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Merced River juvenile Chinook salmon health and physiology assessment, March-May 2012

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SUMMARY

The California-Nevada Fish Health Center performed a health and physiology assessment of juvenile Merced River Chinook salmon during the spring out-migration, 2012. The study consisted of two components including: A sentinel fish exposure using Merced River Hatchery (MRH) juvenile Chinook and monitoring of the health and physiological condition of natural and MRH origin out-migrants. No indications of contaminate exposure or toxicity were observed in histopathology, brain acetylcholinesterase activity or liver lipid peroxidation assays. No indications of immunosuppression were observed in differential blood counts, production of reactive oxygen intermediates in kidney monocytes, or bacterial challenge survival. The most significant health issue observed was *Tetracapsuloides bryosalmonae* (causative agent of Proliferative Kidney Disease, PKD) infection in the out-migrant Chinook during May. Advanced signs of kidney inflammation were observed by histopathology in 50% of out-migrant smolts captured in May. This disease can compromise the fish's performance in many areas (swimming, salt water entry, disease resistance) and decreases its potential for survival during out-migration.

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INTRODUCTION

Health and performance of juvenile salmonid out-migrants (smolts) are major determinants of their survival. Infectious disease will reduce survival both in direct mortality and reduced physical performance (predator avoidance, saltwater adaptation). Contaminants and elevated water temperature are identified as stressors for salmonids in the San Joaquin River and Estuary (USFWS 2001). Both of these stressors have potential for immunosuppressive effects (Rice and Arkoosh 2002). Infection with the myxozoan parasite that causes Proliferative Kidney Disease (PKD), *Tetracapsuloides bryosalmonae*, was observed in 90-100% of naturally produced fish in a 2001 survey of Merced out-migrant salmonid health (Nichols and Foott 2002). Methyl mercury, elevated water temperature, nitrogenous input, agricultural runoff, spring irrigation return and their relationship to river flow are other potential issues for Merced River Salmonids. This study examined smolt health during the critical spring outmigration using a suite of assays to detect pathogens (bacteria, virus, and parasites), biomarkers of contaminates exposure and immunosuppression.

The objectives of this study were to:

- Determine the prevalence and severity pathogen infection including *T. bryosalmonae*
- Examine trends in energy reserves
- Monitor smolt development
- Examine tissues for signs of toxic insult
- Monitor biomarkers of contaminate exposure
- Monitor biomarkers and immune function in sentinel fish exposures conducted in April and May.

METHODS

Sentinel fish exposure study

The sentinel fish study was conducted to assess the effects of exposure to Merced River water on select physiological parameters. This study used Merced River Hatchery (MRH) Chinook salmon (*Oncorhynchus tshawytscha*) fry reared at the California-Nevada Fish Health Center wet lab (Lab). On 7 February, 2012, approximately 400 juvenile Chinook salmon fry (39mm average FL) were transferred from MRH to the Lab and reared on an ozone and UV treated water source until utilized in the study. The fish were transferred from MRH soon after hatch in an attempt to avoid infection with *T. bryosalmonae*. This parasitic infection is observed most years in MRH juvenile Chinook. Fish were reared at ambient water temperature which ranged from 8.6°C to 16.8°C and fed a commercial salmon diet at an estimated 2%-5% of body weight/day. Feed rates were adjusted weekly to target an average fork length of 80mm on 1 May. Sentinel fish exposures were conducted in April and again in May (Table 1).

Fish received either a vaccination with a customized vaccine to *Vibrio spp.* (AquaTactics LLC, Kirkland, WA) or a sham vaccination of brain heart infusion broth (equal numbers Vaccinated and Sham

treatments). Fish were elastomer tagged based on vaccination treatment. Seven days after vaccination, fish were divided into two exposure groups (River or Lab) with equal numbers of Vaccinated and Sham treatment in each group. The River exposure group was transported to the Merced River and held near the Hopeton trap site, while the Lab exposure group was held in the lab. At the end of a 7 day exposure period fish were returned to lab. After an overnight acclimation period, fish either were sampled for physiological assessment (see below) or entered in the *Vibrio* Trial.

Table 1. Timeline summary for dates of *Vibrio* vaccination (Vaccination), start Merced River exposure, end Merced River exposure, start *Vibrio* challenge, end of *Vibrio* challenge for the sentinel fish exposure study.

| Exposure | Vaccination | Start Exposure | End Exposure | Start Challenge | End Challenge |
|--------------|-------------|----------------|--------------|-----------------|---------------|
| April | 12 Apr | 18 Apr | 25 Apr | 26 Apr | 3 May |
| May | 8 May | 15 May | 22 May | 23 May | 30 May |

Vibrio Bacterial Challenge – Fish received a 0.1ml IP injection of either a known titer *Vibrio anguillarum* suspension (Challenge) or sterile brain heart infusion broth (Control). The fish in the April Challenge group received a dose of 8.65×10^3 bacteria per ml (865 bacteria/fish). Due to low mortality in the April trial two doses were used in May. Fish in the May Challenge group received a dose of 1.60×10^4 or 1.60×10^5 bacteria/ml (1600-16000 bacteria/fish). A summary of number of fish in each group is presented in Table 2. Mortality was monitored daily for 7 days.

Table 2. Number of fish in bacterial challenge and physiology assessment groups. The bacterial challenge was conducted in April and repeated in May. Challenge groups were further divided by vaccination treatment (Vaccinated or Sham), exposure location (River or Lab) and challenge dose. Challenge doses were Control (sterile culture media), 10^4 CFU (bacteria)/ml in April, and 10^4 and 10^5 CFU/ml in May.

| Challenge | Vaccination | Exposure | Control | 10^4 CFU/ml | 10^5 CFU/ml |
|--------------|-----------------------|----------|---------|---------------|---------------|
| April | Vaccinated | River | 12 | 37 | - |
| | | Lab | 12 | 38 | - |
| | Sham | River | 12 | 37 | - |
| | | Lab | 12 | 36 | - |
| | Physiology Assessment | River | 10 | - | - |
| | | Lab | 10 | - | - |
| May | Vaccinated | River | 6 | 29 | 29 |
| | | Lab | 6 | 28 | 29 |
| | Sham | River | 8 | 30 | 28 |
| | | Lab | 6 | 30 | 28 |
| | Physiology Assessment | River | 10 | - | - |
| | | Lab | 10 | - | - |

Physiological assessment of sentinel fish- Prior to the bacterial challenge, one sham and one vaccinate fish from each cage were removed and sampled for blood and tissues. Ten salmon exposed to Merced River and ten control fish were sampled for each challenge date. The number of fish for each physiological assay varied. After fork length and weight were measured, a blood sample was obtained by severing the caudal peduncle and collecting blood into a heparinized microhematocrit tube. A blood smear was prepared, fixed in absolute methanol, later stained with Leishman and Giemsa stain, and 100 leukocytes microscopically differentiated to cell type; thrombocyte, lymphocyte, neutrophil, and monocyte (Yasutake and Wales 1983). Gill lamellae were placed into SEI buffer, frozen on dry ice, and later assayed by the enzymatic method of McCormick (1993). Liver (10 – 20mg) was placed into 0.5mL buffer (PBS, 0.5M butylated hydroxytoluene in acetonitrile), frozen on dry ice, and stored at -70°C. Malondialdehyde (MDA), a lipid peroxidation product, was measured in liver tissue by the method of Carney Almroth et al. (2008) using OxisResearch LPO-586 kit (OxisResearch, 1499 Rollins Road, Burlingame CA 94010). Brain was frozen on dry ice and later assayed for acetylcholinesterase activity by the method of Wheelock et al. (2005). Production of reactive oxygen intermediates (ROI) from stimulated kidney monocytes was measured by the method of Chettri et al. (2010). Briefly anterior kidney was aseptically removed, held in cold HBSS buffer (Hanks buffered saline without Ca² and Mg²), a single cell suspension produced with 1cc syringe and 21G needle, centrifuged (300xG, 10°C, 5 min), and the cell pellet suspended in MEM (5% FBS). Cell concentration was determined by hemocytometer counts. The mixed kidney cell population (<5% red blood cells due to blood collection) was added to 96 well plate (100 µL /well) and reacted with nitroblue tetrazolium (10mg in HBSS with 12µL phorbol 12-myristate 13-acetate as a stimulate) for 30 min, plate centrifuge to pellet cells, supernatant removed, and the cells fixed with 70% methanol. After extraction (120 µL 2M KOH and 140 µL DMSO per well), concentration of blue formazin (oxidized tetrazolium) was measured by plate reader at 630 nm. Tissues (kidney, gill, intestine, and liver) were fixed in Davidson's fixative, processed for hematoxylin and eosin slides, and microscopically examined for abnormalities.

Out-migrant Monitoring

Juvenile Chinook salmon were captured at the Hopeton rotary screw trap on the Merced River (RM 38) from 7 March to 15 May, 2012. The Hopeton site was operated by FISHBIO as a salmonid out-migrant monitoring site. Out-migrants juvenile Chinook were grouped into fry (30-45mm), parr (45-75mm) and smolt (75mm+) stages. Chinook fry were targeted on the 7 March collection, parr were targeted 3 March through 21 March, and smolts were targeted 4 April through the last collection on 15 May. The lab assay varied depending on the life stage of the fish (Table 3).

Issues with collection permits for ESA listed steelhead trout (*Oncorhynchus mykiss*) required several changes in this study from the original sampling plan. While steelhead were not targeted or sampled in this study, the permit was required because they were captured in the Hopeton trap. Due to permit delays, trapping did not begin until 7 March. Sampling was suspended between 16 April and 7 May when the *O. mykiss* take permit was exceeded. Due to the condensed sample period, no fish were sampled for energy reserve assessment (whole body triglyceride measurement). The triglyceride assay required the entire fish carcass and due to concerns over limited numbers of fish during the shortened sample period it was decided to divert all fish sampled to multiple assay purposes.

A random selection of live fish in the target size category was provided by the FISHBIO trapping crew after they had finished a preliminary workup. Fish were euthanized by an overdose of MS 222 (Tricaine-S, Western Chemical Inc., Ferndale, WA), measured for fork length, examined for external or internal abnormalities, and tissue samples were collected for lab assays.

Table 3. Target assays and sample numbers at each life stage. Assays include virology (Vir), bacterial culture (Bact), Histopathology (Histo), brain acetylcholinesterase activity (AChE), gill Na⁺/K⁺-ATPase activity (ATPase), liver lipid peroxidation (LPO), direct fluorescent antibody test for *R. salmoninarum* (DFAT), and differential blood leukocyte counts (DiffCt).

| Stage | Vir | Bact | Histo | AchE | ATPase | LPO | DFAT | DiffCt |
|-------|-----|------|-------|------|--------|-----|------|--------|
| Fry | 60 | - | 16 | 24 | - | - | - | - |
| Parr | - | - | 24 | 24 | 24 | 24 | - | - |
| Smolt | - | 48 | 60 | 60 | 60 | 72 | 60 | 72 |

Lab Assays

Bacteriology – A sample of kidney tissue was collected aseptically and inoculated onto brain-heart infusion agar. Bacterial isolates were screened by standard microscopic and biochemical tests (USFWS and AFS-FHS 2010). These screening methods would not detect *Flavobacterium columnare*.

Renibacterium salmoninarum (the bacteria that causes bacterial kidney disease) was screened by fluorescent antibody test of kidney imprints.

Virology – Pooled kidney samples from 5 fish were inoculated onto EPC and CHSE-214 cell lines at 15°C as described in the AFS Bluebook (USFWS and AFS-FHS 2010) with the exception that no blind pass was performed.

Histopathology – Samples of gill, liver, intestine and posterior kidney were rapidly removed from the fish and immediately fixed in Davidson’s fixative. Fixed samples were processed for 5 µm paraffin sections and stained with hematoxylin and eosin (Humason 1979). Sample slides were examined under a light microscope. Infections of the myxozoan parasite *T. bryosalmonae* were rated for intensity of parasite infection and associated tissue inflammation. Intensity of infection was rated as none (zero), low (<10), moderate (11-30) or high (>30) based on number of *T. bryosalmonae* trophozoites observed in the kidney section. Severity of kidney inflammation was rated as normal, focal, multifocal or diffuse. Data analysis was performed using R version 2.15.1 using Fisher’s Exact Test for Count Data.

Gill ATPase activity – Gill lamellae were collected into SEI buffer and frozen on dry ice. In the lab, gill Na⁺/K⁺-Adenosine Triphosphatase (ATPase) activity was assayed by the method of McCormick (1993). Gill ATPase activity is correlated with osmoregulatory ability in saltwater and is located in the chloride cells of the lamellae.

Differential Count – Blood was collected from the caudal peduncle, blood smears were prepared on individual microscope slides, and then slides were fixed with a spray slide fixative. Slides were stained with the Protocol Hema 3 stain kit (Fisher Scientific, Kalamazoo, MI). A Differential blood leukocyte count was performed on the first 200 leukocytes observed (Yasutake and Wales 1993). Because

activated thrombocytes can be difficult to distinguish from some lymphocytes, the differential count was summarized as the ratio of lymphocytes (+thrombocytes) to granulocyte (Lt:G). Higher granulocyte counts result in low Lt:G ratio values and can indicate infection, tissue damage, or seasonal blood cell changes (Modra et al. 1998). A Lt:G ratio of 20 or greater was considered normal; a Lt:G of 10-20 was marginal; and a Lt:G of less than 10 was judged to be low and suggested neutrophilia (abnormally high neutrophil level).

Acetylcholinesterase (AChE) activity – Fish heads were collected and frozen on dry ice in the field. Once at the laboratory samples were stored at -80°C until assayed at a later date. In order to assay the samples for AChE activity brain tissue was removed, weighed, and diluted 1:60 (g:ml) in 0.1M phosphate buffer (pH 8.0) with 0.5% Triton X-100. Samples were homogenized using an ultrasonic cell disruptor for approximately twenty seconds. The supernatant (30 μl) was used in the assay method outlined by Wheelock et al. (2005) using a final assay concentration of 2mM of acetylthiocholine iodide and 0.32mM of DTNB. Plates were assayed for 10mins using a kinetic read on a microplate reader (Synergy H1, BioTek Instruments, Winooski, VT). The amount of protein in the sample was measured using a BCA protein assay kit (Pierce BCA Kit, ThermoScientific, Rockford, IL). AChE activity was normalized to the amount of protein and the final rate was calculated as $\mu\text{moles AChE hydrolyzed}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$.

Lipid Peroxidation (LPO) – Malondialdehyde (MDA), a lipid peroxidation product, was measured in liver tissue by the method of Carney Almroth et al. (2008) using OxisResearch LPO-586 kit (OxisResearch, Burlingame, CA).

RESULTS

Physiological assessment of sentinel fish

No mortality occurred in fish held in the River or Lab groups during the 7d April and May exposure periods. Both River and Lab exposure group fish were of similar size (Table 6). No gill Na-K-ATPase data is reported due to abnormal kinetic profiles. The ADP standard curve was normal indicating that the majority of enzymes and co-factors were functional. Similarly, the pH and magnesium conditions were also normal for the assay. We suspect that the recently purchased Sigma Chemical Adenosine Triphosphate was faulty as this nucleotide is the substrate for the ouabain-sensitive gill $\text{Na}^{+}/\text{K}^{+}$ -ATPase enzyme.

Survival of fish in the *Vibrio* bacterial challenges was much higher than anticipated. Survival in the challenges averaged 99% in April and 97% in May (Table 4). Preliminary trials suggested greater than 50% mortality would be expected in the Sham vaccination treatment. The reason for the decrease in virulence of the *V. anguillarum* isolate between the pilot work and challenges was not apparent. A second, higher dose of bacteria was added to the May challenge, but this change did not significantly increase mortality. Due to the low mortality, no analysis between groups was attempted.

Elevated neutrophil counts (low Lt:G ratios) were observed in 25% (2/8) of fish in April and 50% (3/6) of fish in May River exposure groups, while none of the Lab exposure groups were elevated (Table 5). The difference between River and Lab exposure groups was more significant in the May challenge ($P=0.015$) than in April ($P=0.627$, Fisher's Exact Test). This data is reflected in the lower mean Lt:G ratio seen in the

river fish (Table 7) and suggests that River exposure stimulated the migration of neutrophils into the blood. The high variability in cell types did not allow for valid statistical comparisons. In particular, blood smear quality was deemed poor for the 26 April samples.

Table 4. Percent survival and number of sentinel Chinook salmon in two bacterial (*Vibrio anguillarum*) challenges conducted in April and May. Challenge groups were divided by vaccination treatment (Vaccinated or Sham), exposure location (River or Lab) and challenge dose. Challenge doses were Control (sterile culture media), 10⁴ CFU (bacteria)/ml in April, and 10⁴ and 10⁵ CFU/ml in May.

| Challenge | Vaccination | Exposure | Control | 10 ⁴ CFU/ml | 10 ⁵ CFU/ml |
|-----------|-------------|----------|-----------|------------------------|------------------------|
| April | Vaccinated | River | 100% (12) | 97% (37) | ND |
| | | Lab | 100% (12) | 97% (38) | ND |
| | Sham | River | 100% (12) | 100% (37) | ND |
| | | Lab | 100% (12) | 100% (36) | ND |
| May | Vaccinated | River | 100% (6) | 100% (29) | 100% (29) |
| | | Lab | 100% (6) | 96% (28) | 97% (29) |
| | Sham | River | 100% (8) | 83%(30) | 100% (28) |
| | | Lab | 100% (6) | 97% (30) | 93% (28) |

Table 5. Results of leukocyte differential counts for juvenile Chinook exposed to the Merced River (River) or held as controls (Lab) in challenges conducted in April and May. Results summarize number of sentinel exposure fish with Low (<10), Marginal (10-20) or Normal (>20) Lt:G ratio. Fish with a Lt:G ratio of less than 10 were judged to have an abnormally high number of neutrophils (neutrophilia). The difference between Lab and River in April was not significant (P=0.627), but the difference in May was significant (P=0.015, Fisher's Exact Test)

| Challenge | Exposure | Low | Marginal | Normal |
|-----------|----------|-----|----------|--------|
| April | Lab | 0 | 2 | 4 |
| | River | 2 | 3 | 3 |
| May | Lab | 0 | 0 | 6 |
| | River | 3 | 2 | 1 |

NBT data (mOD/10⁵ cell) was combined for sham and vaccinate salmon per exposure group as no significant difference was detected by T-test (P<0.05). The 26 April River exposed fish had significantly greater ROI production than the laboratory controls (T-test, P=0.007). Conversely, no significant difference in ROI production was observed in kidney cells of all four groups (River and Lab; Vaccinated and Sham fish) in 23 May challenge sample (Kruskal-Wallis 1way ANOVA on Ranks, P=0.233). The AChE activity of the 23 May sentinels (both River and Control) was approximately half that of smolts collected from the river in April and May (Table 12) and there was significant difference between exposure groups. The 26 April brain samples were lost due to an error in defrosting time prior to the assay. None of the sampled fish showed elevated liver lipid peroxidation values (MDA equivalents).

No tissue abnormalities were observed in histological samples of either river-exposed or laboratory control sentinels from either challenge (26 April = 4 River and 2 Lab fish, 23 May = 10 River and 4 Lab fish). A single *T. bryosalmonae* trophozoite was observed in a kidney blood sinus from one fish in both

challenges (26 April River and 23 May Lab exposure fish). No inflammatory response was associated with these parasites. It would appear that the low-grade infection was obtained at Merced River Hatchery prior to the population's transfer to the Lab on 7 February.

Table 6. Mean (SD) size and physiological data of sentinel salmon sampled on 26 April and 23 May. Data includes fork length (FL, mm), condition factor (KFL = wt[g]/FL³x10⁵), reactive oxygen intermediate production of kidney cells in nitroblue tetrazolium (NBT, mOD₆₃₀ / 10⁵ cells), brain acetylcholinesterase activity (AChE, μmoles AChE hydrolyzed/min/mg protein brain), and percentage of liver samples with lipid peroxidation values over background (%LPO>0).

| | <u>FL</u> | <u>KFL</u> | <u>NBT</u> | <u>AChE</u> | <u>%LPO>0</u> |
|---------|-----------|---------------|------------|---------------|------------------|
| 26-Apr | | | | | |
| River | 72 (4) | 0.991 (0.100) | 115 (36) | na | 0 |
| Control | 71 (5) | 1.020 (0.068) | 77 (17) | na | 0 |
| 23-May | | | | | |
| River | 83 (6) | 1.042 (0.074) | 269 (104) | 0.105 (0.026) | 0 |
| Control | 87 (5) | 1.075 (0.074) | 208 (32) | 0.099 (0.022) | 0 |

na = not applicable, defrost error impaired activities

Table 7. Blood leukocyte data from blood smear evaluations of river and laboratory control sentinel salmon sampled on 26 April and 23 May. Data reported as mean (SD). The ratio of lymphocytes + thrombocytes : granulocytes (Lt:G) is reflective of neutrophil counts.

| | <u>Thrombocyte</u> | <u>Lymphocyte</u> | <u>Neutrophil</u> | <u>Monocyte</u> | <u>LT:G</u> |
|--------------|--------------------|-------------------|-------------------|-----------------|-------------|
| 26-Apr | | | | | |
| River, n=8 | 50.8 (18.0) | 40.5 (17.0) | 8.3 (5.1) | 0.4 91.0) | 14.9 (7.9) |
| Control, n=6 | 60.2 (18.5) | 40.0 (15.7) | 2.8 (2.1) | 0.5 (0.6) | 62.2 (53.9) |
| 23-May | | | | | |
| River, n = 6 | 24.7 (0.3) | 68.0 (0.1) | 7.8 (0.5) | 1.5 (1.0) | 25.8 (1.4) |
| Control, n=6 | 20.5 (0.2) | 76.7 (0.1) | 2.7 (0.5) | 0.2 (2.4) | 46.1 (0.6) |

Out-migrant monitoring

A total of 202 juvenile Chinook out-migrants were collected at the Hopeton RST (Table 8). No viral infections were detected in the 60 fry sampled. No obligate bacterial pathogens were detected in the 65 smolts sampled. No external bacterial infection was noted in 130 parr and smolts examined. Clinical signs of PKD (swollen kidneys) were observed in 1.5% (2/130) of the fish. Both of these clinical fish were sampled on 9 May, with one fish also having pale gills (anemia). The vast majority of the juvenile Chinook examined appeared to be in good health.

Histopathology - Tissues of 96 juvenile Chinook were examined by histopathology. Only kidney was examined on small (fry) size fish. The prevalence of infection (POI) of external parasites was very low, with the external protozoan *Ichthyophthirius multifiliis* observed on the gills of a single smolt captured 9 May (2% POI, 1/62). Internal parasites observed included intestinal trematodes in 4 fish (7% POI, 4/59) and *T. bryosalmonae* in kidney, gill and liver of 41 of 91 fish (45% POI). The first *T. bryosalmonae*

infections were observed the week of 18 March when water temperatures observed by FISHBIO trap operators ranged from 10.4 to 16.9°C, and by the week of 13 May water temperatures ranged from 16.8 to 20.1°C (J. Montgomery, email communication, 11 October, 2012). *Tetracapsuloides* infections were observed primarily in the kidney, with associated kidney lesions observed in the 9 May and 15 May samples (Table 9 and Table 10). No histological indications of contaminate exposure was observed. While several parasites were detected, the *T. bryosalmonae* infections were associated with the most serious lesions particularly in the later out-migrant samples (9 May and 15 May).

Table 8 . Number of juvenile Chinook salmon included for health and physiology monitoring at each sample date from the Hopeton trap on the Merced River. Life stages were fry (<45 mm), parr (45-75mm) and smolt (>75 mm).

| Sample Date | Life stage | Number |
|---------------------|------------|--------|
| 1 Mar | Fry | 60 |
| 7 Mar | Parr | 24 |
| 21 Mar | Parr | 24 |
| 4 Apr | Smolt | 24 |
| 11 Apr | Smolt | 24 |
| * break in sampling | | |
| 9 May | Smolt | 24 |
| 15 May | Smolt | 22 |

Table 9. Intensity of *T. bryosalmonae* infection in the kidney by histopathology. Infections were rated as None, Low, Mod or High based on number of *T. bryosalmonae* trophozoites observed.

| Date | None | Low | Mod | High |
|---------------------|------|-----|-----|------|
| 1 Mar | 24 | | | |
| 7 Mar | 10 | | | |
| 21 Mar | 10 | 1 | | |
| 4 Apr | 3 | 8 | | |
| 11 Apr | 3 | 7 | 2 | |
| * break in sampling | | | | |
| 9 May | | 1 | 3 | 7 |
| 15 May | | | 6 | 6 |

Table 10. Severity of kidney inflammation associated with *T. bryosalmonae* infections observed by histopathology. Inflammation was rated as Normal, Focal, and Multifocal or Diffuse.

| Date | Normal | Focal | Multifocal | Diffuse |
|---------------------|--------|-------|------------|---------|
| 1 Mar | 24 | | | |
| 7 Mar | 10 | | | |
| 21 Mar | 11 | | | |
| 4 Apr | 11 | | | |
| 11 Apr | 12 | | | |
| * break in sampling | | | | |
| 9 May | 1 | 4 | 4 | 1 |
| 15 May | 1 | 5 | 5 | 1 |

Gill ATPase Activity - No gill Na^+/K^+ -ATPase activity results are not reported due to abnormal assay kinetic profiles described above.

Differential Count - Blood leukocytes counts were performed on 59 Smolt stage fish and summarized as the lymphocyte + thrombocyte to granulocyte ratio (Lt:G). The normal Lt:G ratio was judged to be 30 or greater, and fish with Lt:G ratio of 10 or less were considered to have an abnormally high number of neutrophils (neutrophilia). The prevalence of neutrophilia ranged from 6-14%. No difference between the sample dates was observed (Table 11, $P=0.402$, Fisher's Exact Test for Count Data).

Acetylcholinesterase (AChE) activity - Brain AChE activity ($\mu\text{moles AChE hydrolyzed} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) ranged from 0.186 to 0.358 in all out-migrant Chinook samples. The greatest mean activity was observed in fish sampled during fry and parr life stages, while the lowest mean activity was observed during in smolts (Table 12).

Lipid Peroxidation (LPO) - The results of the LPO assay are reported in Table 12. Levels of MDA were below the assay background levels, and no evidence of lipid peroxidation was observed.

Table 11. Results of leukocyte differential counts showing number of Merced River Chinook salmon smolts with Low (<10), Marginal (10-20) or Normal (>20) Lt:G ratio. Fish with a Lt:G ratio of less than 10 were judged to have an abnormally high number of neutrophils (neutrophilia).

| Date | Low | Marginal | Normal |
|---------------------|-----|----------|--------|
| 4 Apr | 1 | 4 | 13 |
| 11 Apr | 2 | 1 | 16 |
| * break in sampling | | | |
| 9 May | 2 | 3 | 9 |
| 15 May | 1 | 3 | 4 |

Table 12. Brain Acetylcholinesterase (AChE) activity ($\mu\text{moles AChE hydrolyzed} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) and liver lipid peroxidation (LPO) levels ($\mu\text{M MDA} \cdot \text{mg protein}^{-1}$) in juvenile Merced River Chinook captured at the Hopeton trap. Data presented as Mean \pm SE (N). AChE results with letter subscripts in common are not significantly different (ANOVA, $P<0.05$). All LPO results were at or below assay background levels and were not compared.

| Date | Life Stage | AChE | LPO |
|---------------------|------------|--------------------------|------------------------|
| 1 Mar | Fry | 0.29 \pm 0.008 (24) a | |
| 7 Mar | Parr | 0.29 \pm 0.006 (12) ab | 0.007 \pm 0.001 (12) |
| 21 Mar | Parr | 0.31 \pm 0.005 (12) a | |
| 4 Apr | Smolt | 0.24 \pm 0.012 (12) bc | 0.023 \pm 0.002 (12) |
| 11 Apr | Smolt | 0.28 \pm 0.007 (12) ab | |
| * break in sampling | | | |
| 9 May | Smolt | 0.23 \pm 0.005 (12) c | 0.013 \pm 0.001 (12) |
| 15 May | Smolt | | 0.014 \pm 0.002 (11) |

DISCUSSION

The most significant health issue observed in Merced River Chinook out-migrants was *T. bryosalmonae* infection. By mid-May most of the out-migrant smolts were carrying moderate to high parasite numbers and over half were showing significant inflammation in kidney histopathology sections. A few of the sentinel exposure fish had very light *T. bryosalmonae* infections even though they were transferred from MRH within a few weeks of ponding to avoid infection. Apparently, even a few weeks of exposure in February were enough to achieve light infections. Proliferative kidney disease is progressive and dependent on water temperature (Ferguson 1981; Foott et al. 2007). It was expected that infection intensity and PKD severity would increase during the study period. Hatchery origin Chinook out-migrants (adipose fin clip fish) were first caught in the 15 May sample at the Hopeton trap. Gross clinical signs of PKD (anemia and swollen kidney) were observed in naturally produced fish sampled 9 May (2/24 fish) and histopathology results indicate high numbers of parasites were common in both the 9 May (natural) and 15 May (natural and hatchery) smolts. Infection with this parasite does not necessarily lead to the death of the animal. Hedrick and Aronstien (1987) found that over 90% of *T. bryosalmonae* infected juvenile Chinook salmon transferred to full strength seawater were able to recover under laboratory conditions. Survival in the river is likely much lower due to the anemia, kidney dysfunction and immune suppression linked to PKD (Angelidis et al. 1987, Hedrick and Aronstien 1987). This disease compromises the fish's performance in many areas (swimming, salt water entry, disease resistance) and decreases its potential for survival during out-migration.

Several assays originally proposed for this study were did not occur as originally envisioned including monitoring energy reserves by whole body triglyceride, growth trends by RNA:DNA, and smolt development by gill Na^+/K^+ -ATPase. The decision to drop energy reserve measurement was driven by the condensed sample period and most efficient use of the fish captured. The RNA:DNA lab assay requires further bench testing before it can be implemented. A problem with the gill ATPase assay in the lab resulted in the loss of that measurement.

No indications of toxic insult or contaminate exposure were observed by histology, brain AChE activity or liver LPO assays. Tissue abnormalities observed by histopathology were associated with parasite infection, primarily *T. bryosalmonae*. It is not known if contaminate exposure during the break in sampling due to permit issues would have been detected in fish captured later in the season. The April sentinel fish exposures did occur during the break in sampling and those fish provided an indication of out-migrant health. No indications of problems were observed in the April sentinel exposure fish; however AChE activity was not available for the April sentinel exposure (see results). The lower AChE activity levels in the older smolt fish are consistent with published observations. Phillips et al. (2002) and Durieux et al. (2011) both found that AChE activity levels declined in larger fish. Fulton and Key (2001) observed that a decrease in AChE activity of 70% was associated with mortality. The 27% (0.31 to 0.23, Table 12) decline observed in this study was likely the normal decline associated with growth. Low AChE activity levels were observed in the May sentinel fish compared to the natural out-migrant fish captured two weeks earlier. Both the River and Lab exposure groups for the May sentinel samples had similarly low AChE activity levels, so the low activities were not a result of the exposure. We have no explanation for this difference

In the sentinel fish exposures, River exposure appeared to stimulate the innate immune system as seen in an increase in blood neutrophils (May challenge), and kidney NBT (April challenge). No indications of overt immunosuppression were observed in differential blood counts (leukocyte numbers), production of reactive oxygen intermediates in kidney monocytes, or bacterial challenge (high survival in all groups). No biomarker responses indicating oxidative stress (MDA assay) or organophosphate inhibition of AChE were observed in sentinel fish.

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