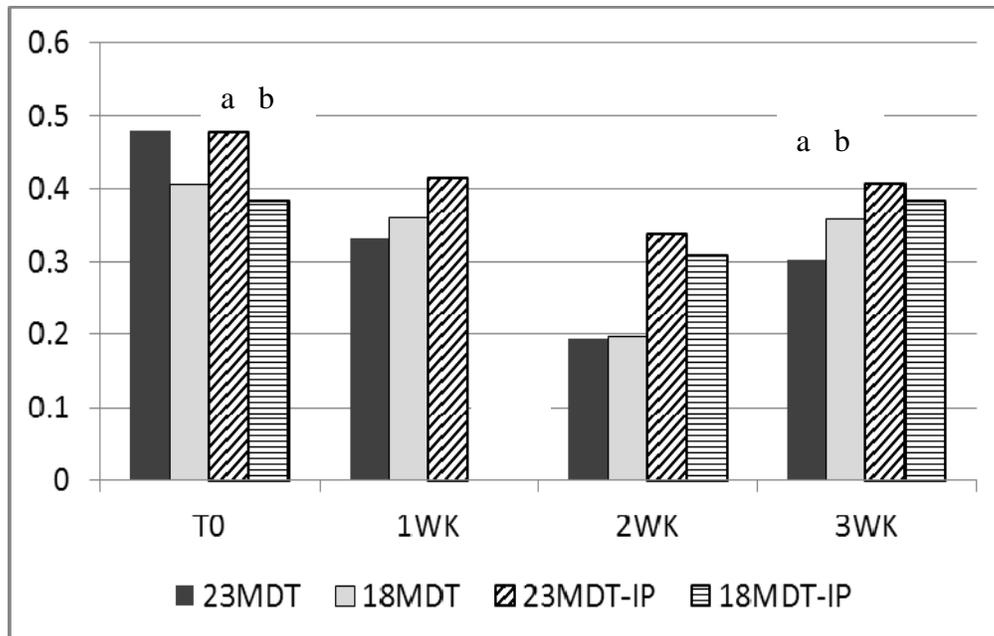


Figure 9. Mean albumin to globulin ratio (A/G) of plasma samples of 23° and 18°C MDT salmon sampled at time zero (T0) and at weeks 1 – 3 (1-3wk) after elevated temperature profiles. Samples came from the tank population as well as cohorts challenged on the same date with $1.5 - 5.1 \times 10^4$ cfu *Vibrio anguillarum* by intra-peritoneal injection (IP). There is no sample for 1wk 23MDT-IP group. Significant difference between 2 sample groups of a given week indicated by different letters (t-test, $P < 0.05$).

Plasma lysozyme activity was not significantly different (T-test, $P < 0.05$) between temperature groups sampled at the same time (Fig. 10, Table 6). Activity ranged from 121 to 1248 mOD/min/mL in samples demonstrating lytic action. Samples collected 24h post-challenge did not show a consistent elevated trend when compared to the tank population of the same sample date. No activity was measured in 8 – 85% of the sample groups with an increasing trend over time (Fig. 11). It is unclear if this phenomenon was an artifact of specific sample collection, storage, and assay conditions or if it accurately reflects the fish's plasma lysozyme concentration and functionality. Hen egg white standard curves were linear and performed in a similar manner on all lysozyme plates.

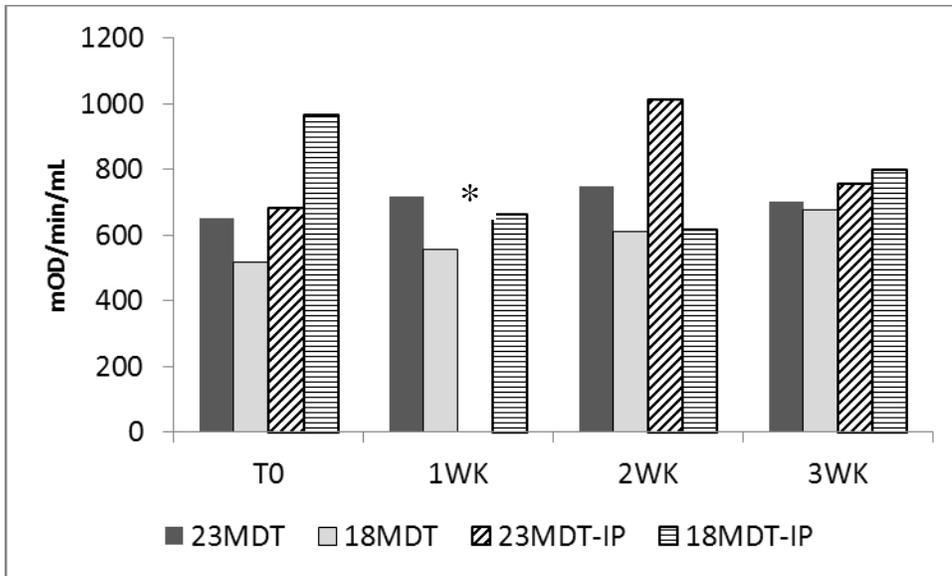


Figure 10. Mean lysozyme activity (mOD/min/mL) of plasma samples with lytic activity of salmon in the 23° and 18°C MDT groups sampled from the tank population at time zero (T0) and at weeks 1 – 3 (1-3wk) after elevated temperature profiles began as well as cohorts challenged on the same dates with $1.5 - 5.1 \times 10^4$ cfu *Vibrio anguillarum* by intra-peritoneal injection (IP). There is no sample for 1wk 23MDT-IP group (*).

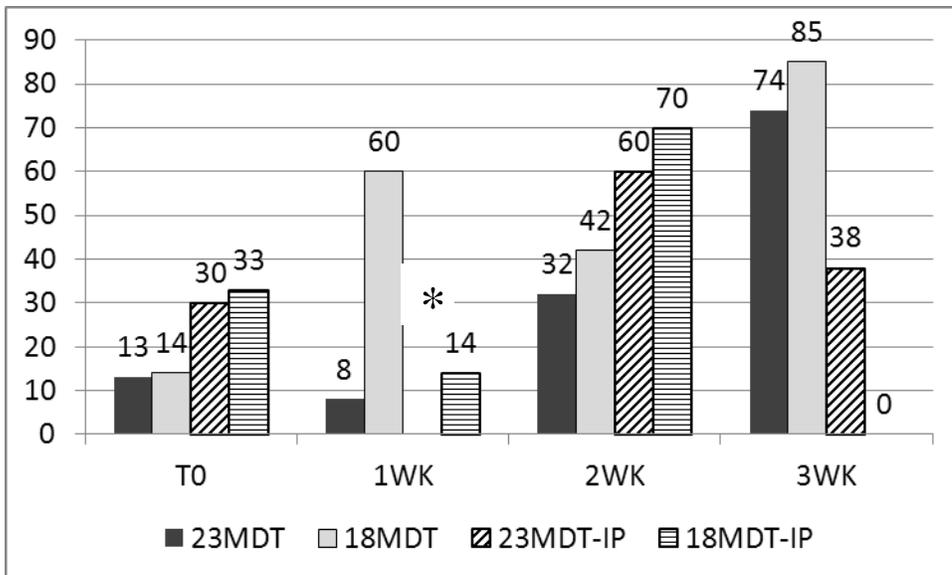


Figure 11. Percent of lysozyme samples demonstrating no activity of salmon in the 23° and 18°C MDT groups sampled from the tank population at time zero (T0) and at weeks 1 – 3 (1-3wk) after elevated temperature profiles began as well as cohorts challenged on the same dates with $1.5 - 5.1 \times 10^4$ cfu *Vibrio anguillarum* by intra-peritoneal injection (IP). There is no sample for 1wk 23MDT-IP group (*).

Alternative complement pathway activity- Complement activity was similar between temperature groups on any specific sample date. Mean values for both groups declined at 1 week and subsequently increased to or above time zero level in the week 3 sample (Table 6, Fig. 12).

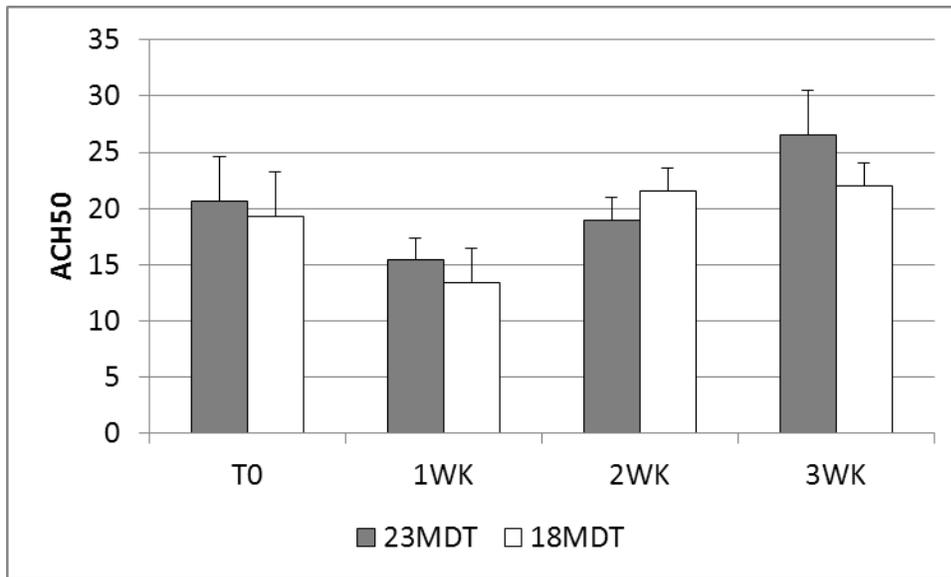


Figure 12. Mean ACH50 values for plasma of salmon in the 23° and 18°C MDT groups sampled from the tank population at time zero (T0) and at weeks 1 – 3 (1-3WK) after elevated temperature profiles. Bars signify standard error.

Spleen cytokine gene expression - Salmon in the 23°MDT group had consistently lower cytokine expression than 18°MDT cohorts (negative fold changes in Table 7). Elevated temperature appears to down-regulate gene expression of the pro-inflammatory cytokines IL-1 β and TNF α , T-cell interleukin IL-6, and the regulator cytokine TGF β . Interleukin 1 β expression was reduced in 23°MDT salmon from both 2 and 3 week samples. Samples from 24 h post-*Vibrio* challenge (24hpi) fish did not demonstrate a consistent pattern for up-regulation of pro-inflammatory cytokines when compared to non-challenged fish collected the day prior (Fig. 13). It is possible that ramping error on 29June influenced the marked difference in IL-6, TGF, and TNF levels observed between 23° and 18° MDT 24hpi challenged fish at time zero. The housekeeping gene, beta actin, appeared to be a suitable target for normalizing gene expression (Li et al. 2010). There was no significant difference between beta actin C_T values (T-test, P>0.05) and the coefficient of variation was only 12%. Small sample numbers per group (2-4) and mixed cell types within the spleen limits the sensitivity of this data in describing subtle changes in cytokine expression.

Table 7. Fold change ($2^{-\Delta\Delta Ct}$ method) of gene expression of spleen cytokines from 23°MDT salmon compared to 18°MDT cohorts. Number of samples in the 23° and 18°MDT reported as (no. 23°, no. 18°). If no gene amplification occurred, results given as “no data”. Fold changes ≥ 2.0 are in bold text. Fish were sampled from the tank population at time zero (T0) and at weeks 1 – 3 after elevated temperature profiles began as well as cohorts challenged on the same dates with $1.5 - 5.1 \times 10^4$ cfu *Vibrio anguillarum* by intra-peritoneal injection and sampled 24 h later (24hpi). There were no 23° samples for comparison in the 1 week *Vibrio* challenge (24hpi).

	IL-1 beta	IL-6	TGF beta	TNF alpha
T0	No data	-1.9 (3,3)	No data	-1.7 (3,3)
T0 24hpi	-1.1 (2,4)	-6.1 (2,4)	-3.4 (3,2)	-3.5 (2,4)
1 week	-1.1 (2,2)	-1.4 (3,3)	-1.0 (3,2)	-1.6 (3,3)
2 week	-2.7 (3,3)	-1.3 (3,3)	-0.1 (3,2)	-1.5 (3,3)
2 week 24hpi	-0.8 (4,4)	-1.3 (4,4)	-1.2 (4,4)	-1.6 (4,4)
3 week	-4.2 (3,3)	-2.6 (3,3)	-0.4 (3,2)	-2.0 (3,3)
3 week 24hpi	-3.6 (4,4)	-0.4 (4,4)	-3.6 (2,2)	-0.3 (4,4)

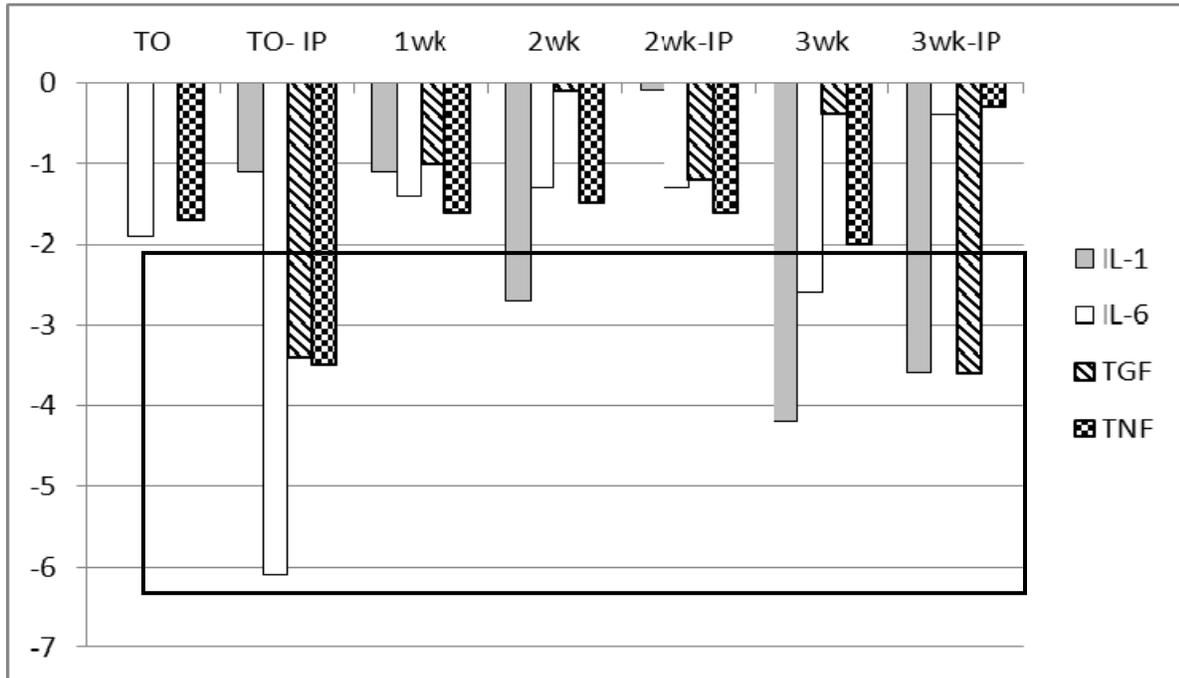


Figure 13. Expression level of cytokine genes displayed as fold change between 23°MDT and 18°MDT fish. Square highlights those changes ≥ 2.0 .

Discussion

Elevated water temperature did not exert an obvious effect on the immune functions or general health of juvenile Klamath R. Chinook salmon. Basal levels of plasma cortisol values in 23°MDT salmon sampled at week 2 and 3 suggest that they had acclimated to the elevated temperature. Additionally, 23°MDT salmon had a positive (albeit slight) growth rate over the experiment. The observed decline of gill Na-K-ATPase activity in 23 °MDT salmon was similar to previous studies at our wetlab. Trinity R. juvenile Chinook, reared for 21 days at a mean daily temperature of 21°C, demonstrated the plasticity of Na-K-ATPase activity. Despite depressed levels, these Trinity R. smolts were able to osmo- regulate during 96h saltwater challenges and showed rapid increases in enzyme activity after saltwater exposure (unpublished data). Overall, the Klamath River Chinook used in the experiment adapted to the elevated temperature regime under our laboratory conditions and no “fitness” threshold at 23 °MDT was observed over the 3 week period.

Except for the first two days of the experiment, diurnal temperature profiles were \leq 0.5°C of the hourly target temperatures with a daily span of 2.8 – 3.1 °C. This profile was very similar to that reported for the lower Klamath River in late June and July (Sutton et al. 2007, Perry et al. 2011). The rapid temperature increases on 29 and 30 June are the likely cause of the elevated cortisol values observed in both groups at time “zero” and may have influenced the 23 °MDT salmon elevated values in week 1. One open question of this study is whether the 6 day acclimation period, that moved fish from 16°C to the 23°MDT profile (~1.2C per day), was sufficient to avoid biased responses. Salmon in the wild are unlikely to see mean temperature changes at the 1-2°C per day rate. Future studies should approximate the temperature regimes over a longer period to avoid this potential experimental bias.

Protective immunity, of the immersion vaccination against artificial challenge, was the primary evaluation for temperature effects on specific immune function. No clear temperature trend on challenge survival was observed in this study. Both groups showed a pattern of protection similar to that reported in the literature for juvenile salmon. Depending on water temperature, juvenile sockeye had maximum protective immunity between 5 and 10 days post-vaccination (Johnson et al. 1982). The lowest mortality (best protection) occurred at 15 days post-vaccination (week 1 after high temperature profile began) despite the slightly higher challenge dosage (5.1×10^4 cfu / mL compared with 1.5 – 1.9 cfu /mL for other weeks). Elevated plasma cortisol levels in high temperature fish did not correspond to increased challenge mortality at time 0 and week 1. While the high temperature group experienced higher cumulative mortality in the week 2 challenge (22 days post-vaccination, 14 days after high temperature profile began), this trend was reverse a week later. The lipopolysaccharide layer of *V. anguillarum* is highly immunogenic to salmonids and protection is reported to be antibody-mediated ((Evelyn 1984, Harrell et al. 1975). Conversely, Raida and Buchman (2008) report rainbow trout, vaccinated by immersion to another gram-negative bacteria (*Yersinia ruckeri*) and demonstrating protection from artificial challenge, showed little humoral protection but instead a heighten CD8 alpha gene expression. This T-cell marker indicates that protection could be more cell-mediated than by immunoglobulin.

Immersion vaccination provides a lower level of immunity than intra-peritoneal injection but is still protective (Lillehaug 1989). Intra-peritoneal challenge is unnatural as it bypasses external defenses (mucus proteases, lectins, immunoglobulin, lysozyme, etc.) however, it is the most reliable method of inducing specific bacterial challenge in fish (Nordmo et al. 1997). The challenge survivors likely managed the infection by a combination of humoral (immunoglobulin acting as both an opsonin and complement activator) and cellular mechanisms (phagocytosis). Numerous reports have described the rapid elimination of systemic bacteria in immune fish and the relationship of rapid clearance with challenge survival (Russo et al. 2009, Reyes-Becerril et al. 2011, Zhang et al. 2011). The inability to detect specific antibody following immersion vaccination was similar to the results of Palm et al. (1998). They reported that serum antibody was undetectable by ELISA in trout given a single immersion vaccination but was still protective in challenges. Recent studies have begun to characterize the mucosal antibody IgT (Zhang et al. 2010), which may facilitate this protection, and could explain why antibodies were not detected in the serum.

Immune cell profile and functions were not markedly different between the temperature groups. While high assay variability limits the interpretation of anterior kidney cell oxidative burst and phagocytosis activity, no overt decline was observed in the 23°MDT salmon. Similarly, both groups had declines in blood lymphocytes over the experiment which may indicate a general response of the salmon to smoltification or culture conditions. It is unclear why a large proportion of plasma samples had no detectable lysozyme activity. Additionally, the mean activities (mOD/min/mL plasma) of the tank population samples ranged from 518 - 748 which is much lower than the 1000 – 2200 reported for Iron Gate hatchery juvenile Chinook reared at 20°C for 17 days (Foott et al. 2004). Lysozyme is produced primarily by macrophages and has bactericidal properties through its hydrolysis of bacterial peptidoglycans, opsonin function for phagocytes, and ability to activate the complement system (Paulsen et al. 2003). Chronic stress and smoltification are reported to decrease plasma lysozyme in Atlantic salmon (Melamed et al. 1999, Mock and Peters 1990, and Muona and Soivio 1992). Alternately, sample collection and storage could have resulted in loss of activity. Low confidence in data validity limits any interpretation of the plasma lysozyme activity. In contrast, the plasma complement assay performed well with values (20x dilution %hemolysis data) similar to that reported in Foott et al (2004). There was no significant difference in the ACH50 values between the groups at any specific sample period indicating no temperature effect on this humoral defense. Carlson et al. (1993) reported that elevated cortisol is associated with reduced serum hemolytic activity (alternate complement pathway). Complement activity data in our study further supports the low plasma cortisol trend. The albumin measurements (as well as the calculated globulin and A/G ratios) must be viewed as relative data given that bromocresol green reagent binds to both alpha-globulin proteins and albumin (Jacobs et al. 1990). Maillou and Nimmo (1993) report a similar overestimation of albumin with bromocresol green in Atlantic salmon sera. Several group trends were observed in the plasma protein data. First, globulin concentration was elevated at week 3 in the 23°MDT salmon which was reflected in the increased total protein and lower A/G ratio of this group. Second, 24hpi fish of both temperature groups tended to have reduced globulin levels and associated increased A/G ratios. As

with the other plasma constituents, plasma protein values were not strongly altered in the 23°MDT salmon.

Small sample numbers and mixed cell types extracted from the spleen limits the sensitivity of the cytokine gene expression data. All four genes assayed had lower expression in 23°MDT salmon with significant reductions (≥ 2 fold) of the pro-inflammatory cytokine IL-1 β occurring in the tank population at weeks 2 and 3. As expected, the C_T values of pro-inflammatory genes were lower (greater expression) in 24 hpi fish compared to the tank population of both temperature groups. Weins and Vallejo (2010) report an increase in both TNF α and IL-1 β transcripts in trout 24 h post-injection of *Yersinia ruckeri*. Carp sampled 4 to 12h post-challenge with *Aeromonas hydrophilia* are reported to have >2.0 fold increases in IL-1 β and TNF α (Tanekhy et al. 2009). Expression of both TNF α and T-cell interleukin IL-6 were also significantly reduced in 23°MDT salmon by week 3. We question whether the lower gene expression values for the time zero, 23°MDT, 24hpi salmon could be an artifact of the 29June ramping error as a similar trend in 23°MDT challenged fish did not occur again until week 3 (> 2.0 fold changes in both IL-1 β and TGF- β). Both IL-1 β and TNF α have been identified in teleosts and act as pro-inflammatory cytokines. They are produced by immune cells in response to acute infection and mediate leukocyte proliferation, migration, activation, and apoptosis (Secombes et al.2009). Similarly IL-6 has also been documented in fish and is produced by T-cells in response to inflammatory stimuli while TGF- β acts to inhibit macrophage activity (Secombes et al.2009). The reduced cytokine expression in week 3 did not correlate with increased mortality in the *V.anguillarum* challenge. Reduced cytokine expression may be a biomarker for temperature alteration of the immune system however we were unable to link it with impaired immune function.

Disease in Klamath River salmon is often associated with elevated water temperature (California Department of Fish and Game 2004, Foott et al. 2004). The prevalence and virulence of bacterial (e.g. *Flavobacterium columnare*) and some parasitic infections (*Ceratomyxa shasta* and *Ichthyophthirius multifiliis*) have a positive relationship with water temperature (Groberg et al 1978, Holt et al. 1975, Udey et al. 1975). Elevated water temperatures will tend to favor microbial replication rates over host defenses. This study supports the hypothesis that juvenile Chinook salmon immune function is not significantly impaired under a summer temperature profile of 23°MDT. Furthermore, parasitic and bacterial disease in the Klamath River salmon population is influenced more by infectious pressure (pathogen quantity) and rapid multiplication, than host immunosuppression.

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Appendix 1. Initial proposal (2009) and brief statements on changes and / or assay failures not reported.

PROJECT TITLE: Summer water temperature effects on the immune system of Klamath River juvenile Chinook salmon.

BACKGROUND: A high incidence of infection by the myxosporean parasites, *Ceratomyxa shasta* and *Parvicapsula minibicornis*, has been documented in Klamath River juvenile Chinook salmon during the spring and summer. These infections lead to lethal disease in a large portion of the infected population. The incidence of external bacterial and parasitic infections increase in smolts as water temperature rises above 21°C. River temperature often reaches in excess of 23°C during the outmigration period. Numerous references state the above temperatures are stressful to salmon however there are few studies that have examined the relationship between elevated temperature and the juvenile salmon immune system.

OBJECTIVE:

1. Rear juvenile Chinook salmon (Iron Gate Fall –run) under a 2-3° C diurnal fluctuating water temperature regime that simulates the Klamath River during June and July (Mean Daily Temperature (MDT) of approximately 23°C). Dissolved oxygen and food will be maintained at optimal levels. A control group will be reared at a MDT of approximately 18°C.
2. Document how innate and specific immune function indicators of the test and control fish change over a 5 week experiment.
3. Perform bacterial challenges on test and control fish.

FUNDING SOURCE: USFWS Pacific Southwest region FONS project and internal FHC funds

COOPERATORS: USFWS Arcata FWO, CDFG Iron Gate Hatchery

Methods: In early April 2010, 1500 juvenile Fall-run Chinook will be transported from IGH to the FHC wet lab and reared to a 75-80mm FL size by late May. Treatment and control fish will be held in 4 replicate tanks, acclimated to a mean daily temperature (MDT) of 18°C and vaccinated with formalin-killed *Vibrio (Listonella) anguillarum* at the beginning of the 5 weeks experiment. Treated fish will then be reared for 1 week at 21°C MDT followed by 4 weeks at 23°C MDT. Control fish will remain at MDT of 18°C for the entire period. Blood, anterior kidney, and spleen will be collected weekly for immune function assays (specific = plaque forming cell assay or ELISPOT, anti-vibrio titer; innate = bacteriocidal assay using kidney cells, plasma complement activity) and hematogram profile. Every 10-12days, a bacterial challenge will be conducted with *V.anguillarum* (IP). All assays will be evaluated as percent of control values.

PRODUCTS: Oral presentation at annual Klamath R. Fish Health conference and other scientific meetings, technical report in late 2010 / early 2011.

Failures/ Changes:

1. 3 week experiment not 4wk (only 4 replicate tanks per system available and the decision to obtain a time 0 sample resulting in only 3 tanks (weeks) for the remaining sample).
2. NBT plate method (Secombes 1990 -FITC) – poor adherence of AK cells – NBT color too faint to measure (possible factor was use of ELISA 96 well plate, not tissue culture plates?)
3. PFC and ELISPOT - pilot work in 2008- 2010 unsuccessful
4. Bacteriocidal action of plasma – 20uL of plasma had no inhibition of *V.anguillarum*.
5. Peroxidase AK suspensions - poor morphology of preparations
6. Histology apoptosis (TdT) – pilot work with Trevigen kit unsuccessful with fish tissues
7. IgM ELISA / agglutination assay – problem with assay or effect of immersion vaccination ?
- 8 Cortisol in tank water – interference with SEPAK filter extract in Neogen EIA.
9. Time 0 – controller initiation jumped too rapidly