FY96 Investigational Report :

Physiological Effects of *Nanophyetus* Metacercaria Infection in Chinook Salmon Smolts (Trinity River)

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Summary: In September 1996, yearling chinook from Trinity River Hatchery were exposed for 27 days to *Juga sp.* snails carrying an infective stage of the trematode *Nanophyetus salmincola.* Little mortality occurred to the exposure group and all fish became infected with the metacercaria stage of the parasite. Severity of infection ranged from 32 to 10,220 metacercaria per gram of kidney tissue with over 50% of the infected fish having > 5,000 metacercaria / g. No significant difference in blood cell numbers or composition, or plasma concentrations of protein, glucose, and triglyceride was detected between infected and control fish. Infected fish showed an increase in immunoglobulin and a decrease in saltwater osmoregulation ability. This study demonstrates that juvenile chinook can withstand severe infections of *N. salmincola* in freshwater, however, saltwater survival is likely to be impaired. A direct correlation between the number of metacercaria in the kidney (threshold level) and plasma sodium was not identified in the short-term saltwater challenges.

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Introduction

The California - Nevada Fish Health Center (FHC) surveyed Trinity River chinook salmon (*Oncorhynchus tshawytscha*) juveniles and adults for health and physiological measurements in 1991-1994 and again in 1996. Both natural and hatchery juveniles have been examined in the spring and fall. A common and significant fish pathogen detected in both natural and hatchery out-migrants is the trematode *Nanophyetus salmincola*. Trinity River Hatchery (TRH) chinook are exposed to the parasite soon after their release into the river and can sustain heavy infections within days. Both the incidence of infection and severity tend to be higher in fish collected in the fall compared with the spring. The severity of infection can range up to 33,000 metacercaria per gram of kidney in juvenile chinook. Similar high numbers of parasites are detected in adult chinook spawned at TRH. *Nanophyetus salmincola* is found in salmonids and other associated freshwater fish (cottids, cyprinidae, lamprey) throughout the Pacific Northwest (Millemann and Knapp 1970). Its range is limited to waters which are habitat for its intermediate *Juga* sp. snail host. The pathogenicity of the trematode to fish is reported to vary considerably and appears to be a function of rate of accumulation, fish size, species and stock susceptibility, infection site(s), and absolute number of metacercaria (Necomb et al. 1991, Wood and Yasutake 1956, Baldwin et al. 1967 , Milleman and Knapp 1970). Human cases of nanophyetiasis have been reported (Harrell and Deardorff 1990). The trematode is itself parasitized by the rickettsia, *Neorickettsia helminthoeca*, which is responsible for salmon poisoning disease of dogs (Schmidt and Roberts 1981)

The life cycle of *N. salmincola* starts with the release of eggs from the adult trematode into the intestine of its final host, a piscivore such as an otter, bear, raccoon, heron, merganser, etc, and pass out into the water with feces. . A ciliated miracidium stage hatches from the egg , penetrates a snail host (*Oxytrema = Juga sp.)*, asexually multiples, and eventually produces a xiphidiocercaria (cercaria with oral sucker stylus which is motile by use of its tail). The cercaria will seek out a fish host and rapidly burrow into the skin, lose its tail, and migrate through the circulatory system to various tissues such as the gill, heart, liver, muscle, optic nerve, and kidney. The parasite (now referred to as metacercaria) tends to concentrate in the posterior kidney, probably due to the migration path through the renal portal system (Milleman and Knapp 1970). The metacercaria will remain with the salmonid fish throughout its salt water phase and will complete its lifecycle when the fish is eaten by a final host. The longevity of the metacercarial stage has been used as a biological tag for steelhead caught in the central Pacific ocean (Dalton 1991).

The high incidence of severe metacercarial infections and the acute challenge conditions (to TRH fish) prompts the question of whether there is a □threshold□ level of infection which will significantly impair the performance of the chinook smolt and decrease its chance of survival. This study was designed to examine several performance measures of infected smolts.

Method and materials

Snail Collection and cohabitation challenge- Few *Juga sp.* snails were found during June. Two days (3 persons) of searching along the Trinity River from below the hatchery to Junction city failed to produce more than 10 *Juga* sp. snails. Both snorkeling and wading were done to search for the snails. Mark Magneson (USFWS, Coastal Calif. FWO) provided 15 snails from the New River on 13June. Uninfected juvenile chinook (65 mm Fork Length, Coleman National Fish Hatchery = CNFH) were co-habitated with snails from either New River or the Trinity R. in aerated 17 °C, static aquariums for 2, 4, and 24 hrs. Chinook exposed to the New River snails for 24 hrs showed significant infections (18-28 metacercaria in the kidney with hemorrhaging at the fin bases). On 17June, approximately 50 snails were collected by snorkeling at the New River rotary screw trap (3.75 rkm above the confluence with the Trinity river). After confirming their infective nature with CNFH chinook as described above, these snails were moved to TRH on 19June and placed in 30L aquaria with 65 Fall-run chinook (FCS) from TRH. The aquaria were aerated and held in ambient 11 °C water. After 2 days the chinook were removed and placed in a flow-through hatchery tank. Water quality was poor due to fecal matter and 75 % of the aquarium□s water was replaced (waste water was chlorine-disinfected). Another 65 chinook were co-habitated for 4 days as above with a partial water change on day 3. Unexposed chinook were maintained as negative controls.

On 04September, several sites on the Trinity R. between Junction city and Willow creek were snorkeled for snails. A high concentration of *Juga* sp. were found in a shallow, mud-bottomed site near Cedar flats (76.4 rkm) and approximately 1,000 snails were transported to TRH. These snails were placed in a shallow hatchery trough (275 m^3 total volume, 15.2 cm depth) supplied with 11 - 20 L / min. of 9 - 10 °C hatchery water. The effluent from the trough went directly to the river without contacting any other hatchery component. After adding 120 yearling FCS, a black plastic cover was installed over the trough. A negative control group of 60 FCS were held in a similar but snail-free trough. The fish were not fed and dissolved oxygen in the tail-section of the trough was 9.3 ppm on 06 September. The infection status of 6 exposed fish were checked on September 6th and 10th. The maximum number of metacercaria seen from an entire kidney was 6 and it was decided to extend the challenge. On 01October (27 days of exposure), high numbers of metacercaria were detected in 4 exposed chinook. The two groups were moved to deep tanks (2700 L vol.) and fed daily up to the time of sampling on 16 October. A total of 13 snail-exposed (infected) and 4 control fish died prior to 15October.

Fish necropsy - On October 16th, ten fish from each group were netted from their tanks and 5 were rapidly euthanized with an overdose of a benzocaine solution, examined for external organosomatic parameters (see below), weighed, measured for total and fork length, and bled from the caudal peduncle into heparinized microhematocrit tubes. After centrifugation (10,000 RPM, 10 min.), hematocrit (% packed erythrocyte volume) and leukocrit (% white blood cell volume) were

measured and the plasma was frozen on dry ice . Leukocrit was determined by measuring the buffy coat above the RBC band with an eyepiece micrometer (Bausch and Lomb binocular dissection microscope, 1 eyepiece unit = 0.017 mm) at 30X magnification. The buffy coat length (mm) was divided by the total fluid length (mm from clay-RBC interface to top of plasma layer) and multipled by 100 (McLeay and Gordon 1977). A blood smear was also made, air dried, and fixed in absolute methanol for 10 minutes. After a brief examination of internal organosomatic parameters, various tissues were collected for histological examination as described below. After 30- 40 minutes, the next 5 fish (stress glucose measurements) were sampled in a similar manner. Fish were evaluated by a modified organosomatic assay (Goede and Barton 1987, 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 condition of skin, eyes, gill, quantity of visceral fat, and any abnormalities of the internal organs. Fulton's condition factor was calculated from total length (K = weight (g) / length (mm) ^{3} x 10 ^{5}) .

Organosomatic analysis criteria scores

Skin	0 = normal scale number, no lesions
	1 = some scale loss, 5 - 20 % of body surface
	2 = focal hemorrhages, scale loss 21 - 40 % of body
	3 = open wound, scale loss > 40 % of body surface
Eye	0 = no abnormalities
	1 = missing 1 eye, diminutive, external abrasion, some opacity
	2 = exophthalmic 'pop-eye', cataract, bubbles, parasites
	3 = hemorrhage, rupture
Gill	0 = normal condition, color
	1 = pale
	2 = clubbed, frayed, nodules, mild parasite load
	3 = necrotic zones, fungi or bacterial lesions, hemorrhagic
Vfat	0 = no visceral fat in peritoneal cavity or on pyloric ceca
	1 = < 50 % coverage of ceca and/or cavity fat dia. < ceca vol.
	2 = >50 % but not covering ceca and/or cavity fat dia. = ceca vol.
	3 = ceca and cavity completely filled with fat, organs obscured by fat
Internal	Notes about any hemorrhagic organs , abnormal size or color. Organs examined included spleen, liver, lower intestine,and kidney.

Saltwater challenge- A 30 ppt saltwater (SW) static aquarium (bucket) was produced by adding 900 g Instant ocean □ salt to 30 L of freshwater from the fish□s environment. Salinity was checked by salinity refractometer. The buckets were held in ambient water to maintain temperature, supplied with bubbled air, and a lid was securely fasten. Test fish are quickly captured and placed in the SW buckets (6-10 fish / bucket). After 24 hrs, the surviving fish are quickly captured, euthanized in a saltwater-MS222 solution, weighed, measured for total and fork length, and bled from the caudal peduncle into heparinized tubes. The tubes are centrifuged soon after collection and the plasma frozen for later sodium analysis (Johnson & Johnson DT60 clinical chemistry analyzer). A series of SW challenges conducted in late June were not analyzed because of inadequate metacercaria infections. On October 15th, three groups of 10 snail-exposed (infected) fish and one group of 10 control fish were placed into separate 30 L saltwater challenge aquaria held in ambient 9.3 °C water.

<u>Vibrio anguillarum</u> challenge - One hundred and 11 fish (37 / group) were transported to the UC Davis Fish Pathology wet laboratory on 28June. These fish were to be used in a *V. anguillarum* challenge on 02July, however, the discovery of poor metacercial infection during the spring canceled this experiment and the fish were destroyed.

Sixty fish from snail-exposed (infected) and control groups were transported to the FHC on October 16 and held in separate 350 L aquaria equipped with aeration / chiller units (Frigid Units Inc., Toledo, OH). Water temperature was held at 8- 10 °C. All but 3 of the control fish were killed prior to the challenge when the screen over the chiller impeller fell off. Late-fall run chinook (LFS) juveniles from Coleman NFH (mean FL 109mm) were substituted for the lost TRH fish. These fish had been exposed to *Nanophyetus* in the hatchery□s Battle creek water supply and carried light metacercaria infections.

Vibrio anguillarum was chosen as the challenge agent to reflect its pathogenicity to salmon in salt and estuarine waters around the world (Inglis et al. 1993). A virulent, *Vibrio anguillarum* isolate obtained from Dr. Ron Hedrick at UC Davis (Norwegian isolate VA-32) was injected into a juvenile chinook held in a 15 °C static aquarium. The bacterium was re-isolated from the spleen of the moribund chinook and aliquots frozen at -80 °C in Trypic Soy broth without NaCl (TSB). Previous experience with VA-32 has shown that salt addition was not necessary for culture. Prior to the challenge, several 1 mL aliquots of VA-32 were inoculated into a flask containing 40 mL of TSB. The culture was mixed with a sterile stir-bar rotated on a magnetic stirrer and kept at 25 °C for 16 hrs.

Immediately prior to the challenge, control LFS were lightly anesthetized with 0.5 ppm MS-222 and marked with Alcian blue dye at the base of the anal fin with a 21G needle. Snail-exposed FCS were handled in a similar fashion but without dye. No mortality occurred to fish of either group until 6 days post-challenge. The Alcian blue mark was

difficult to read after 6 - 8 days and required that the number of metacercaria be determined for each sampled fish to identify its group affiliation. Forty control and 40 snail-exposed (infected) fish were challenged with 3.7 x 10^3 CFU bacteria / mL in an aerated 30L bucket of 12 °C water for 20 minutes and placed into a 350 L aquaria. A unchallenged group composed of 20 control and 20 snail-exposed (infected) chinook were handled as above except they received TSB only and were held in their own 350 L aquaria. The temperatures were maintained between 7 - 12 °C over the 12 day challenge period. Fish were not fed and dissolved oxygen concentration ranged from 9.1 - 11.0 ppm. Mortality and temperature was checked daily. Tryptic soy agar was inoculated with kidney tissue from the mortalities and isolates were identified by standard biochemical tests.

Histology - Brain, anterior and posterior kidney, gill, intestine, and liver tissue was rapidly removed from the fish, fixed for 24 hrs. in Davidson's fixative (Humason 1979), transferred to 70 % ethanol, processed for 5 μm paraffin sections, and stained with hematoxylin and eosin. Tissue abnormalities and metacercaria were evaluated by light microscopy.

Metacercaria counts - The entire kidney was dissected from the fish, frozen in cyrovials at -20 °C, later defrosted and placed onto a tared glass slide (50 x 25 cm). After the weight of the tissue was recorded to the nearest 0.001 gram, another glass slide was press down on top of the tissue and the number of metacercaria in the squash preparation counted with a binocular dissection microscope at 50 - 70X magnification . Kidney tissue remaining after histological sampling of the anterior and posterior portions from 10 snail-exposed fish was also processed as above. The standardized measurement of metacercaria per gram of kidney examined was determined for each sample.

Hematology and plasma chemistry - Differential leukocyte counts were performed on diff-quik ☐ stained blood smears. The number of lymphocytes , neutrophils, monocytes, and thrombocytes observed at 1000X magnification out of 100 leukocytes was recorded. Plasma was assayed for total protein, triglyceride, sodium, and glucose using a Johnson & Johnson DT60 Clinical Chemistry Analyzer and reagents. Albumin content of the plasma was tested by the bromcresol green method (Sigma chemicals). Plasma protein electrophoresis was performed with a 7 μL sample run on a CIBA 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 Seprascan☐ 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 electrophoretic albumin and F1-F7 % area measurements . Pre-albumin fractions were not included in the A/G ratio calculation. 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.

Statistics - Group data was tested for normality and either analyzed by the parametric (T-test, 1-way ANOVA) or non-parametric tests (Mann-Whitney rank sum test, Kruskal-Wallis ANOVA on ranks). If significant differences among the groups were detected in the ANOVA tests, Student-Newman-Keuls multiple comparison (pairwise) tests were performed to identify which group was different. An alpha (type I "false difference" error) value of $P \leq 0.05$ was chosen for all tests. Both Lotus 1-2-3□ spreadsheets and SigmaStat□ software was used for data manipulation and analysis.

Results and Discussion

The lifecycle of *Nanophyetus salmincola* has 2 intermediate hosts: the *Juga* sp. snail and a fish. The abundance of the parasite is influenced by the density of its intermediate hosts (Paperna 1995). In the spring of 1996, we recorded the lowest incidence and severity of metacercarial infection in out-migrant chinook smolts since health monitoring work was begun in 1991 (Foott et al. 1994). Despite considerable effort, it is likely that our inability to collect many snails in the spring was due to their low abundance in the upper Trinity river. The failure to produce metacercaria infections in the spring TRH challenge was not due to uninfected snails, as Coleman chinook test fish were infected by the same snails. Water temperature was one major difference between the 2 sites with Coleman challenges occurring at 15 -17 ° C and TRH at 11 ° C. Lyholt and Buchmann (1996) report that *Diplosotmum spathaceum* cercaria shedding was strongly temperature dependent with maximum shedding rates occurring at 20 °C. Poor water quality, resulting from fecal material, could have also had a detrimental effect on cercaria shedding from the snails. We observed that the snails did not crawl about the 30 L aquaria during the spring challenge, however, they were alive at the end of the co-habitation period.

In the fall challenge, the flow-through container provided the snails (and fish) with adequate water quality, however, the rate of infection appeared to be slow. This slow infection rate could be attributed to a low incidence of trematode infection in the 1,000+ snail population or a function of low water temperature and reduced cercaria release. We did not observe any sign of snail predation by fish, shell debris, or obvious snail mortality.

Parasite infection / histological results- Metacercaria were detected in all fish from the September snail-exposed group and are henceforth called the "infected" group . In spite of the stressful conditions (27 day starvation, darkness, cercaria attacks, shallow tank), few mortalities (10%) occurred to the snail-exposed fish during the experiment. The severity of infection ranged from 32 to 10,220 metacercaria per gram of kidney tissue. A bi-modal distribution of metacercaria severity was observed in the infected fish (Figure 1). Over 50 % of the infected fish had a severity of infection greater than 5,000 metacercaria per gram kidney. The mean level of infection for the 10

fish sampled for plasma chemistry, organosomatic analysis, and histology was 1,073 (± 602) and tended to be lower than the saltwater challenged fish. Data for those fish tested in the saltwater and *Vibrio* challenges are discussed below.

The highest concentration of metacercaria were observed in histological sections (in order of intensity of infection) of: 1) posterior kidney, 2) gill, 3) anterior kidney, and 4) heart (Table 1). The intensity of metacercarial infection did not correspond between histological sections of gill, kidney, and heart, and the mid-kidney region of the same fish examined with a dissection microscope (Table 2). This data highlights the uneven distribution of the parasite between and within the tissues. Bennington and Pratt (1960) reported that the posterior kidney was the primary site of encystment after invasion of the fish and entry into the venose system. No significant inflammatory responses to the parasites were observed in the tissues. Wood and Yasutake (1956) also found little tissue reaction to the encysted metacercaria in coho salmon. Kidneys from both control and infected fish had diffuse melanin deposits indicative of the previous starvation period (Agius and Roberts 1981). No parasites or abnormalities were seen in the intestine, spleen, or brain. Cytoplasmic vacuoles, indicative of mild fatty change, were seen in the hepatocytes of fish from both groups. This slight abnormality may have been associated with the lipolytic metabolism induced by the earlier starvation period. No metacercaria were detected in the liver sections.

fig1

Table 1 . Histological observations.

	Infected	Negative control
Kidney		
Metacercaria < 10	1 / 10	0 / 5
Metacercaria > 10	9 / 10	0 / 5
Mean metacercaria (SD)	28.0 (14.7)	NA
Assoc. Inflam. / lesion	0 / 10	0 / 5
Gill		
Metacercaria < 5	6 / 10	0 / 5
Metacercaria > 5	2 / 10	0 / 5
Mean metacercaria (SD)	2.8 (3.7)	NA
Assoc. Inflam. / lesion	1 / 10	0 / 5
Heart		
Metacercaria	7 / 7	0 / 4
Mean metacercaria (SD)	3.4 (1.4)	NA
Assoc. Inflam. / lesion	0 / 7	0 / 4
Liver		
Metacercaria	0 / 10	0 / 5
Hepatocyte vacuolization	7 / 10	5 / 5
Intestinal tract		
Metacercaria	0 / 10	0 / 5
Assoc. Inflam. / lesion	0 / 10	0 / 5
Brain		
Metacercaria	0 / 8	0 / 4
Assoc. Inflam. / lesion	0 / 8	0 / 4
Spleen		
Metacercaria	0 / 2	ND
Assoc. Inflam. / lesion	0 / 2	

ND Not done

Table 2. Number of metacercaria (Metac.) observed in different tissues of infected fish by histology and wet mount. Samples from fish collected for organosomatic examination (ORG) and saltwater challenge (SW).

Fish	Mid-Kidney Metac./ g	Histological sections Post. Kidney	Gill	Heart
ORG1	1754	30	2	6
ORG2	533	45	0	ND
ORG3	6323	16	2	ND
ORG4	487	21	6	2
ORG5	267	59	3	4
ORG6	300	29	0	ND
ORG7	327	23	1	3
ORG8	454	20	1	2
ORG9	251	29	1	4
ORG10	32	8	12	3
SW1	4704	ND	2	ND
SW4	8292	ND	3	ND
SW8	10221	ND	4	ND
SW15	1856	ND	0	ND

Organosomatic / Plasma chemistry / Hematological data - There was no significant difference in size or condition factor between fish of the 2 groups (Table 3). The relatively low condition factors and visceral fat scores (mean 1.2) reflected the starvation conditions during the 27 day co-habitation period. However, the presence of some visceral fat showed that severe depletion of energy reserves did not occur to either group. The level of circulating triglyceride was similar in both groups and normal for fed hatchery fish (unpubl. 1994 - 95 Trinity R. studies, FHC). This data also indicated a normal energy balance (Table 4). There was no significant difference between the plasma glucose response of fish from either group (Table 4). While the glucose values were within normal ranges for salmonids, they tended to be less than we

typically observe in TRH fish (80 - 120 mg / dL in stressed hatchery fish). This data indicated both groups may not have had adequate glycogen reserves or had impaired corticosteroid metabolism. All chinook were quite silver in appearance and had extensive scale-loss after netting. No other abnormalities were observed in the exterior features or interior organs of either group.

Table 3. Weight, length, and Fulton□s Condition (K= Wt. / Total length^3 x 10^5) of infected and negative control chinook (n=10 each). Reported as mean ± S.E.M..

	Infected	Negative Control
Weight (g)	17.8 ± 1.3	19.8 ± 1.3
Total length (mm) Fork length (mm)	137 ± 2 126 ± 2	139 ± 3 129 ± 3
Condition factor (K)	0.679 ± 0.031	0.732 ± 0.017

Table 4. Plasma triglyceride and glucose data (mean ± SEM). Glucose data subdivided into samples from fish held < or > 30 minutes (stress response).

	Infected		Negative control	
Triglyceride (mg / L)	52 ± 5	n=10	52 ± 6	n=10
Glucose (All fish)	77 ± 4	n=8	80 ± 5	n= 5
< 30 min.	76 ± 7	n=5	84 ± 5	n= 4
> 30 min.	78 ± 5	n=3	68	n= 1

Neither the quantity or composition of the blood cells differed significantly between infected and control chinook (Table 5). The hematocrit, leukocrit, and differential leukocyte count values were within normal ranges for juvenile salmonids (Wedemeyer and Yasutake 1974). The hypothesized lymphocytemia (elevated numbers in the blood due to infection) did not occur in the metacercaria- infected fish. While total protein concentration and albumin : globulin ratio (A/G) of the plasma did not differ significantly between the groups, the presumptive immunoglobulin fraction (f6 and 7) did increase in the infected fish (Table 6). This data indicates that the fish were responding to the parasite infection by producing antibody (immunoglobulin) and were not compromised in their immunodefenses. Rainbow trout are reported to have a humeral antibody response to infection with cercaria of *Diplostomum spathaceum* and demonstrate a cell-mediated immunity to re-infection (Hoglund and Thuvander 1990).

Table 5. Blood cell data.

	Infected	Negative Control
Hematocrit n = 10	38.2 ± 1.3	37.5 ± 0.7
Leukocrit n = 10	1.082 ± 0.065	0.971 ± 0.080
Differential counts	n = 8	n = 6
% Lymphocyte	84.4 ± 3.7	84.0 ± 4.9
% Thrombocyte	13.6 ± 3.9	14.1 ± 5.2
% Neutrophil	1.0 ± 0.6	1.3 ± 0.4
% Monocyte	0.6 ± 0.3	0.3 ± 0.2

Table 6. Plasma protein data. Total protein (7 fish each) determined by DT60 analyzer. Electrophoretic data and derived A/G ratio from 9 snail - exposed and 6 control fish expressed as mean (± standard error of the mean) percent area.

	Infected	Negative control
Total protein (g/dL)	2.9 ± 0.1	3.3 ± 0.1
Albumin / globulin ratio*	0.95 ± 0.11	0.90 ± 0.05
Pre-albumin	21.5 ± 1.8	19.3 ± 2.3
Albumin	36.9 ± 0.7	38.6 ± 2.5
F1	7.7 ± 0.5	8.4 ± 0.9
F2	5.3 ± 0.6	5.1 ± 0.7
F3	6.9 ± 0.6	9.5 ± 0.7
F4/5	15.6 ± 1.7	15.7 ± 1.6
F6/7	6.0 ± 0.5 **	3.4 ± 0.6

* derived from electrophoretic data, not including pre-albumin fractions

** significantly different at $P < 0.05$.

***Vibrio anguillarum* challenge -** Metacercaria infection did not comprise the resistance of the fish to bacterial infection. Only 2 of the 40 metacercaria-infected fish (5%) died in the 12 day *V. anguillarum* challenge. While the controls had a slightly higher mortality rate (7 of 40 = 18%), the different genetic characteristics (Sacramento Late-fall run) and low-level metacercaria infection do not allow for direct comparison with the infected Trinity chinook. *V. anguillarum* was isolated from 5 of the 6 mortalities (one fish was too decomposed for successful testing). The low challenge dosage of 3.7×10^3 cells / mL and 20 minute exposure time did not produce high mortality in either group, however, these conditions were chosen to reflect environmental conditions a smolt could face in the estuary. The average metacercaria / g value of the eleven "snail-exposed" survivors examined was 12,510 ($\pm$ 3211). One of the 4 control LFS survivor examined had 10 metacercaria in its kidney (454 metacercaria / g). No chinook (either LFS or TRH) in the sham-challenged tank died during the 12 day challenge. In conclusion, this challenge had several flaws (switch in control fish, low virulence in challenge conditions, static aquarium) but did demonstrate that chinook with high numbers of metacercaria had adequate, and perhaps heightened, resistance to bacterial infection.

Saltwater challenge- While all infected and control fish survived the 24 hr. challenge, the infected chinook had poor osmoregulatory control of their plasma sodium levels (Table 7). This group had both a significantly higher mean sodium concentration (Mann-Whitney test, $P < 0.01$) and over half of the fish (55%) with sodium levels above the upper normal range of 170 $\pm$ 4 mmol / L (Blackburn and Clarke 1987). The selection of 174 mmol /L as the upper normal plasma sodium cutoff took into account assay variability. Infected fish also appeared to have also undergone dehydration (drop in wet wt. and condition factor). In comparison, the control' s condition factor (mean 0.758×10^5) was similar to the value of the freshwater organosomatic control fish (mean 0.733×10^5) which indicates successful osmoregulation. Mean metacercaria / g values for the infected group was 5,616 which equates to 500 - 1000 metacercaria in a kidney weighing < 0.2 grams (Table 7). The severity of metacercarial infection was similar to that seen in captured out-migrant TRH chinook during the fall (unpubl. data of FHC monitoring of Trinity R. chinook). In 1993 and 1994, the *metacercaria / g kidney* values measured from yearling TRH chinook captured near Willow creek (rkm 34) over a 4 week period post-release was:

	mean	maximum
1993	306	4,094
1994	5,412	33,954

No significant relationship between metacercarial infection severity (No. / g kidney) and plasma sodium concentration was detected by linear regression (r. sq. = 0.018). Necomb et al. (1991) reported a direct correspondence with the number of metacercaria found in the posterior kidney of coho smolts and their survival over 10 weeks in 29 ppt saltwater. In their study, a mortality rate of 50% or greater occurred in smolts with 80 or more metacercaria with significant mortality beginning after 5 weeks. Given their poor sodium regulation in the our 24 hr. test, it is likely that long-term survival of heavily-

infected TRH fish would be poor after reaching salt water.

Table 7. Twenty-four hour, 30 ppt saltwater challenge data. Percent survival, condition factor (K = weight (g)/ length (mm)^3 x 10^5), mean plasma sodium concentration (± SEM), % of survivor□s > 174 mmol / L plasma sodium, severity of metacercarial infection from 30 snail -exposed and 10 control fish.

	Infected	Negative control
%Survival	100	100
Condition factor (K)	0.665 ± .010 *	0.758 ± .015
Plasma Na+	177 ± 3	160 ± 2
% > 174 mmol/L Na+	55 %	0 %
Metacercaria / g Mean (± SEM) Maximum	 5,616 (± 485) 10,221	 0 NA

* Significantly less than controls, T-test P<0.01.

Juvenile chinook must undergo hormone-directed, developmental changes referred to as smolt transformation ("smoltification") prior to gaining seawater tolerance. In the marine environment, they must ingest seawater to balance the osmotic loss of water across the gills and skin (Evans 1993). Water uptake occurs in the small intestine as it passively follows an active transport of NaCl into the blood. The excess NaCl is excreted by the chloride cells of the gill. The kidney' s osmoregulatory functions are different in freshwater compared with saltwater. While in freshwater, excess water in the blood is excreted as dilute urine and NaCl is actively reabsorbed in the kidney tubules. In saltwater, there is a marked decrease in urine flow (now isosmotic to the blood) and an active excretion of the divalent ion magnesium by the kidney tubules (Clarke and Hirano 1995).

It is not clear why the infected fish showed impaired osmoregulation. The light metacercaria infection seen in the gills would not have physically impaired the chloride cells, however, infection could have altered the hormone or hormone-receptor profile from that of a successful smolt. Elevated cortisol, levels in response to parasite infection, could have affected smolt development. Patino et al. (1986) reported that coho smolts reared under stressful conditions (high density) had lower gill Na-K-ATPase activities than low-density cohorts. Neither cortisol or gill ATPase activity was

measured in the present study. Osmoregulatory disfunction could not be attribuated to kidney damage due to the parasite. The kidney nephron (glomerulus, tubules, associated blood vessels) appeared normal in the infected fish. It is possible that compression from the metacercaria cysts could have impaired the nephrons. Another possibility may be the inability of infected fish to restrict urine production upon entry into saltwater. Water loss under these conditions would result in the observed dehydration (weight loss). Further work is warrented to identify the osmoregulatory mechanism(s) impaired by metacercaria infection and the significance to Trinity river chinook survival in the marine environment.

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Future studies to determine whether there is a threshold severity of metacercarial infection which elicits impaired osmoregulatory ability should include:

1) Evaluation of survival, ion regulation (plasma NaCl, Mg++), plasma protein and osmolarity, hematocrit, and gill ATPase activity of infected smolts held for weeks in saltwater.

2) Monitor the incidence and severity of metacercaria infection of chinook smolts captured in the Klamath estuary. Are heavily infected fish found in the saline regions of the estuary?

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