Physiological responses of juvenile chinook salmon held in the Lower Klamath River and thermal refugia (June – August 2000).

Laboratory analysis component of Cooperative study with Yurok Tribe Fisheries

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Summary : Replicate groups of juvenile Iron Gate Hatchery chinook were held for 4 to 12 days in either the mainstem Klamath River or within 2 thermal refugia (mouths of Cappel and Pecwan Creek). Control groups were held in Cappel creek water. All groups experienced the same stress of handling, direct transfer into the exposure site, and confinement within a live cage. The additive stress of elevated temperature in the mainstem site was the primary focus of the study. Mean daily water temperatures (MDT) for the mainstem exposures ranged from 19 – 23 °C and were 3- 7 °C higher than the corresponding thermal refugia (MDT). Mainstem exposure groups incurred significantly higher mortality than the thermal refugia cohorts. Mortality began at 4 days post-exposure and was associated with symptomatic *Flavobacterium columnare* infections. Plasma globular protein concentrations were elevated in exposure survivors particularly in mainstem exposure fish. Gill Na-K-ATPase activities of mainstem fish tended to be lower than thermal refugia groups. Results from this study point out the survival benefits for juvenile salmonids that reside in thermal refugia along the Klamath River during the spring and summer months.

INTRODUCTION

Declining chinook populations in the Klamath basin have prompted an intense restoration effort of this valuable resource and a key element of the State's aquatic biodiversity.   Both infectious and non-infectious diseases are an important factor in smolt survival. Ceratomyxosis and columnaris disease are 2 common and significant infectious fish diseases during the spring – summer period in the Klamath River (Foott et al. 1999, Williamson and Foott 1998, Hendrickson et al. 1989). Elevated water temperatures, often in excess of 20° C during the late spring and summer, have been identified as a stressor for anadromous fish in the Klamath River Basin (Klamath R. Basin Fish. Task Force 1991, Zedonis and Newcomb 1997).

As per a cooperative agreement (fws#1937-0012, $14,150), the California – Nevada Fish Health Center (FHC) provided the Yurok Tribe Fisheries office with written study design recommendations, initial fish health sample collection training (14June2000), field collection of physiological sample groups, diagnostic laboratory analysis of specimens, and this narrative report of the laboratory results. Fish husbandry, daily evaluation of live boxes, temperature data, mortality data on one live box per exposure (YF) and a comprehensive analysis of the study was the agreed responsibility of the Tribe.

This study examined the health and physiological response of Iron Gate Hatchery chinook salmon juveniles held for 4 – 12 days in either the mainstem Klamath River (temperature range 15 – 23 °C) or thermal refugia within the mouths of 2 lower Klamath River tributaries (range 15 - 21°C). Mainstem exposure groups incurred higher mortality and tended to show larger physiological responses than refugia groups. This report is focused on the health and physiological assay results of the study.

MATERIALS AND METHODS
The exposures took place at two sites located along the lower Klamath River.
The first site was at the mouth of Cappell Creek that is approximately 10 river
miles downstream from the confluence of the Klamath and Trinity Rivers. The
Yurok Tribe Fishery Office hatchery is adjacent to lower Cappel creek. The
second site, Pecwan Creek, is approximately 8 miles downriver of Cappel Creek.
On 21June2000, approximately 2,000 Broodyear 1999 Iron Gate Hatchery fall-
run chinook salmon juveniles (*Oncorhynchus tshawytscha*) were graciously
transported by California Department of Fish and Game hatchery personnel to
the Yurok Tribe's Cappel Creek hatchery. These fish were held in 2.5 m^3
rectangular tanks supplied with 11 – 14 °C creek water and fed at 2 – 3 % BW / d
with semi-dry salmon diet. Replicate groups of 25 fish were held in 0.12m^3 live
boxes for evaluation of survival (Yurok Fisheries replicate) and physiological
responses (FHC replicate). The live boxes were constructed of one ft. diameter
PVC pipe with mesh netting attached to both open ends. The exposure locations
are listed below in Table 1.

Table 1.   Site locations and group identifier for exposures.

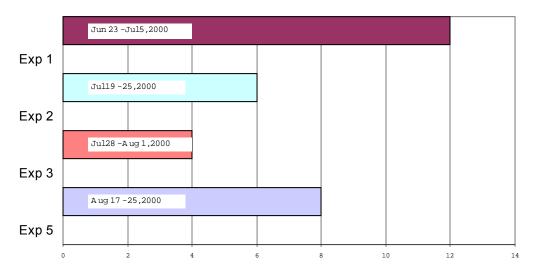| Site	Group identifier	Exposures
Cappel hatchery	CH	1 - 5
Refugia, Mouth of Cappel creek	CR	1 - 5
Mainstem Klamath River above Cappel creek	CM	1 - 5
Refugia, mouth of Pecwan creek	PR	2 - 4
Mainstem Klamath River above Pecwan creek	PM	2 - 4

Fish were moved directly from the hatchery to the exposure site without
acclimation and were not fed for the duration of the exposure. Each live box was
checked daily for mortality and hourly temperatures were recorded by Onset
Computer Corp. Stowaway ® or TidbiT® probes . The original study plan called
for 14 day exposures prior to physiological sample collection. High mortality rates
in the mainstem groups forced us to sample the FHC replicates between 4 and
12 days post-exposure (Fig. 1).

       Necropsy – Physiological assay live box survivors (FWS) were evaluated
by a modified organosomatic assay (Goede and Barton 1990, Foott 1990). The
organosomatic assay is a method for ordered observation and reporting of the
gross morphology of selected organs, hematological parameters, and size
criteria of each individual. Features evaluated included skin condition (scale
loss), gill, visceral fat, and any gross abnormalities of internal organs. Hematocrit
(% packed erythrocyte volume) and leukocrit (% white blood cell volume) were
determined from centrifuged microhematocrit tubes containing heparin. Plasma
was frozen at - 70°C for later analysis. Condition factor was calculated from the
fork length (K = {weight (g) / length (mm)^3 x 10^5}). Tissue samples for percent
lipid, gill ATPase, histology, and kidney phagocyte assay were collected from
subsets of each exposure survivor group. . Imprints of gill and spleen tissue from

moribund fish were gram stained and evaluated for filamentous gram-negative rods as presumptive *Flavobacterium columnare* infections.

Figure 1.

Exposure Days

Percent lipid- Analysis of body lipid content was assayed using a modification of the Bligh and Dyer (1959) chloroform:methanol (CM) method. Fish carcasses with head and tail removed were frozen at –70°C, crushed while frozen into a powder-like consistency, homogenized and a 1 gram subsample placed into a 50 mL screw cap test tube. Ten milliliters of a 2:1 CM mixture was added to the 1 g tissue homogenate, allowed to extract for 1 hour, and then deionized water was added to the mixture at 40% of the CM volume. To speed phase separation, the tubes were vortex mixed and then centrifuged at 3000xg for 5 minutes.   A volume of the chloroform layer was removed by pipette, evaporated in a weighing pan and the lipid residue weighed to the nearest 0.001 g. Percent lipid was calculated with the formula: Percent lipid = [lipid wt / sub-sample wt] x [original chloroform volume / volume extracted] x 100.

Histology – Gill, intestinal tract, pyloric ceca, posterior kidney, and liver tissue was rapidly removed from the fish after death and immediately fixed in Davidson's fixative, after 24 hours transferred to 70 % ethanol, later processed for 5 μm paraffin sections and stained with hematoxylin and eosin (Humason 1979). All tissues for a given fish were placed on one slide and identified by a unique code number. Each slide was examined at both low (40X) and high magnification (400X) without knowledge of treatment group.

Kidney Phagocyte Activity - Respiratory burst capacity of anterior kidney cells, as a marker for viable phagocytes, was performed on cell suspensions immobilized in agarose by the nitroblue tetrazolium (NBT) dye method (Ellsaesser et al. 1984). Briefly, the anterior kidney ("cephalic horn" region) was aseptically removed from the fish and placed into pre-weighed tubes of chilled MEM with antibiotics plus 5% FBS (MEM5) for transport. Within 24 h of

collection, the tissues were weighed to the nearest 0.001 g, disrupted into a single cell suspension by titration with a 3 mL syringe and 21G needle, washed by centrifugation, and re-suspended in 300 μL of MEM5. Triplicate wells of a 96 well NUNC plate received 10 μL of the suspension for later cell enumeration by DNA quantification (Molecular Probes, Inc. CyQuant C-7026 kit) and another 10 μL drop was mixed with 100 μL of warm 0.4% agarose. The mixture was immediately spread onto a microscope slide, solidified by cooling on a ice pack, and immersed in 1 mg / mL solution of NBT for 15 minutes, fixed in cold absolute methanol, and counterstained with 0.2% fast green stain. The dried preparations were examined microscopically at 1000x oil immersion and the number of NBT (+), erythrocytes, and non-erythrocyte NBT(-) cells counted from a total of 200 cells to derive the percentage of NBT (+) cells for each fish.

Plasma protein analysis – Plasma protein electrophoresis was performed with a 1 μL sample run on an agarose gel (1M barbital buffer, 90V for 45 min.). The electrophoresed samples produced up to 11 bands including 1-3 pre-albumin fractions, albumin, and 5 - 7 globulin fractions (F1 - F7). The stained gels were scanned and the percent area of each fraction determined with Scanalytics ™ One-Dscan software. Analysis of variance was performed on the arcsine of the percent area for each fraction (or combined fractions).   An albumin: globulin protein ratio (A/G) was calculated from the % area measurements of the pre-albumin + albumin fractions divided by the sum of the F1-F7 globulin fractions. The A/G ratio is an index used to track relative changes in the composition of serum or plasma (Jacobs et al. 1990). A drop in A/G can indicate a shift from albumin production to globular proteins in response to infection. Total protein concentration was assayed with a Pierce BCA kit (no. 23225). The 30X diluted sample (5 μL plasma: 145 μL water) was placed into replicate wells, reacted with BCA reagent and the color intensity measured at 540 nm. Albumin (20 mg / mL) was used as the standard in the total protein assay. Plasma osmolarity was measured with a Wescorr ™ vapor osmometer.

ATPase - Gill Adenosine Triphosphatase activity (ATPase = μmoles ADP / mg protein / hr) was assayed by the method of McCormick and Bern (1989). Briefly, gill lamellae were dissected and frozen in sucrose-EDTA-Imidazole (SEI) buffer on dry ice. The sample was later homogenized, centrifuged, supernatant sonicated, protein content assayed, and the ATPase activity determined by the decrease over time in optical density (340 nm) as NADH is converted to NAD+. This activity was reported as μmole ADP / mg protein / hr as 1mole of NAD is produced for each mole of ADP generated in the reaction. Gill Na-K-ATPase activity is correlated with osmoregulatory ability in saltwater and is located in the chloride cells of the lamellae.
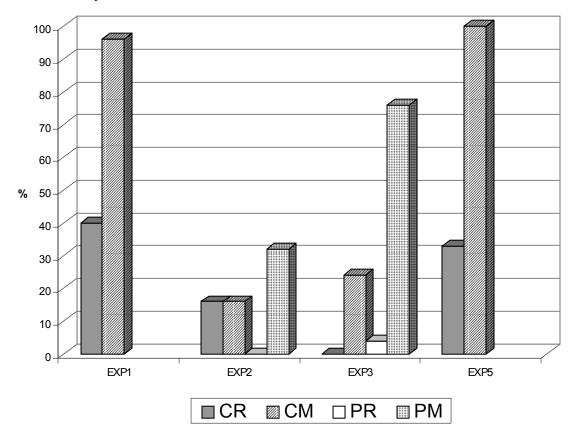
RESULTS

Water Quality –Temperature data for exposure 4 was not recovered. Inability to correlate temperature with physiological measurements necessitated the removal of this exposure from the study data set (appendix X). Physiological assay live box survivors from exposures one, two, three and five were sampled after 12, 6, 4 and 8 days post-exposure, respectively (Fig. 1). The range of dissolved oxygen concentrations measured at the end of each exposure did not go below 8.0 mg /L (mainstream 8 – 9.7 mg/L, refugia 8.7 – 10.4 mg/L, and the hatchery ranged from 10.7 – 11.7 mg/L). The mean daily temperature (MDT) of the thermal refugia sites were 2.7 – 7.1 °C lower than their associated mainstem sites (Table 2). The MDT of the CM site ranged from 18.9° to 22.7 °C with a maximum hourly reading of 25.5°C occurring during exposure 3. Mainstem exposure groups, held above Pecwan creek, experienced MDT of 22.2° and 23.3°C compared with the Pecwan refugial group MDT of 16.9° and 16.1°C. Maximum temperature of 25 °C at the PM site was similar to the CM site during exposure 3. If 21 °C is selected as a stressful temperature threshold, exposure groups in the mainstem experienced much longer periods above this threshold than their respective thermal refugia cohorts (Table 2). The number of hours above 21°C at the CM site ranged from 0 to 133 compared to a maximum of 3 h at the CR site. Similarly, the chronically high temperature profile at the PM site was shown by the long periods above 21 °C (142 and 141 hours compared with 4 and 1 hours at the PR live box).

Mortality – Due to incomplete daily mortality records, the cumulative mortality figures for the FWS live boxes are based on the number of live fish sampled at end of each exposure divided by the total (25or 15). Mortalities recorded during periods between the stated exposures calls into question the start dates for some exposure groups. Excluding exposure 5, four of five mainstem FWS exposure groups experienced higher mortality than their thermal refugia cohorts (Fig. 2). In exposure 2, both CM and CR groups had 16% cumulative mortality over the six day period. Mean daily temperature for the CM group was 18.9 ºC and 16.9ºC for the CR group. No comparison of mortality was possible in exposure 5 as the entire CM group was lost after an otter(s) broke into the live box on 8/24/2000. No mortality occurred to any control fish held within live boxes at the hatchery. Few mainstem or refugia fish died until 4 days post-exposure and mortality was often associated with eroded lesions of the skin and gill. Gram-stain imprints of gill and spleen from a subset of these moribund fish with lesions contained filamentous gram-negative bacteria characteristic of *Flavobacterium columnare*. Contact with the live box netting appeared to cause many fish to develop abrasions on the tail and snout. *Flavobacterium columnare* infections were probably accelerated by these abrasions, however, CH fish with abrasions did

Table 2.   Water temperature minimums, maximums, mean daily temperature (MDT), and hours above 21° C measured during exposures 1,2,3 and 5 in Cappel hatchery (H), Cappel mainstem (CM), Cappel refugia (CR), Pecwan mainstem (PM), and Pecwan refugia (PR) live boxes.

		M.D.T.(ºC)	Min	Max	Hours > 21°C
Exp 1	H	13.68	11.29	16.27	0
	CR	16.47	14.09	19.02	0
	CM	20.97	15.73	23.55	29
Exp 2	CR	16.12	14.03	18.00	0
	CM	18.91	14.64	20.58	0
	PR	16.88	12.91	21.71	4
	PM	22.24	21.31	23.88	142
Exp 3	CR	19.38	17.34	21.91	2
	CM	22.77	14.97	25.47	84
	PR	16.12	13.66	21.02	1
	PM	23.26	22.14	25.04	141
Exp 5	H	13.94	11.90	17.69	0
	CR	18.76	15.27	21.07	3
	CM	21.43	18.70	22.31	133

Figure 2. Cumulative percent mortality for fish held live boxes at Cappel mainstem (CM), Cappel refugia (CR), Pecwan mainstem (PM), and Pecwan refugia (PR) during exposures (EXP) 1,2,3,and 5. No mortalities occurred in the Cappel Hatchery control groups and salmon in CM-5 were attacked by a river otter .

not develop columnaris. It is unclear why fish without external lesions died. No parasitic or other bacterial infections were detected in these fish, however, many of the survivors had altered plasma ion concentrations. This observation is based on the combination of normal plasma protein concentrations (see below) and low plasma osmolarity seen in many exposure survivors. Osmolarity (mmol / kg) is a measure of the total dissolved particle concentration without regard to charge or particle size. Protein and ions are the main constituents in fish plasma. A reference plasma osmolarity range of 298 – 330 mmol / kg (mean of 314 $\pm$ 1SD or 16 mmol /kg) for this juvenile chinook population was determined from thirty CH samples. Of the 28 CM, CR, and PR survivors tested for osmolarity, 36 % were below the 298 mmol/kg threshold.   Half of these fish had osmolarity values below 2 SD (283 mmol / kg).

Histology – Seventy-three fish representing a subset of the FWS survivors from each exposure group were sampled for histological examination. No parasites or tissue lesions were observed in any of the fish.   Most fish showed the following signs of food deprivation: reduced intestinal villi height, vacuolated intestinal epithelium, reduced acinar cell size and zymogen granule content, and low hepatocyte glycogen content (lack of vacuoles). Despite the Ceratomyxosis mortality that was observed in Klamath R. out-migrant smolts during the summer, this parasite was not seen in the exposure groups (Manji. 2000. CDFG memo Oct. 25, 2000). It is likely that either the duration was too short for infection or the live box fish were not exposed to the infectious stage of *Ceratomyxa shasta*.

Organosomatic data - Salmon reared at the hatchery grew during the two month study period as indicated by increased length and weight for each exposure (Table 3). No significant loss in weight, condition factor, or percent body lipid was detected between the live box groups for each exposure. Hematocrit data was normal for all groups and only exposure 5 showed a statistically significant difference (Mann-Whitney Rank sum test, P= 0.001) in leukocrit between the CH and CR fish (Table 3). The refugia fish in this 8 day exposure having approximately half the circulating leukocytes as the control fish. The refugia and mainstem fish of exposure 3 (4 days) showed an increased leukocrit compared to the hatchery controls, however this difference was not statistically significant (Kruskal-Wallis ANOVA, P= 0.0635).

Kidney Phagocyte counts –The effect of temperature on the number of viable anterior kidney phagocytes (Nitroblue tetrazolium positive, NBT+) was variable among the different exposures and appeared to be influenced by duration of the exposure (Table 4). For example, the refugia and mainstem groups from the 4 day exposure 3 showed elevated NBT(+) cell numbers in comparison to the Cappel hatchery controls. The opposite trend was seen in the 6 day exposure 2.   No statistical differences were detected between the sites for each exposure. Variability in the % NBT positive cell (phagocytes) was judged to be relatively high in the sample groups with coefficient of variation ranging from 27 – 130%. No obvious correlation between animal health and % NBT(+) was

Table 3.	Mean (± SEM) fork length (FL,mm), weight (WT,g), percent body lipid (% lipid), condition factor (KFL), leukocrit (LCT), and hematocrit (HCT) of hatchery controls(H), Cappel thermal refugia (CR), Cappel mainstem (CM), Pecwan thermal refugia (PR), and Pecwan mainstem (PM) live box fish for exposures 1,2,3,and 5. The number of samples (N) is listed for each measurement.

		FL	N	WT	N	% LIPID	N	KFL	N	LCT	N	HCT	N
EXP 1	H	96.4 ± 1.49	16	8.8 ± 0.43	16	3.62 ± 4.35	4	0.97 ± 0.02	16	0.64 ± 0.16	16	43.34 ± 2.15	16
	CR	91.3 ± 1.53	15	6.6 ± 0.29	15	5.16 ± 4.24	4	0.87 ± 0.03	15	0.74 ± 0.06	12	41.80 ± 1.69	12
	CM	94.0 ± 2.00	2	8.5 ± 1.50	2	nd		1.01 ± 0.12	2	1.29	1	33.85	1
EXP 2	H	99.0 ± 2.90	13	10.7 ± 0.78	13	nd		1.08 ± 0.03	13	0.61 ± 0.08	13	41.84 ± 1.10	13
	CR	98.7 ± 1.39	21	10.2 ± 0.53	21	2.26 ± 1.34	3	1.05 ± 0.02	21	0.62 ± 0.09	11	38.62 ± 1.44	11
	CM	97.5 ± 1.48	22	9.4 ± 0.45	21	nd		1.00 ± 0.02	21	0.57 ± 0.10	11	33.12 ± 1.07	11
	PR	97.6 ± 1.65	25	9.5 ± 0.49	25	4.70 ± 8.05	4	1.00 ± 0.03	25	0.61 ± 0.07	6	39.95 ± 0.99	6
	PM	97.7 ± 1.92	19	10.0 ± 0.54	19	6.12 ± 7.23	4	1.06 ± 0.02	19	0.43 ± 0.12	5	47.00 ± 6.52	5
EXP 3	H	105.1 ± 1.62	25	11.4 ± 0.52	25	4.02 ± 3.02	4	1.00 ± 0.02	25	0.61 ± 0.11	8	40.84 ± 0.94	8
	CR	102.4 ± 1.44	24	11.0 ± 0.52	24	3.78 ± 4.41	4	1.01 ± 0.02	24	0.85 ± 0.04	8	33.89 ± 5.02	8
	CM	102.3 ± 1.42	20	10.6 ± 0.45	20	5.36 ± 7.92	4	0.99 ± 0.03	20	1.22 ± 0.21	8	32.47 ± 0.97	8
	PR	103.4 ± 1.55	25	10.8 ± 0.47	25	3.53 ± 2.83	4	0.97 ± 0.03	25	0.92 ± 0.06	6	38.27 ± 0.82	6
	PM	106.2 ± 0.98	6	12.7 ± 0.68	6	4.08 ± 2.84	4	1.06 ± 0.05	6	0.91 ± 0.24	5	38.67 ± 2.05	5
EXP 5	H	108.1 ± 2.25	13	12.5 ± 0.89	13	3.88 ± 2.95	4	0.97 ± 0.02	13	0.99 ± 0.06	8	39.89 ± 0.73	8
	CR	113.6 ± 2.04	14	15.1 ± 1.07	10	4.15 ± 5.44	4	0.98 ± 0.02	10	0.49 ± 0.13	8	40.07 ± 1.59	8

observed in the sample groups, however, several aspects of the assay were sub-optimal for valid testing. The anterior kidney cell suspensions produced approximately 10^7 cells / mL (as counted by both hemocytometer and CyQuant DNA kit). Cells remained intact throughout the procedure and viability was greater than 90% (as judged from sub-samples in 0.2% trypan blue solution). The percentage of red blood cells in the NBT slides ranged from 3 – 21%. Our original plan was to standardize values by calculating the number of NBT (+) cells per gram of anterior kidney (%NBT x total cell number of suspension / grams of Anterior kidney tissue collected). Unfortunately, small tissue weights (average 0.02 grams) and minimum loss of transport media in the field (due to carry over from forceps) resulted in many samples having a "negative" weight. Mixing of agarose and cell suspension produced uneven distribution of kidney cells in some slide mounts. It was critical for the reader to examine a minimum of 20 fields to prevent a bias due to cell clumping.

Table 4　　　Mean ($\pm$ SEM) percent Nitroblue tetrazolium positive cells in anterior kidney preparations from survivors at Cappel (8 fish per group) and Pecwan creek sites (6 fish per group).

	CH	CR	CM	PR	PM
Exposure 1	5.0 $\pm$ 1.1	12.6 $\pm$ 4.0	ND **	ND	ND
Exposure 2	15.8 $\pm$ 1.6	11.3 $\pm$ 1.6	8.3 $\pm$ 2.8	4.7 $\pm$ 0.5	2.7 $\pm$ 0.7
Exposure 3	5.6 $\pm$ 1.3	11.5 $\pm$ 4.9	9.0 $\pm$ 3.5	19.2 $\pm$ 5.9	9.8 $\pm$ 1.3
Exposure 5	9.6 $\pm$ 2.0	7.9 $\pm$ 2.9	ND	ND	ND

**　　　The anterior kidney sample was lost from the single CM survivor
ND　　Not done

Plasma protein - Total plasma protein concentrations of the PM survivors from exposure 3 were significantly lower than the PR fish ($P<0.05$, T-test). All other exposure groups had similar protein values (Table 5). Electrophoresis of the same plasma samples revealed that while the total concentration remained similar, there was a shift in production from albumin to beta globulin proteins by the liver (Table 5).   This shift resulted in lower Albumin / Globulin (A/G) ratios. Survivors from the Cappel sites demonstrated lower Albumin / Globulin (A/G) ratios in the order: Mainstem < Refugia < Hatchery (Fig 3). These differences tended to be more pronounced the longer the fish were exposed to the elevated temperatures (i.e. the 12 day exposure 1 groups had greater differences than the 6 day exposure 2 groups). The Pecwan creek exposure groups showed a similar response (Fig 4).   The globulin fractions 4 + 5 correspond to the zone where human beta globulins such as complement proteins and transferrin are found (Burtis and Ashwood 1994) as well as fibrinogen. We have observed similar

Table 5   Plasma protein concentration (g/dL) and electrophoresis fractions (albumin-like proteins (Alb), beta globulin fractions 4+5 {F4/5}) and Albumin / globulin {A/G} ratio of livebox survivors sampled from Cappel Hatchery (CH), Cappel creek refugia (CR), mainstem Klamath R. upstream of Cappel creek mouth (CM), Pecwan refugia (PR), and upriver of Pecwan creek mouth (PM). Fraction data reported as mean (+ SEM) of percent area of electrophoretic bands.

	CH	CM	CR	PM	PR
Exposure 1					
no.	3	1	4	ND	ND
g/dL	2.2 + 0.4	2.2	2.6 + 0.7		
Alb	65.8 + 0.7	55.5	60.3 + 2.2		
F4/5	16.6 + 0.5	21.5	19.9 + 2.2		
A/G	1.925+ 0.061	1.2447	1.545 + 0.158		
Exposure 2					
no.	8	8	8	8	6
g/dL	3.0 + 0.1	2.6 + 0.2	3.2 + 0.2	2.4 + 0.3	2.1 + 0.3
Alb	61.6 + 1.6	58.3 + 2.2	60.8 + 1.9	56.9 + 2.1	65.4 + 1.0
F4/5	17.7 + 0.7	20.5 + 1.1	19.9 + 1.5	20.3 + 1.2	16.4 + 1.1
A/G	1.633 + 0.103	1.443 + 0.118	1.591 + 0.121	1.355 + 0.114	1.904 + 0.089
Exposure 3					
no.	8	8	8	4	4
g/dL	3.2 + 0.1	3.1 + 0.2	3.5 + 0.3	2.0 + 0.6 a	3.8 + 0.4 b
Alb	62.2 + 1.9	51.3 + 2.3	60.8 + 1.3	40.2 + 6.2	55.6 + 4.2
F4/5	19.2 + 0.7	19.9 + 0.9	20.8 + 0.9	26.1 + 2.7	18.3 + 1.5
A/G	1.684 + 0.116	1.090 + 0.108	1.567 + 0.080	0.731 + 0.186	1.307 + 0.186
Exposure 5					
no.	8	ND**	8	ND	ND
g/dL	1.6 + 0.1		1.7 + 0.2		
Alb	58.9 + 1.2		52.9 + 1.9		
F4/5	18.0 + 1.2		20.2 + 1.2		
A/G	1.447 + 0.068		1.145 + 0.08		

ND   Not done
ab   Significant difference (P<0.05) between protein concentrations of a specific tributary livebox group
**   All fish were lost to otter predation

Figure 3.   Albumin-like proteins : globulin protein ratio (A / G) of plasma from Cappel creek groups (M= mainstem river above mouth, R = refugia at mouth, and H= hatchery controls) in exposures1,2,3,and 5. Significant differences (P<0.05) indicated by different subscripts for a given exposure.
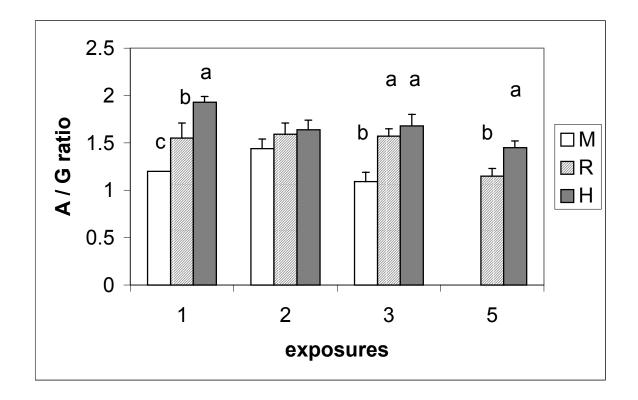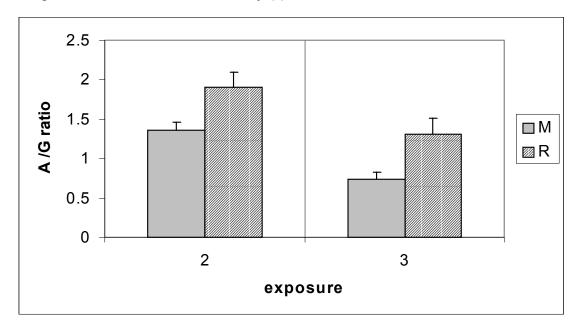
Figure 4.   A / G ratio for Pecwan exposure groups. Legend similar to figure XX
with significant difference indicated by (*).

locations for these human proteins in chinook plasma that is spiked with them. Both Cappel and Pecwan exposure groups demonstrated the same pattern of Fraction 4+5 increases in the order: Mainstem > Refugia > Hatchery (Fig. 5 & 6).

Gill Adenosine Triphosphatase activity – Gill ATPase activities tended to be lower in mainstem groups compared with their refugia exposure cohorts at both Cappel and Pecwan sites (Fig.7 -10).   Except for exposure 5, hatchery control ATPase levels were either greater than or equal to the refugia and mainstem fish. It is unclear why the exposure 5 CH fish had lower activities than their CR cohorts. The CH salmon showed a general decline in ATPase activity between exposure 1 and exposure 5. The population may have been reverting back to a parr state in August. Statistically significant differences ($P< 0.05$, T-test or ANOVA) were only detected between the following groups:

exposure 1   CH > CM
exposure 2   CH > CM, CR, PM, and PR
exposure 5   CR > CH

No samples were available for the CM groups in exposure 1 and 5 due to mortality or otter predation. No correlation between fish size (fork length) and ATPase activity was observed in the data set (r^2 = 0.01).

Figure 5. Beta globulin fraction 4+5 of plasma from Cappel creek groups (M= mainstem river above mouth, R = refugia at mouth, and H= hatchery controls) in exposures1,2,3,and 5. Data reported as mean percent area of densitometer reading with SEM bars. Significant differences (P<0.05) indicated by different subscripts for a given exposure.
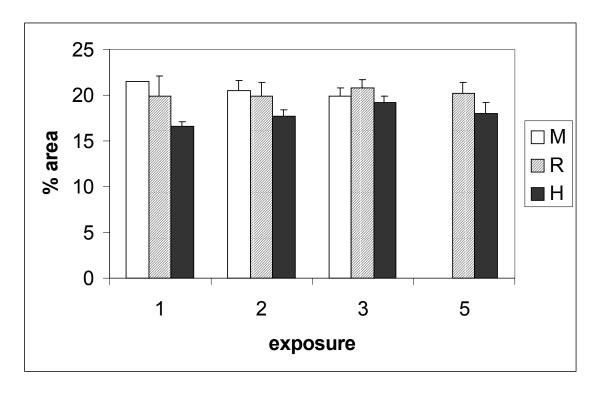
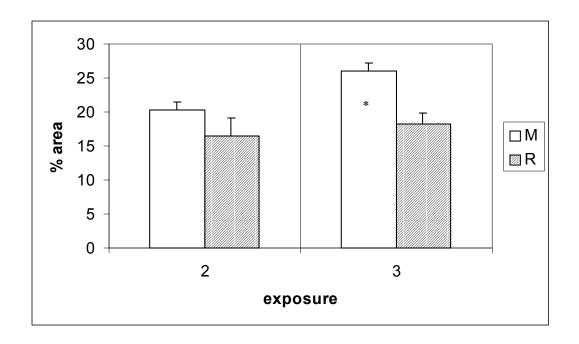Figure 6. Beta globulin fraction 4+5 of plasma from Pecwan creek exposure groups. Similar legend as figure AA. Significant difference between groups indicated by (*).

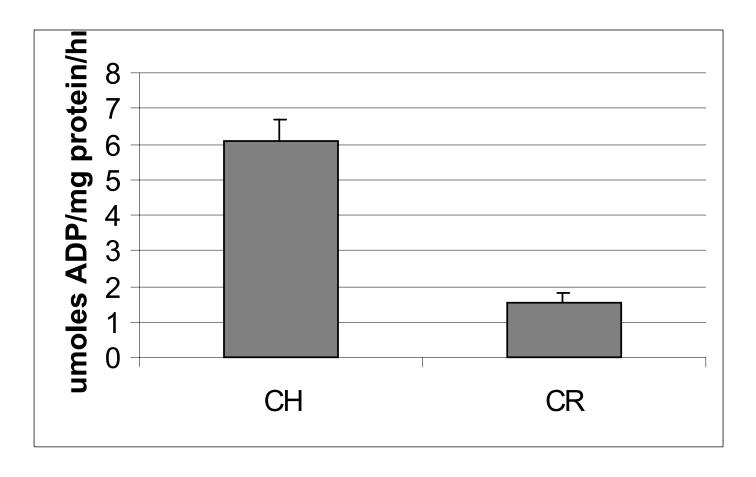Figure 7. Gill ATPase activities (mean +SEM bar) from exposure 1.

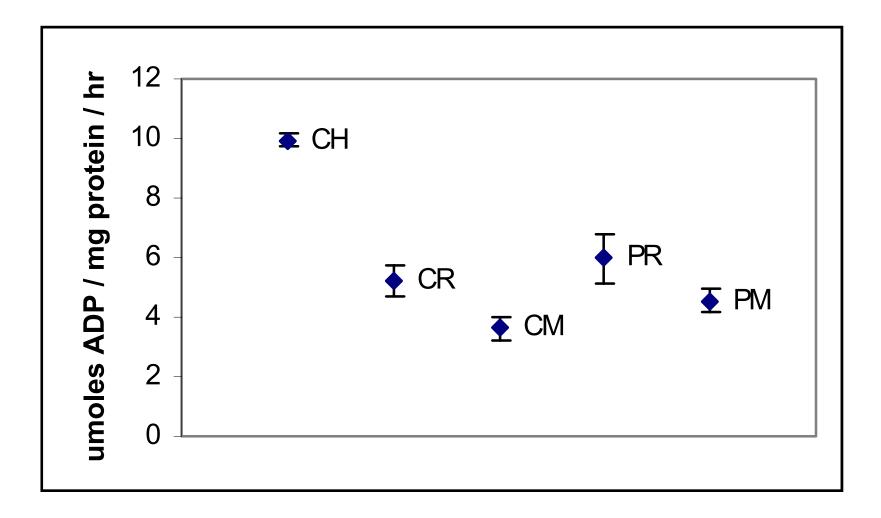Figure 8. Gill ATPase activities (mean +SEM bar) from exposure 2.

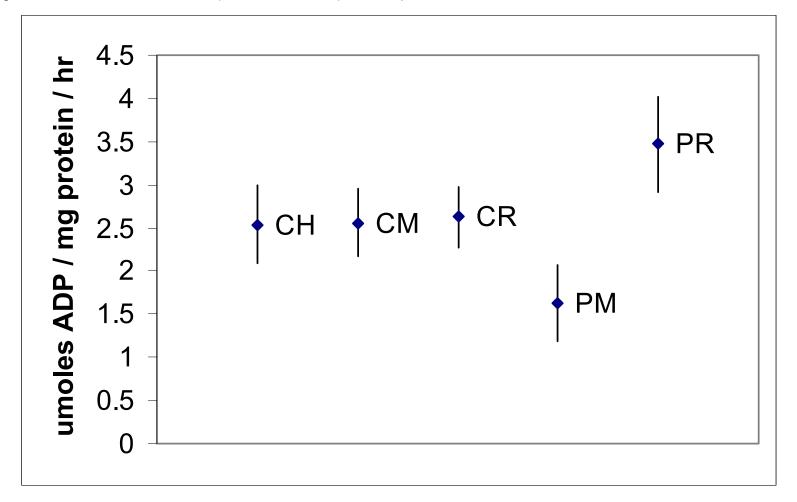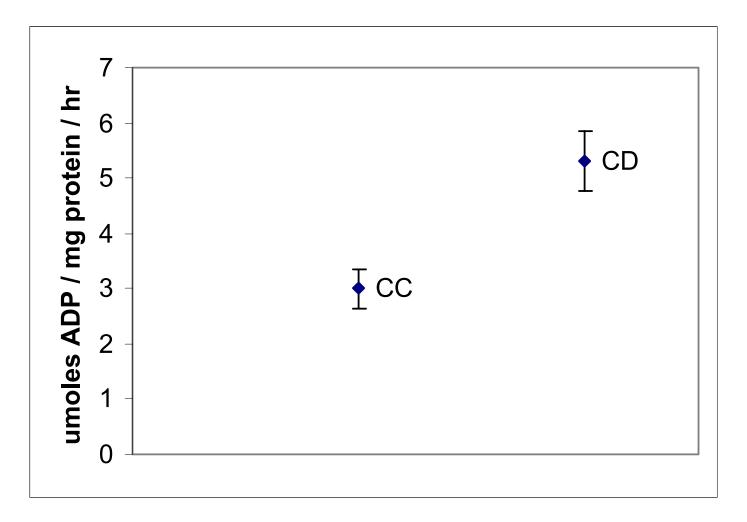Figure 9. Gill ATPase activities (mean +SEM bar) from exposure 3.

Figure 10. Gill ATPase activities (mean +SEM bar) from exposure 5.

DISCUSSION

Elevated water temperature is only one of many interacting biotic and abiotic factors (e.g. dissolved oxygen, food availability, predation, infection, smolt development) influencing smolt survival in the Klamath R. basin. Sub-lethal high water temperatures would act to impaired a fish's performance capacity and thereby decrease its probability of out-migration survival. Salmonids will actively seek favorable thermal habitat when faced with elevated water temperatures (Torgenson et al. 1999, Biro 1998). In a 1995 study on Klamath R. out-migrant chinook health, we observed smolts using thermal refugia during the spring-summer migration (Foott et al. 1999).

The upper temperature threshold for juvenile chinook salmon survival varies by the method of analysis. Brett et al. (1982) experimentally identified 18.5 ° - 21°C as the zone of sub-lethal growth stress for juvenile chinook salmon. They also reported that the upper incipient lethal temperature (UILT) for acclimated salmon to be 25°C. Baker et al. (1995) modeled a 23 °C UILT from recapture data on marked juvenile chinook moving through the Sacramento- San Joaquin R. Delta. The high mortality of mainstem exposure fish and stress responses seen in thermal refugia groups suggest that significant health effects occur at mean water temperatures less than 23 °C.

The lack of temperature acclimation and stress of live box confinement are two factors in the study design which limit application of this data to natural chinook salmon populations. Given that the control live box group held in the hatchery would experience a similar degree of stress from confinement and starvation, differences observed between the controls and both the mainstem and refugia groups were largely due to temperature. The high incidence of columnaris seen in the river groups was probably related to impaired host defenses (abrasion, immunosuppression) and optimal pathogen conditions (temperatures above 15°C). Salmonid mortality in the Klamath basin is typically associated with high water temperatures and infection from the endemic pathogens, *Flavobacterium columare* and *Ceratomyxa shasta* (Foott et al. 1999, Manji 2000). We do not known whether the controls were also exposed to *F. columnare* in Cappel creek water. While thermal refugia provided marked survival benefits in comparison to the mainstem Klamath river, refugial groups demonstrated heighten stress responses and higher mortality than the hatchery controls. This data indicates that MDT of <19°C can cause health problems when in association with other stressors (confinement, abrasion). An upper MDT threshold would be influenced by the duration of exposure however, both mortality and altered physiology appeared to be negatively affected in the range of 19 - 20°C.

Both temperature and exposure duration appeared to influence several of the measured physiological responses (gill ATPase, A/G shift to beta globulin proteins, responsive phagocyte numbers).   There was a general trend where the refugia fish were in between the controls and the mainstem group. Barton and Schreck (1987) reported that juvenile chinook salmon held at 21°C had

significantly greater plasma glucose responses to stress than cohorts held at either 7.5° or 12.5°C. Mortality was also much higher in salmon stressed at the higher temperature. Juvenile coho salmon have been reported to show elevated stress responses (plasma cortisol and glucose) when maintained for 20 d at a fluctuating regime of 6.5 - 20°C in comparison to groups held at 9 - 15°C (Thomas et al. 1986). While gill ATPase activity tended to be higher in the controls, this relationship was reversed in exposure 5. It is likely that the juveniles were regressing in smolt development during August and a transient rise in CR salmon ATPase was linked to cortisol effects. Temperature shock and social stress has been linked to transient increases in choloride cells and gill ATPase activity (Schmidt et al 1998, Sloman et al. 2000). We have observed a similar rise in ATPase activity in chinook juveniles held in 24°C waters for 4 – 8 h over a 4 d period (unpubl. data , 1999 Temperature challenge data, CA-NV FHC). Many workers have documented that elevated water temperatures act to accelerated smolt development and hasten smolt-parr reversal (Dustin et al 1991, McCormick 1994, Wedemeyer et al. 1980).

Elevated blood levels of cortisol are one element of the fish's stress response and initially benefit the animal by stimulating a rise in blood glucose and mobilizing energy stores. One outcome of chronic stress on a fish population is an increase occurrence of disease through the immunosuppressive effects of long-term cortisol elevation (Wedemeyer et al. 1976). Cortisol has been shown to inhibit antibody production in salmonid lymphocytes and reduce the numbers of leukocytes in the blood (Tripp et al. 1987, AG Maule and CB Schreck 1990, Ellsaesser and Clem 1986). Weyts et al. (1997) report how cortisol induces apoptosis (individual cell-regulated death) in carp lymphocytes. Cortisol was not measured in this study, however, the exposure periods were probably too short for the specific immune system to react to infection.

The nonspecific immune system (phagocytes, mucus, various plasma factors, etc.) is the first line of defense against microbes for fish.   Phagocytosis is the process where cells engulf, kill, and digest invading microorganisms and is a major element in the non-specific defenses of telests (Secombes 1996). Macrophages and neutrophils are two cells types that perform this defensive action in fish. Intracellular killing is accomplished by both enzymes (e.g. lysozyme) and reactive oxygen species. When activiated by ingestion or other stimuli, phagocytes undergo a "respiratory burst" and produce oxygen radicals such as superoxide anion (O_2^-). The tetrazolium salt, Nitroblue tetrazolium (NBT), is a yellow water soluble substance which is reduced to insoluble blue formazin granules within the cytoplasm of phagocytes undergoing a respiratory burst (Alfoldy and Lemmel 1979). In this study, the number of NBT positive cells (%NBT+) in a suspension of anterior kidney cells was evaluated as a measure of the fish's non-specific defense capability. Stress will tend to stimulate phagocytosis in the short term, however, this stage is followed by a collapse of this non-specific defense mechanism (Ruis and Bayne 1997, Peters et al. 1991). This transient effect may explain why mainstem and refugia fish in the 4 day

exposure (#3) showed higher NBT positive cell numbers than the controls. Longer exposures showed the opposite trend. High variability in the NBT data set limited the use of this assay, in its present form, for correlating general health effects of temperature stress with functional phagocyte numbers.

Relative to the salmon exposed in the mainstem Klamath river, groups held in the thermal refugia had significantly lower mortality and fewer physiological imbalances due to stress. We cannot assign a specific temperature threshold that causes significant health problems for juvenile salmon due to the experimental design of this study, however, chronic exposure to water temperatures above 20°C resulted in high mortality and altered physiology. Controlled laboratory studies, using local chinook stocks, will be necessary to define an upper temperature threshold. Results from this study point out the smolt survival benefits of thermal refugia in the Klamath river during spring and summer.

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