U.S. Fish & Wildlife Service

California Nevada Fish Health Center
FY2004 Technical Report:

Effect of summer water temperatures on growth and bioenergetics in juvenile Klamath River Coho Salmon (*Oncorhynchus kisutch*).

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Summary: Juvenile Klamath R. Coho salmon, reared under a diurnal temperature regime with a mean daily temperature (MDT) of 21.3°C and adequate energy input, had positive growth, normal plasma protein levels and complement activities, and showed no stress response. They were similar to cohorts reared under a diurnal fluctuating profile with a MDT of 16.0°C.

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Background:

Coho salmon *(Oncorhynchus kisutch*) in the Klamath River basin are listed as a threatened species (1997 National Marine Fisheries Service, S. Oregon-N. California ESU). The Klamath River below Iron Gate Dam often has summer maximums of 25-26°C (Bartholow 2005). Numerous workers have observed the importance of thermal refugia for juvenile salmonids in the Klamath R. (Belchik 2003, Sutton et al. 2007) and reported the use of thermal refugia by juvenile coho when river temperature is 19°C or greater (Sutton and Soto 2012). Thermal refuges in this river are formed primarily by cool water tributary. Additionally, lower reaches of cooler tributaries, that remain connected with the main-stem during the low flow summer conditions, can act as thermal refugia. Juvenile coho salmon are reported to have relatively high temperature thresholds for stenothermic salmonids. Konecki et al. (1995) state a critical thermal maximum of 29°C while Thomas et al. report an LT_{50} of 28°C. Sub-lethal physiological limitations dictate the species range. Brett et al.(1958) reports that maximum swimming speed in juvenile coho occurred at 20°C. Actual distribution of juvenile coho in river systems will tend to be closer to optimal temperature for the species. Walsh et al. (2001) state that coho parr, in the Mattole river system of California, were found in reaches that had maximum weekly maximum temperatures (daily peaks) of 18°C or less. Bisson et al. (1988) describe the occurrence of juvenile coho in productive Washington streams with temperatures over 25°C for over 100h during the summer. These authors hypothesized that increased metabolic demands of higher water temperature was met by the abundant food resources in these streams. The importance of adequate energy resources, under elevated temperatures, is exemplified by the positive growth

showed by juvenile Chinook salmon reared at 19°C fed to satiation while cohorts given a 25% satiation diet had negative growth rates (Myrick and Cech 2002). Similarly, McCarthy et al. (2009) modeled positive growth for juvenile Steelhead in the S. Fork Trinity R. at 23°C only under maximum insect consumption rates but not under the observed stream conditions.

In this study, we asked the question of whether Klamath R. juvenile coho salmon, rearing under elevated diurnal temperatures representative of the Klamath R. during July – September and provided adequate energy, will have positive growth and maintain basal stress and immune function. Juveniles, rearing under a lower temperature diurnal profile reflective of thermal refugia, were used as a comparison population.

Methods:

Experiment outline - Five hundred juvenile Klamath River juvenile Coho salmon (mean 76mm FL, 5.2 g) were transported from California Department of Fish and Wildlife Iron Gate hatchery and 325 salmon placed into a 960L circular tank at the CA-NV FHC wetlab on 01July 2004. One hundred and fifty other salmon were given a 30s dip vaccination with a 200x dilution of killed *Yersinia ruckeri* vaccine (Aquahealth Ermogen) and placed into 40L aquaria (3 tanks each of 25 fish in the "main-stem" and "thermal refugia" systems described below). Water temperature at the hatchery was 13 ºC. Over the next 11 days, fish were acclimated to 18ºC at 1ºC per day. On 12July (11d of acclimation), 164 salmon were moved into another 960L circular on the "thermal refugia" system with 235 left in the original acclimation tank now designated "main-stem" system. A 25 fish (acclimation baseline) sample was collected and diurnal temperature patterns began on this date. On 26July (25 days post-exposure to altered temperature {dpe}), the following actions were performed: 1) 25 fish were sampled for physiological assessment from each circular tank population, 2) 100 main-stem fish were moved to 4 thermal refugia system aquaria (25/each) = move fish, 3) 25 fish from each system were challenged by *Flavobacterium columnare* and another 25 non-vaccinated fish by *Yersinia ruckeri*, and 4) 25 vaccinated fish included in the *Y. ruckeri* challenge. A final sample similar to 26July occurred on 6August (36 dpe). Ammonia, dissolved oxygen, and pH was measured twice per week.

Sample protocols – Rapid netting, by 2 persons, captured all salmon used in bacterial challenges (50 put aside in separate aerated container) and another 25 immediately anesthetized for physiological assessment. A team of 4 persons conducted the following actions on anesthetized (50 mg/L MS222) fish: 1)weight (0.001 g) and fork length (mm) was measured for each fish and the condition factor calculated [KFL = (weight / fork length^3) x 10^5], 2) blood was collected into heprinized microhematocrit tubes from 20 fish after severing the caudal peduncle, 3) plasma was obtained after centrifugation and aliquoted into a 12µL "hemolysis assay" tube and the remainder into a separate tube for lysozyme, cortisol, albumin, and total protein assays, 4) plasma samples were frozen on dry ice and stored at -70°C until assayed, 5) viscera (esophagus to rectum without

liver or spleen) removed, weighed (0.01g), and frozen for later triglyceride assay, and 6) a "steak" of caudal muscle frozen on dry ice for RNA:DNA analysis. Five of the 25 fish were fixed in Davidson fixative for histological archive. For bacterial challenges, 25 fish were given 2h static *F.columnaris* (10^5 cfu/mL) bath challenges while the other 25 fish served as unvaccinated fish in a 10^4 cfu/mL intraperitoneal *Y. ruckeri* challenge. Twenty five vaccinated salmon, held in 40L aquaria at the two experimental temperature regimes, were also challenged by intraperitoneal *Y. ruckeri* injection. Challenged fish were held in 18°C aquaria for 10d to monitor mortality.

Fish rearing and temperature profile - All experimental work was conducted within the FHC wet laboratory. The water supply is ozone disinfected and effluent is treated with chlorine (45 min retention time system with free chlorine residual of 0.3 – 0.5 mg / L). Other biosecurity measures include limited access to the locked building, iodophor footbaths, and individual nets with iodophor containers for each treatment tank. Fish were fed 1.3mm BioMoist Grower salmon diet (BioOregon Inc., Longview, WA) by belt feeder. Daily feed ration was adjusted by subtracting the previous sample number from the estimated initial population to obtain a tank specific population number (N_t). The resultant N_t was then multiplied by the average fish weight to obtain the estimated tank biomass (gram). The %BW/d was 3.0 during the acclimation period and 2.0 %BW/d for the diurnal portion of the experiment. The specific growth rate was calculated with mean weights using the formula, SGR = (((e (lnWeight2 – lnWeight1) / (day2 – day1)) -1) x 100) as described by Ornsrud et al. (2004).

Each system consisted of a 960L circular tank, eight 40L aquaria, 500L sump with 3000W immersion heaters (Clepco) on separate timers and 2 Living Stream Chillers, UV filter and 0.75 hp in-line (Delta star) chiller(see photo on cover). Ambient water temperature during the experiment ranged from 18 - 20°C and was introduced at a 10% system makeup rate. The 3-4°C diurnal profiles were based on July – August 2003 main-stem Klamath River and Beaver creek (rkm 263) thermal refugia data supplied by Sutton et al. (2004). This group has published on related thermal refugia studies at this site (Sutton et al. 2007, Sutton and Soto 2012). Target temperatures for the thermal refugia system was 15 – 19.5°C and 20 – 23.5 °C in the main-stem system (Fig. 1). A system base temperature was maintained from 2:00 to 11:00 by the chiller and one heater group. Four separate heaters in each system were activated every 2h to cause a temperature jump between 11:00 - 18:00 followed by 2h decrease pattern resulting from the inactivation of a given heater group until base temperature was reached at 2:00 (am). The chiller was advanced to the middle temperature in each system during the afternoon to prevent overshooting the maximum temperature set point.

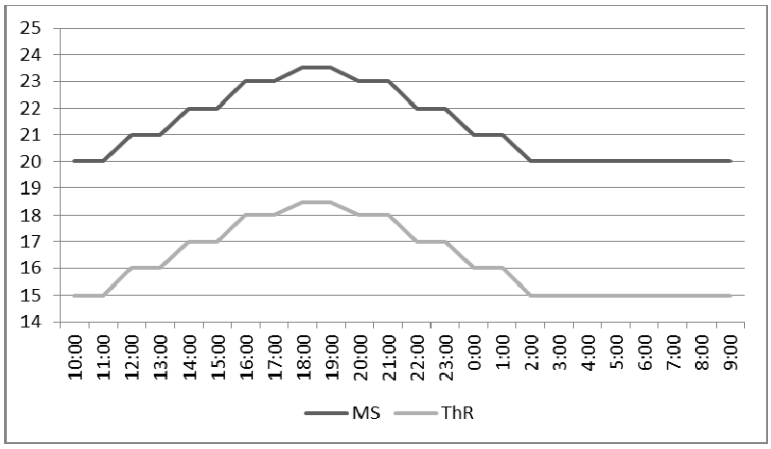

Figure 1. Diurnal temperature targets for main-stem (MS) and thermal refugia (ThR) systems.

Laboratory assays - Hemolytic activity, of the alternative complement system, against rabbit erythrocytes was assayed by the method of Alcorn et al. (2002). Briefly, a 12µL aliquot of plasma was diluted 80X in buffer (0.1% gelatin, 0.1 M EGTA, 0.1 M MgCl2 in veronal-buffered saline), and reacted with 1% Rabbit red blood cells for 60 min at 15°C. The use of a single dilution, due to low plasma volume, precluded calculation of amount of plasma that would lyse 50% of the RaRBC (ACH50). The hemoglobin content of the reaction well was determined by the absorbance at 540 nm. Percent hemolysis was calculated as follows: {(Mean OD sample – Mean OD sample background) – (mean OD neg. control – mean OD Neg. control background) / (Mean OD 100% hemolysis control – mean OD hemolysis background)] * 100. The second plasma sample was assayed for total protein, albumin, lysozyme activity, and cortisol. First, 10µL was tested for lysozyme activity by the method of Ellis (1990) and a 5 µL aliquot was assayed for cortisol 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. Total protein concentration (g / dL) of the same plasma sample was assayed by the Biuret method (Sigma Chemical Co. kit No. 541) and albumin (bromcresol green method, Pointe Scientific kit). Visceral triglyceride content (mg TG / g tissue) was assayed by a modification of Weber et al. 2003. Cold distilled water was added to the 20 mL tube containing the sample (1:1 w / v) and blended for 30 – 90s with a Biospec M133 homogenizer. Absolute isopropanol was added (5x dilution w/v) to an aliquot of homogenate, mixed at room temperature for 20 min, centrifuged at 3220xg for 5 min, and replicate 10 µL samples of the 10x diluted supernatant used in an enzyme assay for triglyceride (Pointe Scientific triglyceride GPO kit). Muscle RNA: DNA ratio was assayed with a Molecular Probes kit (Quant-iT dsDNA and RNA) by the method of Weber et al. (2003) and Kaplan et al. (2001).

Statistical analysis - Analysis was performed with SigmaStat 3.1 software on raw data. 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-Wallace 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. Statistical significance is reported with these corresponding test statistics:

T-test	t	
Mann- Whitney U test on ranks	T	
1-ANOVA	F	(Holm-Sidak MC)
Kruskal-Wallace ANOVA on ranks	H	(Dunns MC)

Results and Discussion:

Water quality and temperature – Unionized ammonia (0.001 – 0.041 mg/L), dissolved oxygen (6.4-7.9 mg/L), and pH (7.24 – 8.36) were deemed acceptable during the experiment. Between 12July - 6August, mean daily temperature were 16.0° and 21.3°C for the thermal refugia and main-stem systems, respectively (Fig. 2). The thermal refugia system had greater variation in temperature particularly in the first 8 days of the experiment (Fig. 2). Warm ambient air temperature likely influenced this daily variation. Salmon in the 2 systems experienced different temperature durations in the experiment. Main-stem fish spent the majority of the day at two temperatures (29% at 23ºC and another 43% at 21ºC). Only 17% of their day was spent at the lower temperatures (19º and 20ºC). In contrast, thermal refugia fish spent 32% of their day at the low temperature (15ºC) with the remaining day evenly spread between the other temperatures (Fig.3).

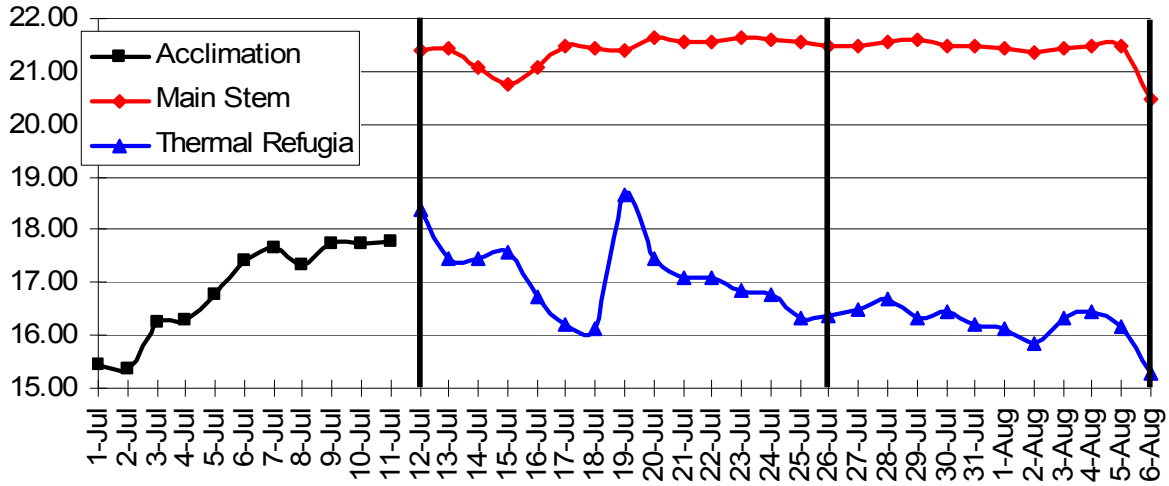

Figure 2. Mean daily temperature (ºC) of the main-stem and thermal refugia systems as well as the common acclimation temperatures.

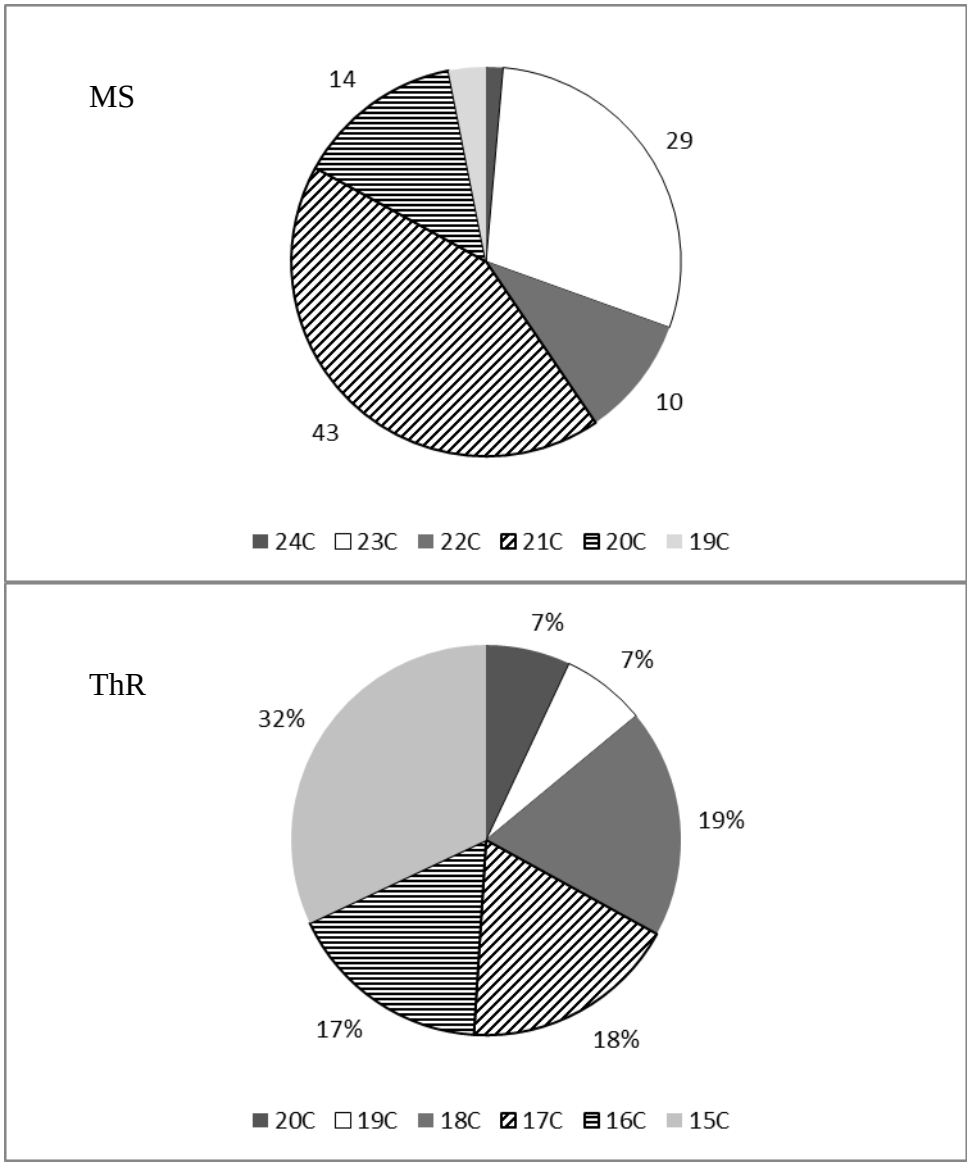

Figure 3. Mean percentage of day spent at 6 temperatures in main-stem (MS) and thermal refugia (ThR) system during experiment.

Growth and morphometrics – After the first 2d of diurnal temperature manipulation, feeding response in the circular tank was consider normal for both groups as no uneaten food was observed on the bottom. No mortalities occurred over the 36d experiment. Little growth was observed during the initial 11d acclimation period (Fig. 4). The specific growth rate increased over the experiment for main-stem fish while thermal refugia fish had a marked decrease between the 2 sample dates (Fig. 4). We have no clear explanation for the latter trend as both groups received the same quantity of food per day. Coho in both regimes increased in size (FL, WT, KFL) over the 36d experiment (Table 1). At the end of the experiment, fish size was similar in both systems.Visceral triglyceride levels was similar throughout the experiment regardless of the temperature. One exception was the decline seen in 26July thermal refugia fish compared to the acclimation sample (Table 1).

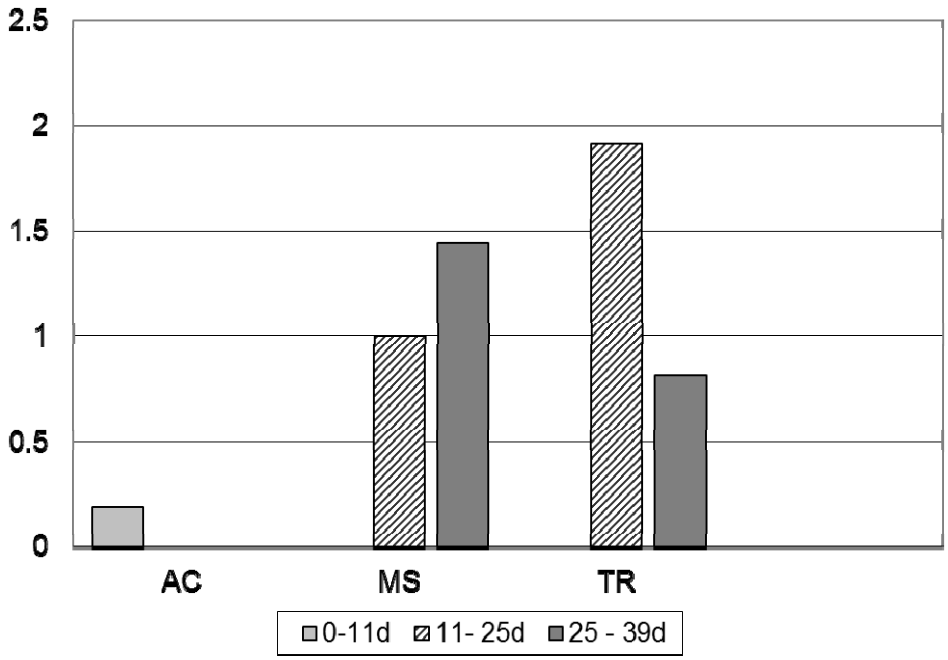

Figure 4. Specific growth rate (%BW/d = natural log ([w2-w1] / [t2 - t1]) - 1) x 100) of Coho salmon sampled at the end of the common acclimation period (ACC), and from the main-stem (MS), and thermal refugia (ThR) temperature regimes.

Table 1. Mean (SD) fork length (mm, FL) ,weight (0.1g, WT), condition factor ($WT/FL^3 \times 10^5$= KFL), Viscera somatic index (Viscera wt / fish wt, VSI), viscera triglyceride (mg TG/ g viscera, V-TG) of juvenile Coho salmon sampled at the end of the common acclimation period (ACC), and from the main-stem (MS), and thermal refugia (ThR) temperature regimes.

	FL	WT	KFL	VSI	V-TG
ACC	76 (9) $_a$	5.3 (1.4)$_a$	1.082$_a$ (0.119)	0.090$_a$ (0.015)	2.62$_a$ (0.44)
26jul MS	80 (6) $_{ab}$	6.1 (1.4)$_{ab}$	1.154$_{ab}$ (0.071)	0.090$_a$ (0.010)	2.57$_{ab}$ (0.21)
26jul ThR	84 (6) $_b$	6.9 (1.5)$_{ab}$	1.153$_{ab}$ (0.074)	0.096$_a$ (0.010)	2.43$_b$ (0.16)
6Aug MS	85 (5) $_b$	7.4 (1.4)$_b$	1.217$_b$ (0.124)	0.114$_b$ (0.015)	2.51$_{ab}$ (0.15)
6Aug Thr	86 (6) $_{bc}$	7.7 (1.6)$_b$	1.202$_b$ (0.060)	0.113$_b$ (0.017)	2.47$_{ab}$ (0.17)

Letters in common indicate no significant difference by Kruskal-Wallis 1-way ANOVA, P> 0.05.

We estimate that the salmon received 3.53 kcal/g of feed using the crude feed analysis supplied by Bio-Oregon and fuel values reported for the nutrient classes as reported by Phillips and Brockway (1959) in Smith (1988) (Table 2).

Table 2. BioMoist 1.3 mm Grower guaranteed analysis.

crude protein 42.5% x 4.5 = 1.91 kcal/g,
crude fat 14% x 8.0 = 1.12 kcal/g
carbohydrate (CHO = 100-sum of protein, fat, ash, and fiber)
31% x 1.6 = 0.50 kcal/g.
3.53 kcal/g feed

Using the 2%BW/d feeding rate, we estimate that the 4.9g fish (pound count data of circular population not sample data) at the beginning of the diurnal regime (12July) could have each received 0.10g/fish of feed per day. This quantity would provide 0.353 kcal of energy. After the 26July sample, the 6 – 7 g fish could have consumed 0.41 – 0.47 kcal per day. MacCarthy et al. (2009) report that juvenile Steelhead in the South Fork Trinity R. (in Klamath basin) consumed aquatic larvae and nymphs with an mean energy density of 0.80 kcals/g. Applying this 0.80 kcal/g insect value to the 0.35 – 0.47 kcal intake per day data for the appropriate size (weight) salmon we can estimate that experimental fish would have had to consume approximately 8.3 – 8.9%BW/d (0.4 – 0.6g) of aquatic insects to equal the pellet diet. This feeding quantity seems reasonable in a productive system like the Klamath River, however, consumption data for this stock was not found for comparison. Higgs et al. (1995) reports that coho fry feed predominantly on chironomid larvae and pupae as well adult insects in the drift.

Plasma cortisol, protein, and complement activity - Plasma cortisol levels ranged from 1.4 – 18 ng/mL and were within the basal level reported for the species (Barton et al. 1985). An example of acute stress response is demonstrated by plasma cortisol values of 119 and 165 ng/mL detected in 2 coho juveniles held in a bucket for 25 minutes prior to sample. The 11d acclimation and 2 week post-temperature regime samples (25 dpe) showed no elevated stress cortisol response and were similar (Kruskal-Wallis 1-way ANOVA, P= 0.132). While main-stem fish tended to have lower levels than thermal refugia cohorts sampled on the same date (Fig. 5), no significant difference was detected by (Mann-Whitney Rank sum test, P$\leq$ 0.074). Cortisol levels rose slightly in both temperature groups between 25 and 36dpe.

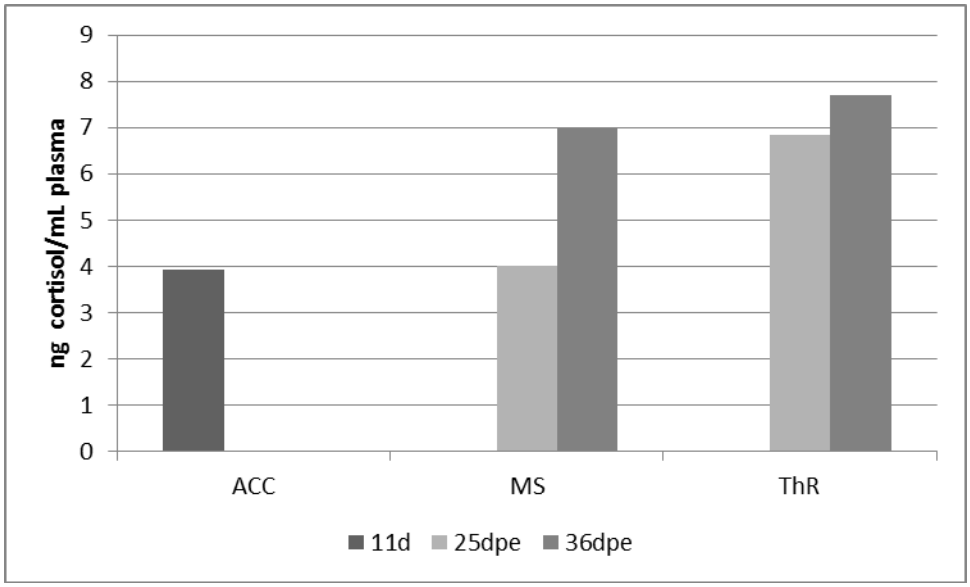

Figure 5. Mean plasma cortisol (ng/mL) from Coho sampled at the end of the 11d common acclimation period (ACC), and from the main-stem (MS), and thermal refugia (ThR) temperature regimes at 25 and 36d into the experiment. Standard errors are not shown but ranged from 0.5 – 2.4.

Plasma protein (mg/mL) content rose slightly over the experiment but was within normal ranges (Fig.6). The 36dpe thermal refugia fish had significantly higher values than the acclimation group sampled at 11 d (1-ANOVA, P=0.002). These values are slightly higher than those seen in juvenile Chinook salmon (3 mg/mL) held at 20°C (Foott et al. 2004) but within the 1.4 – 4.3 mg/mL range reported by Wedemeyer and Chatterton (1971) for juvenile coho. Plasma complement activity, as measured by Rabbit RBC hemolysis, was not influenced significantly by either the acclimation period or the 2 temperature regimes (Kruskal-Wallis ANOVA, P=0.138). While not significant, main-stem values tended to be higher than thermal refugia samples with a general increase in both temperature groups between 25 and 36dpe (Fig. 7). The mean activities observed in the coho

sample groups were similar or slightly higher to those in juvenile Chinook reared at 20°C (mean activities of 42- 72%) (Foott et al. 2004).

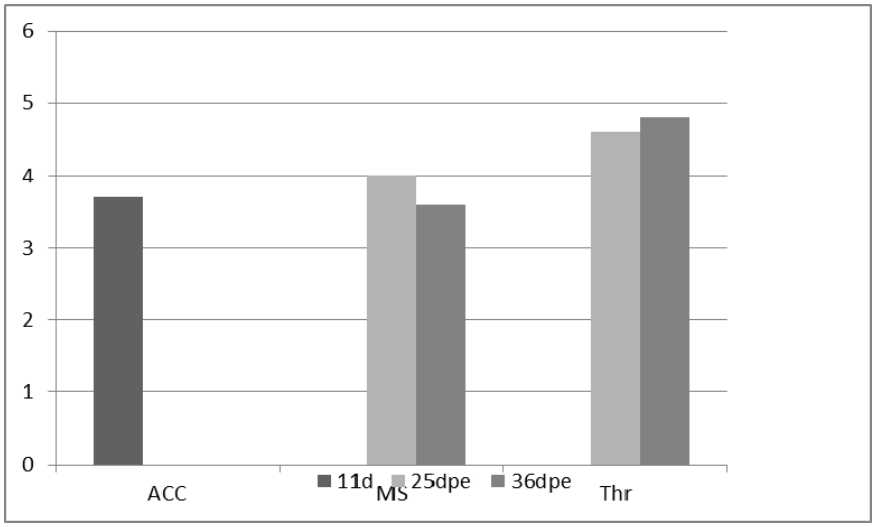

Figure 6. Mean plasma protein (mg/mL) from Coho salmon sampled at the end of the 11d common acclimation period (ACC), main-stem (MS), and thermal refugia (ThR) temperature regimes at 25 and 36 dpe. Standard error is not shown and ranged from 0.05 – 0.57.

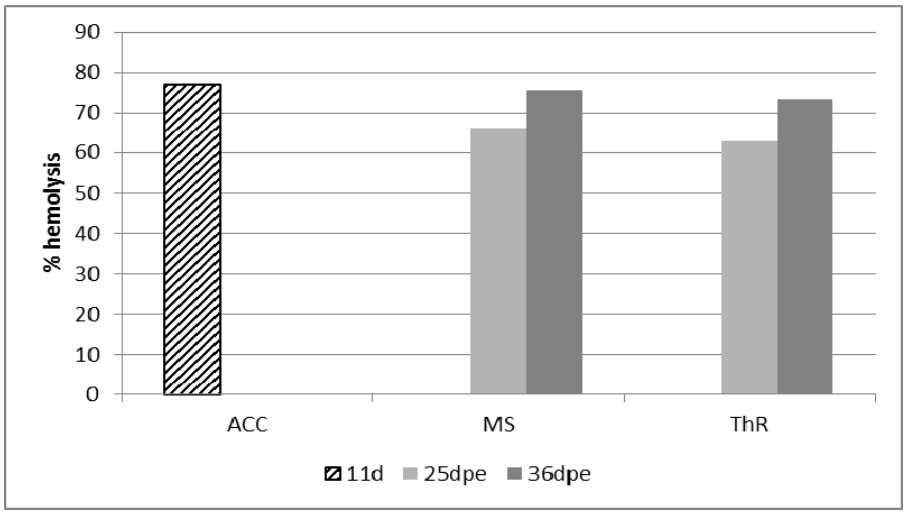

Figure 7. Mean percent rabbit RBC hemolysis activity of plasma from Coho salmon sampled at the end of the 11d common acclimation period (ACC), main-stem (MS), and thermal refugia (ThR) temperature regimes at 25 and 36 dpe. Standard error is not shown and ranged from 2.7 – 5.0%.

Conclusions: Juvenile Klamath R. coho salmon, rearing under a mean 21.3°C diurnal temperature regime with adequate energy input, had positive growth, normal plasma protein levels and complement activities, and showed no stress response. They were similar to cohorts reared at a mean temperature of 16.0°C. Given the upper thermal thresholds reported for the species, we conclude that growth should not be impaired during the summer if there is adequate feeding opportunity and particularly if juveniles have access to thermal refugia when temperatures are ≥ 23°C. Use of thermal refugia can have negative impacts on juvenile salmonids. Concentration of juveniles within thermal refugia increase the likelihood of avian predation and the horizontal transmission of infectious pathogens such as the endemic bacteria *Flavobacterium columnare* and ectoparasite *Ichthyophthirius multifills* associated with the 2002 adult salmon mortality event (California Department of Fish and Game, 2003).

Our wetlab system of immersion heaters on mechanical timers along with chillers did not produce consistent profiles for the thermal refugia group due to the effect of high and variable ambient air temperatures. We have since converted to a temperature controller system that blends hot and cold water. Feeding behavior may have changed as the density of fish decreased over time during the experiment as dominance could have occurred in the tank populations. It is possible that this dominance effect could be involved in the deceased growth rate observed in the thermal refugia population over the last 11d of the experiment. Several measurements (plasma lysozyme activity, albumin:globulin ratio, bacterial challenge mortality, immersion vaccination and antibody titer, and muscle RNA:DNA ratio) conducted during the experiment did not provide accurate data and are listed only in Appendix 1.

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Contribution of authors:
JS Foott
study proposal, wetlab design, laboratory assays, data analysis, report

Rick Harman and Ron Stone
wetlab system design and construction, fish culture, laboratory assays, data entry.

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Appendix 1. 2004 experiment methods / data not discussed in report.

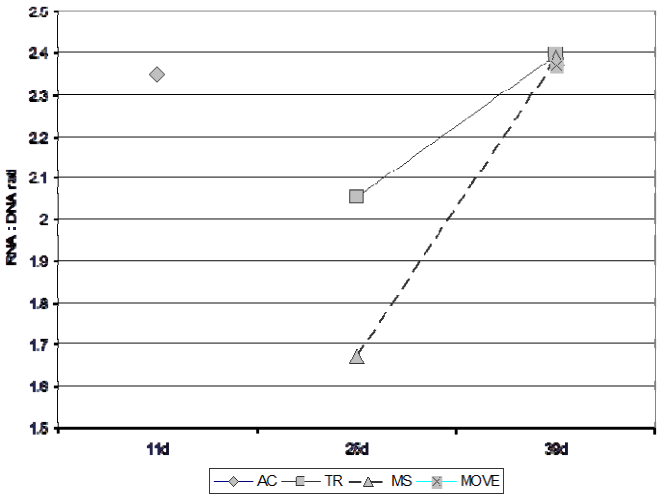
1. Moving 100 fish sublot on 25dpe from MS to ThR system were placed into 40L aquaria. Different rearing condition may have confounded feeding and growth response. <u>Appeared to induce stress.</u>
    a. Different conditions than circular tank– high cortisol values in fish 11d post-move.
    b. Higher %H than both MS and ThR circular salmon but not significant
    c. Protein = Move fish > both ACC and 7/26MS, no values>6.0
    d. Plasma cortisol was variable (3.8 – 47.5 ng/mL) with 3 fish having elevated values.

2. Two hour static *F columnaris* (10^5 cfu/mL) bath challenges killed all fish within 24hs – dose too high on both 25 and 36 dpe sample dates.

3. *Y.ruckeri* vaccination (Ermogen 200x , 30s dip)– 25 salmon moved into six 40L aquaria (3 MS, 3 ThR)
    a. 27July 10^4 cfu/mL IP challenges – very low mortality response in all groups, no data
    b. Plasma antibody agglutination titer at 36 dpe – 4 to 8x dilution similar for controls and vaccinates indicates insignificant titer induced by immersion

4. Plasma lysozyme activity – Plasma (10µL) lysozyme activity was not detected as the samples were thawed during sorting by accident prior to the assay.

5. Nitroblue tetrazolium dye assay of anterior kidney cell suspension (reactive oxygen response) assayed on 25 dpe, fish too small to obtain enough cells and assay dropped from remainder of study.
    .
6. RNA:DNA muscle - **<u>all fish similar by 36dpe</u>**.
    a. Later extraction work shows that RNA data is suspect due to RNAase effects.



7. A:G = Total protein - BCG albumin = globulin estimate. Albumin values very low and questionable.
    a. A/G data seems too low (means 0.25 – 0.45), literature is typically in the 0.5-0.6 range. We reported protein data only.
    b. Plasma samples (2µL albumin and 4 µL 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 (AG) was calculated as (Albumin (mg/mL) / [protein (mg/mL) – albumin]). Bovine serum albumin was used as the standard in both assays. The bromcresol green reagent used in the albumin assay is reported to slightly overestimate albumin concentrations.