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Juvenile Lost River Sucker sentinel survival in Upper Klamath Lake mesocosm netpens (July - October 2013), overwinter survival, and laboratory hypoxic challenge.

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Summary

Survival and tissue responses were evaluated in 6 sentinel groups of Lost River sucker (LRS) 0+ fry and 1+ yearlings exposed in Upper Klamath Lake between 29 July – 16 September 2013. Replicates were sampled every 3 – 4 days post-exposure (dpe) from 0.37m^3 hoop net pens that spanned the entire water column. LRS 1+ held for 14dpe experienced 21 and 25% mortality between12-26 August. Hypoxic conditions ($\leq$ 1.50 mg/L) preceded these sample dates by 3- 6 d. Other LRS 1+ cohorts had low mortality (0 – 4%) and LRS 0+ mortality was $\leq$ 5% over the summer. Bi-weekly sampling of LRS0+ held at the Williamson R. mouth showed high survival ($\geq$ 90%) except for fish held over the entire summer (93d). This sentinel group had low survival (11%), no growth, low whole body triglyceride content (TG), and heavy metacercaria infections. Both yearling and LRS0+ placed at this site on 3September had $\geq$ 93% over-winter survival. Rearing captive fry with lake benthos (mud) was judged to be slightly beneficial for growth and health compared to standard tank environments. No conclusive effect on growth was observed in a pilot experiment.

Two histological observations were common to all exposure groups. Hyaline droplet (excess protein from the filtrate) was seen in proximal tubules of the kidney and positive immunohistochemistry staining for microcystin toxin (MCLRa) was seen in digesta and intestinal epithelium. No such reaction was observed in the liver or kidney of these fish. No other tissue abnormalities or parasite infection was seen in North lake sentinels. Sub-lethal stressors likely affected North lake sentinel fish during August. The lowest TG values, in 14dpe sentinels, occurred in early August during low oxygen conditions. Depressed liver protein phosphatase 2A (PP2A) activity were measured in the 19-26 August period. Yearling sentinel PP2A showed this depression more often than LRS0+ cohorts. Laboratory studies indicate that hypoxia does not affect PP2A which implies that microcystin toxin concentration could have induced the observed PP2A depression. Generally fish exposed for 14dpe had higher PP2A activities than 7dpe cohorts. Laboratory hypoxic challenges demonstrate that both LRS 0+ and 1+ can survive the published 96h LD_{50} oxygen concentration of 1.58mg /L as well as > 28h exposure to a combination of elevated ammonia (UIA 0.312 – 0.547mg/L, 33 - 40% of the LD_{50} value) and 2 – 4mg/L oxygen. The critical oxygen minima, for both LRS 0+ and 1+, is between 0.75 and 1.03mg/L. Our currently working hypothesis is that synergistic effects of adverse water quality, parasitic infection, and ingestion of toxic algae may substantially increase predation rates for juvenile suckers in Upper Klamath Lake during the summer.

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Introduction

Lost River Suckers (*Deltistes luxatus*) are listed as an endangered species and inhabit eutrophic waters of Upper Klamath Lake in Southern Oregon. Summer blooms of the cyanobacterium *Aphanizomenon flos-aquae* are associated with chronic high alkalinity (pH> 9.5) as well as transient periods of hypoxia (dissolved oxygen < 2.0mg/L) and elevated ammonia (Wood et al. 1996). Low recruitment success has been observed in skewed age data (Scoppettone and Vinyard 1991, Janney et al. 2008). Burdick et al. (2008) stated that 0+ fry catch declines significantly between August and October each year. Several hypotheses concerning the causes of poor age-0 to age-1 survivorship have been suggested including emigration out of the lake (Harris and Markle 1991; Gutermuth et al. 2000) and poor summer water quality conditions (Martin and Saiki 1999). Another cyanobacterium in Upper Klamath Lake, *Microcystin aeruginosa*, poses a health threat to suckers due to its hepatotoxin, microcystin-LR (Gilroy et al. 2000.). Microcystin bind to protein phosphatase (PP) 1 and 2A and inhibit their multiple functions that often lead to liver necrosis (Fisher et al. 2000). Monitoring the activity of PP2A could provide insight into microcystin effects on juvenile sucker health. Intermittent hypoxia during the summer is another health threat faced by juvenile suckers in Upper Klamath Lake. Critical water quality thresholds for these fish, including LD_{50} concentrations for dissolved oxygen, have been determined in laboratory studies (Sakai et al. 1999, Meyer and Hansen 2002). These controlled studies were run under base conditions of pH7.5 to 8.0, 20 -22°C without acclimation. Investigation, of juvenile sucker responses to hypoxia under summer alkalinity (> pH 9.5), would simulate summer lake conditions. Sentinel fry studies in 2011 – 2012 have observed highly variable mortality rates with hypoxia (Foott et al. 2012 and 2013).

We focused this study on LRS fry survival during the late July through October "first summer" period as well as over winter. The study design used captive reared progeny, from Upper Klamath Lake Lost River Sucker adults, held in replicate mesocosm net pens. Cultured juveniles provided experimental fish of known age, health, similar size, and energy content. The mesocosm represented a link between observational field studies in the lake and controlled laboratory experiments (Sala et al. 2000).

Specific objectives were:

1. Develop early rearing techniques.

2. Determine temporal survival of sentinel fry in the North region of Upper Klamath lake and Williamson River mouth during the summer and over winter.

3. Perform histological examination of select tissues for abnormalities and pathogen infection in sentinel fry and yearlings as well as presence of microcystin toxin. Determine liver protein phosphatase 2A activity (enzyme inhibited by microcystin

toxin) of sentinel and experimental LRS. Compare condition and triglyceride content of sentinels held for 14d.

4. Monitor temperature, ammonia, dissolved oxygen, and pH of water at the two sites in order to observe relationships to survival.

5. Design, test, and validate a wetlab hypoxic system to challenge suckers under defined pH, temperature, low oxygen, and ammonia conditions. Determine, mortality threshold of 0+ fathead minnows, LRS 0+ and 1+ for hypoxic conditions.

Methods

Fish rearing LRS 0+ – On 4 April 2013, gametes were obtained from 4 male and 4 female Lost River suckers captured by USGS at Sucker Springs, Upper Klamath Lake OR. Fish were immediately released after a small portion of gametes were collected (~30g/3 female wet weight of eggs, [1 female at ~4g wet weight of eggs] and 4 mL of semen per male). We estimate that 25,000 eggs were collected based on an egg count estimator of 275 eggs/ g (pers. comm. S. Vanderkooi, USGS) (275/g x 30g x 3 females +1000 eggs from 1 female). This egg collection represents a small percentage of the 50,000 – 100,000 eggs per female reported for 500 – 750mm FL female LRS (Buettner and Scoppettone 1990). Gametes from 2 females were expressed directly into a plastic Tupperware container with semen from 2 males immediately added to the same container. This process was repeated with an additional 2 females and 2 males into a separate container. During the second collection it was noted the first male had "watery" milt and the 2nd female only gave 4g of eggs. Gametes were gently mixed for 15 minutes, excess fluid poured off, the container filled with lake water and then placed into an ice chest for transport to the CA-NV Fish Health Center. Water quality at Sucker Springs at spawning was 15.4°C, pH 8.55, and dissolved oxygen of 7.24mg/L. Upon arrival at the wetlab, lake water was poured off and eggs disinfected in a 100-mg/L iodiphor solution for 5min. This process was repeated once more to ensure adequate surface disinfection of fertilized eggs. Virological assay of ovarian and seminal fluid did not detect virus (USFWS and AFS-FHS 2007).

Eggs were incubated in 4 upwelling cylinders (12.7cm PVC with 250µm mesh screen bottom) divided into 2 tanks (2 upwellers per tank) and held at 13.3 - 13.7˚C until larvae were actively swimming and observed feeding. There was no manipulation of eggs or detection of fungus until 7 days post-spawn when cleaning occurred and fungus was observed in 2 of the 4 upwellers. Contaminated upwellers were treated with 1600mg/L formalin for 5min for two consecutive days. During the treatment period approximately 6,936 eggs with visible fungus were removed from the two upwellers. First hatch was observed 13April (10 days post hatch). By 14April, 95% of all larvae had hatched and were actively swimming; the remaining 5% hatched on 15April. Larvae were subsequently reared in 2.8L baskets (16.5cm x 12.7cm x 13.3cm frames covered by a

500µm mesh bag) suspended within aquaria. Newly hatched artemia nauplii were introduced 19April, however, no feeding response was observed. Nauplii were offered each day thereafter. On 22April (9dph) larvae could be seen actively hunting and feeding on artemia. At 13dph larvae were transferred from rearing nets to 40L aquaria where they were fed a compilation of artemia nauplii and decapsulated artemia cysts. On 3May (20dph) a larval diet (Otohime^{TM} A diet; granule size 250µm, manufactured by Marubeni Nisshin Feed Co., Ltd, Tokyo, Japan, and purchased directly from Reed Mariculture, Inc. Campbell, CA, 95008, USA) was introduced . Feed size was increased approximately every 9 to 18 days depending on feeding response. Granule size was 250µm - 1410µm over the experiment. During the first month of feeding fish were hand fed 2 times per day; thereafter belt feeders were used allowing for constant access to feed (feed was estimated 5-6% bodyweight/d). In both 2011 and 2012, LRS fry developed gill hyperplasia prior to the July deployment into Upper Klamath Lake net pens. We observed some positive effects on survival and growth when the 2012 fry were reared over lake mud. To maximize chances of producing healthy fry that were >35mm long by late July 2013, we split the population into two 450L rectangular tanks on 16May. One tank contained sediment (mud) from Upper Klamath Lake. Fish were fed at the same rate (3-5%BW/d) a diet of B1 and B2 Otohime pellets (250-650µm) and sub-sampled for weight and length on bi-weekly basis until July26. This aspect of rearing was not a controlled study. Due to the expanding size variation of the diets there was approximately 1-2 weeks of overlap for each new diet with the previous diet type. By 25May all larvae had been moved into 450L fiberglass rearing troughs. The fish from both rearing troughs were transferred to a 900L fiberglass rearing trough on 26July. We estimate that the population experienced a 28% loss from egg to 20mm fry. No virus was detected in a 60 fry sample performed in July prior to the first movement to Upper Klamath Lake. Fry were transported to the lake within plastic bags containing 2 - 2.5L of water adjusted to pH 9.0 at ambient lake temperature and filled with oxygen. Bags were then placed inside coolers to minimize movement and temperature change. Our intent was to provide some acclimation to lake conditions during the 3h transport to the lake.

Fish rearing LRS 1+ – A portion of the 2012 LRS population was reared over the winter and used as **1+** sentinel fish in 2013. Fish were held in a 900L D&T trough on ambient water at a flow rate of 18.9 – 26.5L/min and fed a dry pelleted diet (OtohimeTM, manufactured by Marubeni Nisshin Feed Co., Ltd, Tokyo, Japan, and purchased directly from Reed Mariculture, Inc. Campbell, CA, 95008, USA). Feed sized ranged from 840µm – 1.7mm over the holding period. No virus was detected in a 60 fish sample performed in July prior to the first movement to Upper Klamath Lake. Yearling suckers were transported to the lake in plastic bags containing 2 - 2.5L of water adjusted to pH 9.0 at ambient lake temperature and filled with O2. Bags were then placed inside of coolers to minimize movement and temperature change. Our intent was to provide some acclimation to lake conditions during the 3h transport to the lake. For additional rearing information refer to the Foott et al. (2013).

Exposures – Six exposures were conducted between 29July and 16September at two sites (Table 2). The sites were 1) Northern Upper Klamath Lake near Modoc point and 2) in the Williamson River approximately 0.4km upriver from the mouth (Figure 1). In contrast to the 2012 study, net pens extended the full depth of the water column (similar to the 2011 study). Water depth at both the Northern exposure site and Williamson River ranged from 2.5m at each location in July to 2.3m & 1.9m, respectively, in August. Net pens were sampled in triplicate every 3, 7, 10 and 14 days at the Northern exposure site. Williamson River net pens were sampled every 7 and 14 days. The number of live and dead fish were recorded from each net pen, live fish anesthetized in MS222, a subset of 4 - 5 fish dissected for PP2A liver samples (north lake) and TEM liver archive (Karnovsky fixative for 6h and stored at 4°C in cacodylate buffer), 4 - 5 fish were fixed in Davidson's fixative for histological examination, and the remainder frozen for whole body triglyceride and/or toxin analysis (archive). The primary purpose of sentinel fish in the Williamson River was to examine survival and over summer growth of LRS should this site be used for in-situ rearing. Both fry and yearling suckers were held in 0.58 m^3 net pens that rested on the Williamson R. mouth bottom from 30October2014 to 10March2014 (131 d).

Each net pen consisted of an outer and inner hoop net. Outer hoop dimensions were 3.66m high x 0.6m diameter enclosed with 5mm UV treated netting. The approximate volume was 53m^3. Each hoop net was anchored to the lake bottom and two buoys attached by D-rings at the water's surface to ensure each net pen maintained its vertical integrity. Each buoy was given a unique number to identify sampling groups. Smaller inserts were placed into each net pen where fish were held during each exposure period. The smaller inner hoop nets were weighted, in order to have contact with the lake bottom, with a high density polyethylene jug acting as a buoy to maintain vertical integrity. This allowed fish to be sampled by removing the net insert while the outer net pen remained in its original location. A new insert could then be added to the net pens along with new sentinel fish for the following exposure. Overall dimensions of the inner hoop nets were 3.66m high x 0.5m diameter enclosed with 1.6mm netting. The approximate volume was 0.37m^3. Water quality measurements (temperature ˚C, dissolved oxygen (mg/L), and pH) were logged hourly with YSI data sondes suspended from a buoy located in the center of the net array. Surface measurements were obtained at 1m depth and at the bottom by another data sonde at 1m above the bottom. Ammonia (Hach low range kit, DR800 colorimeter), pH, and temperature were obtained from a composite water column sample (5.1cm PVC pipe with stopcock 0.5m above bottom) to calculate unionized ammonia. This spot measurement occurred prior to fish sampling.

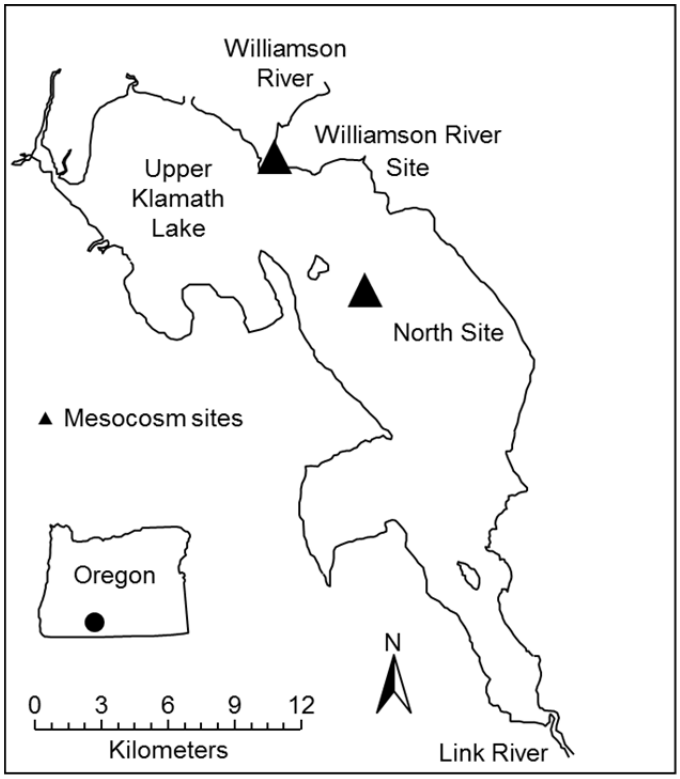


Figure 1. Map of the study area in Upper Klamath Lake, Oregon, showing the Northern and Williamson River exposure sites.

Whole body triglyceride - Standard length and weight were recorded for each frozen fish and its condition factor calculated (KSL = WT/ SL^3x 10^5). Konig and Borcherding (2012) report freezing has less effect on morphometrics than preservation in ethanol or formalin. Cold distilled water was added to a 55mL tube at a 1:1 ratio containing each fish and blended for 30 – 90s with a Biospec M133 homogenizer. Tissue triglyceride content (mg TG/g tissue) was assayed by a modification of Weber et al. (2003). Absolute isopropanol was added (5x dilution w/v) to an aliquot of homogenate, mixed at room temperature for 20min, centrifuged at 3220xg for 5min, and replicate 10μL samples of the 10x diluted supernatant used in an enzyme assay for triglyceride (Pointe Scientific triglyceride GPO kit).

Protein phosphatase 2A activity - Protein phosphatase 2A activity, in both 0+ and 1+ LRS livers, were assayed by an immunoprecipitation method (Millipore 28820 Single Oak Drive, Temecula CA 92590, catalog #17-313, PP2A kit). Liver (0.02 – 0.08g) was sampled from 7 and 14d fish, frozen on dry ice in 0.5mL base buffer (20mM imidazole-HCL, 2mM EDTA and EGTA, pH 7.0 in Tris saline buffer), defrosted with the addition of 0.5mL of inhibitor buffer (base buffer with 10μg/mL aprotinin, leupeptin, antipain, soybean trypsin inhibitor; 1mM benzamidine and $PhMeSO_4F$ (Sigma Chemicals), aliquots held at -70°C), sonicated for 5s (Microson XL2005 ultrasonic cell disruptor, Heat Systems Inc, #3 setting), centrifuged at 2,000xg 5°C for 10min, 100μL of

the supernatant placed into the assay. Briefly, polyclonal antibody to human PP2A and protein A agarose beads were reacted with the sample for 2h at 15°C, pellet washed 4 times, threonine phosphopeptide reacted with bound enzyme, and phosphate production detected in a multiwell plate (650nm) using malachite green reagent. Protein content of the supernatant was assayed by the BCA method (Pierce BCA protein assay kit, Thermal Scientific) using bovine serum albumin (2mg/mL) as a standard. Activity is reported as pmoles P/mg protein liver homogenate.

A pilot trial was performed with LRS1+ of similar weight (mean 8g) in which fish were given an intra-peritoneal injection of cortisol (0.1µg/g fish in PBS), PBS (control) or MCLRa toxin (0.25 and 1.0µg / fish in PBS). Additionally 2 fish were challenged with 1.0µg of MCLRa by oral gavage. After 18h, the fish were sacrificed for histology and liver PP2A assay. In addition to sentinel suckers, liver tissue from control 0+ and 1+ LRS held at pH 9.0, 20°C in the wetlab for 7d without food and hypoxic trial fish were also assayed. The dataset was examined for activities ≤ 50% of the mean control values to identified significantly impaired individuals.

Histology - Histological samples were held in Davidson's fixative for 48h, processed for 5µm paraffin sections, and stained with hematoxylin and eosin. Sagittal sections were made of smaller fish with tissues examined including skin, muscle, gill, thymus, olfactory pit, eye, brain, liver, intestine, adipose tissue, acinar cells, kidney, and the peritoneal cavity. Fish larger than 70mm were dissected and specific organs (kidney, gastrointestinal tract, gill, and liver) processed for histology. Abnormalities were rated on an ordinal 0, 1, 2, 3 scale based on distribution (none, focal, multifocal, and diffuse). A subset of Davidson's fixed specimens were examined for the presence of microcystin-LR antigen in the liver, kidney, and intestine by the immunocytochemistry method of Djediat et al. (2010) using a monoclonal antibody to microcystin-LR (MC10E7, Enzo Life Sciences ALX-804-320) and the universal VECTASTAIN kit (Vector laboratories). Positive (MCLR treated Chinook) and negative (wetlab LRS) control sections were processed with the sample set.

Laboratory hypoxic trials - Three separate hypoxic trials were conducted at the CA-NV Fish Health Center wet lab in November and December 2013, and January 2014. Two separate recirculation systems (normoxic (N) and hypoxic (H)) were used in the trials. Each system consisted of a 300L mixing sump, four 10L aquaria, heater and chiller, recirculation pump, and either aeration or a nitrogen input-%saturation controller. Water in each sump was maintained at a set level to adjust for evaporation but each system was largely static. Alkalinity was maintained by peristaltic pump input of 4-6mL/min 6N NaOH once every hour. Sodium hydroxide pumps were controlled using the APEX controller and Energy Bar with 8 programmable outlets for 24h pH maintenance. ChlorAm-X® (AquaScience Research Group, sodium hydroxymethanesulfonate) was added (20-30g as needed to maintain low ammonia levels) to both systems for control of ammonia. The hypoxic aquaria had Styrofoam lids that prevented fish from using the air-water interface and drainage tubes that led

directly into the sump to minimize additional aeration. Hypoxic system oxygen concentration was controlled using the Dissolved Oxygen Module PM3 that was linked directly to an APEX controller (Neptune Systems, Morgan Hill CA, Figure 2) and recorded every 5 minutes from inside one aquarium using a HACH HQ40d LDO probe. Temperature and pH data in the hypoxic system was recorded using the Apex controller every 10min. Normoxic data (temp, D.O. and pH) was monitored utilizing a separate HACH HQ40d meter and recorded at 5min intervals. Ammonia nitrogen was measured twice daily (except on weekends when it was measured once in the morning) with a HACH low range ammonia kit (0.01-0.50mg/L NH3-N) and DR850 colorimeter.

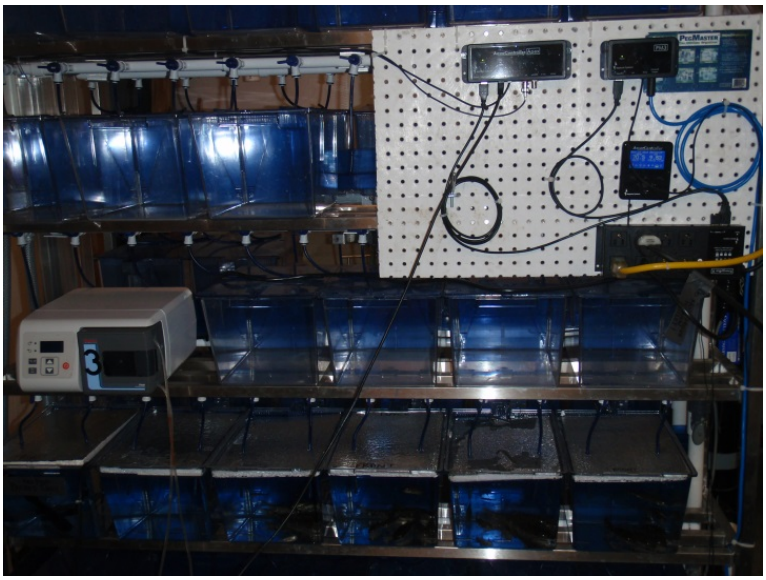


Figure 2. Hypoxic aquaria with nitrogen controller on peg board (sump, N2 diffusers, and heat pump is below) and NaOH metering pump (labeled #3).

Three or four of either LRS 0+ or LRS 1+ were loaded into separate 10L aquaria housed in the hypoxic (H) and normoxic (N) recirculation systems. Each aquaria was also loaded with two 0+ fathead minnows. During the 3d acclimation period for both systems, pH was increased from ambient conditions (~7.8) to 9.0 for the first day, 9.5 on the second, and 9.8 for the remaining days of the experiment. The test fish had been reared for 2 weeks at 18°C and later experienced 21 - 22°C (mean 22.4°C) in the experiment.

In trial 1 (15-22November 2013), a target dissolved oxygen concentration of 2 mg/L was chosen based on our observations of Upper Klamath Lake conditions in August of 2012 and the mortality response between 7 and 14dpe at this level. Trial 2 (6-13December 2013), hypoxic DO target was set for 1.6 mg/L based on the 96h LD_{50} values reported by Saiki et al. (1999) as well as 48h LD_{50} value reported by Meyers and Hanson (2002). In Trial 3 (10-18January 2014), hypoxic system DO concentration was decreased incrementally over 24h intervals in a “stair-step” manner ranging from a target of 1.6mg/L on 13January to 0.80mg/L by 17January.

In trial 1, length (SL) and weight was recorded for each sampled fish, caudal peduncle severed and blood collected in heprinized microhematocrit tubes, plasma was stored at -70°C until assayed for total protein (1µL sample in BCA protein assay kit) and sodium (5µL diluted in 5mL distilled water assayed by flame photometer). Tissues were fixed in Davidson for histological examination. In trial 2, all fish within an aquarium were sampled on 9Dec (N0 and H0= both groups prior to experimental pH and oxygen change), 11Dec (H48 = hypoxic fish at 48h), and 13Dec (N96 and H96= both groups at 96h end of experiment). In addition to the samples collected in trial 1, hematocrits were recorded and a portion of the liver was collected for PP2A assay and archived for TEM (Karnovsky fixative). Similar samples were collected in trial 3.

Laboratory data statistical analysis - Analysis was performed with SigmaPlot 12 software on raw data. Normality was tested by the Kolmogorov – Smirnov method at the P= 0.05 level. One-way ANOVA or T-test (data with normal distribution, reported with F or t value) or Kruskal-Wallace ANOVA or Mann-Whitney U test on ranks (non-parametric analysis) with subsequent multiple comparison procedures (Holm-Sidak or Dunns method respectively, alpha $\leq$ 0.05) was used to compare groups.

Permits- Permits for this project are as follows: sub-permit FWSKFFWO-7 (USFWS Klamath Falls Office) under the USFWS Region 8 regional blanket permit (TE-108507), Section 10 Federal authorization number TE007907-15 (adult brood stock, TE007907-15), Oregon Scientific Take Permit Number 17010 M1, and California DFG Standard Importation Permit number 2013-2564.

Results

Fish Culture and net pen - In contrast to 2012, there were no significant mortality events in the wetlab population (LRS 0+ and 1+) throughout the study period. We speculate that incorporation of soybean vegetable oil and vitamin premix into the Otohime diet in August enhanced growth and fitness over 2012 rearing. The outer and inner hoop nets were relatively easy to sample, allowed fish to feed on benthos and still have access to surface waters.

Initial fry mortality was low in the control (no substrate) and mud troughs for the first 2 weeks of culture. Control mortality climbed to 208 on 9-10June. On 14June, the mid-trough screens were removed from both tanks to accommodate the growing fry. Unfortunately, sediment in the mud tank plugged the drain filter resulting in a tank overflow and loss of a least half of this population. Approximately 4,000 control fry were moved into the mud tank on 16June. Several mortalities from both tanks were examined by histology in early June and found to have moderate gill hyperplasia. LRS0+ reared over benthic sediment had significantly higher weights than control trough cohorts (exception being 26July, t-test, P = 0.620) in 4 of 5 sample counts (Table 1). Similarly, condition factors of mud trough fry were greater than controls except on 12July (T-test,

P>0.05). Standard length of mud trough fry was greater than controls on 14June (prior to mixing) and again on 12July (T-test, P>0.05). Growth rate over the 71d period was similar for both trough populations (0.35mm/d and 0.32 mm/d). Immediately prior to sorting for the initial lake deployment, a mortality increase occurred in the mud trough population for several days. For this reason, only fry from the control trough were used in the sentinel study.

Table 1. – Mean length, weight and condition factors for fry reared in troughs with mud (sediment) or without (control) obtained from six weigh days 16May – 26July, 2013. P-values calculated from raw data utilizing two tailed t-test assuming equal variance.

	Date	16May	4June	14June	28June	12July	26July
	Day Post transfer	33*	11	21	35	49	63
Mean Standard Length (mm)							
	Sediment		19.19**	21.64	26.53	35.03	39.21
	Control	16.49*	18.50	18.30	25.84	33.27	41.12
	P-value		0.070	**<0.001**	0.320	**0.030**	0.128
Mean Weight (g)							
	Sediment		0.083	0.137	0.288	0.750	0.963
	Control	0.019*	0.073	0.069	0.242	0.633	0.922
	P-value		**0.008**	**<0.001**	**0.012**	**0.016**	0.620
Mean Condition Factor (KSL)							
	Sediment		1.216**	1.342	1.542	1.745	1.597
	Control	0.433*	1.153	1.129	1.403	1.719	1.326
	P-value		**0.032**	**<0.001**	**0.002**	0.750	**<0.001**

*Values reported as Days Post Hatch (dph), not Days Post Transfer (dpt).

** Values reported as Fork Length (FL), not Standard Length (SL).

Sentinel survival – The only significant mortality at the North lake occurred to 14dpe LRS 1+ (21 and 25%) sampled during the 12 - 26August period of low dissolved oxygen (Table 2, Figure 3). LRS 0+ cohort mortality was ≤ 5% regardless of exposure time. Bi-weekly survival of LRS0+ held at the Williamson R. mouth from 29July to 3

September was high and ranged from 90 – 100%. In contrast, LRS 0+ held over the summer (93d) had an 11% survival (9 of 80). Additional LRS 0+ and 1+ were placed at this site on 3September for use in the overwinter survival trial. They had 88% and 90% survival after 57d total exposure. Overwinter survival, after 131d of exposure, was high in both the LRS0+ (48 / 53, **93%**) and LRS1+ (37 / 38, **97%**).

Water quality – High alkalinity (29July-12August, mean pH 9.5, maximum pH 10.09) and low dissolved oxygen concentrations occurred at the North lake site in August (Table 3, Figure 3). Dissolved oxygen concentrations were less than 2 mg/L between 12 – 19August for 52 and 71h at the surface and bottom, respectively (Figure 4 and 5). Oxygen levels did not exceed 4mg/L during this period; which was associated with limited mortality of 14dpe LRS 1+ (Table 3). It is likely that fish experienced periods of dissolved oxygen levels at or below LD_{50} values of 1.58mg/L (Table 5). On 13-14Aug, 17-18Aug, and 23August bottom DO levels were $\leq$ 1.50 mg/L for 3- 10h per day (Figure 5) while the surface water DO similarly hypoxic on 13-14Aug and 23Aug for 3- 8h per day (Figure 4). These conditions indicate that sentinels experienced hypoxia throughout the water column (possible exception of the surface interface?) for periods $\geq$ 3h during several days in August. These hypoxic periods precede 2 sample dates (19 and 26Aug) in which we observed mortality in LRS1+ juveniles. Sentinel survival at this time was likely enhanced by access to surface waters without the threat of avian predation. Water temperature averaged 19 - 21°C during the summer. While total ammonia reached 0.44mg/L on 19August, the moderate pH (7.0) resulted in low unionized (UIA) values (Table 4). The highest UIA measurement of 0.09mg/L occurred on 5August which is much less than LD_{50} values for the species (Table 5). Excluding the early to mid-August period, water quality was relatively mild for Upper Klamath Lake. Similarly, we judged water quality to be good at the Williamson River mouth site (UIA $\leq$ 0.012mg/L, 01August – 16September means of 16°C, 9.3mg/L D.O., and pH 8.6,Table 6).

Table 2. Exposure period, site location, sample dates, number (n) of mortalities observed per net pen and mean percent mortality for each exposure at North Lake for 0+ LRS and 1+ LRS

Exposure	Site	Date	Mortality Per 0+ LRS Net pen (n)			Mean Percent Mortality	Mortality Per 1+ LRS Net pen (n)		Mean Percent Mortality
			A	B	C		A	B	
Exposure 1 29 July – 12 Aug	North Lake	1 Aug (3 dpe)	0	0	0	0% (0/80)			
		5 Aug (7 dpe)	2	0	0	5% (3/61)	0	0	0% (0/24)
		8 Aug (10 dpe)	0	1	0	2% (1/61)			
		12 Aug (14 dpe)	0	0	1	5% (3/60)	0	0	0% (0/24)
Exposure 2 5 Aug – 19 Aug	North Lake	8 Aug (3 dpe)	0	1	0	3% (2/60)			
		12 Aug (7 dpe)	0	0	0	2% (1/60)	0	0	0% (0/24)
		15 Aug (10 dpe)	0	0	0	2% (1/60)			
		19 Aug (14 dpe)	0	0	0	3% (2/60)	2	3	**21% (5/24)**
Exposure 3 12 Aug July – 26 Aug	North Lake	15Aug (3 dpe)	0	0	0	2% (1/60)			
		19 Aug (7 dpe)	1	0	0	3% (2/60)	0	0	0% (0/24)
		22 Aug (10 dpe)	n/a *						
		26 Aug (14 dpe)	1	0	0	0% (0/61)	2	4	**25% (6/24)**
Exposure 4 19 Aug – 3 Sept	North Lake	22 Aug (3 dpe)	n/a *						
		26 Aug (7 dpe)	0	0	0	0% (0/60)	0	0	0% (0/24)
		29 Aug (10 dpe)	1	1	0	5% (3/60)			
		3 Sept (14 dpe)	0	0	0	3% (2/60)	0	0	0% (0/24)
Exposure 5 26 Aug – 3 Sept	North Lake	29 Aug (3 dpe)	0	0	0	0% (0/60)			
		3 Sept (7 dpe)	0	0	0	2% (1/60)	0	0	0% (0/24)
		5 Sept	0	0	0	2%			

		(10 dpe)				(1/60)			
		9 Sept (14 dpe)	0	0	0	0% (0/60)	0	1	4% (1/24)
Exposure 6 3 Sept – 16 Sept	North Lake	5 Sept (3 dpe)	0	0	0	0% (0/62)			
		9 Sept (7 dpe)	0	0	0	2% (1/60)	0	0	0% (0/23)
		12 Sept (10 dpe)	0	0	0	3% (2/60)			
		16 Sept (14 dpe)	0	0	0	2% (1/60)	1	0	4% (1/24)

. n/a * Data not collected due to storm.

Table 3. Maximum, mean, and minimum readings for temperature (˚C), dissolved oxygen (mg/L) and pH obtained from 1m below the water *surface* and 1m above *bottom* at the North Lake exposure site over a six week study period. Standard error ranged from 0.04-0.06 for temperature, 0.04-0.14 for dissolved oxygen and 0.01-0.04 for pH. The number of readings taken by each datasonde ranged from 319-391. Sondes were suspended from buoys located in the center of the net array.

			Exposure Periods					
			Exp. 1	**Exp. 2**	**Exp. 3**	**Exp. 4**	**Exp. 5**	**Exp. 6**
Constituent	**Site**		**July 29 - Aug 12**	**Aug 5 - Aug 19**	**Aug 12 - Aug26**	**Aug 19 – Sept 3**	**Aug 26 - Sept 9**	**Sept 3 – Sept 16**
Temperature (˚C)	**North Lake Surface**	max	24.08	24.08	23.32	23.11	22.42	22.14
		mean	**20.66**	**20.83**	**20.89**	**20.46**	**19.75**	**19.70**
		min	18.77	18.77	18.58	18.11	17.72	17.72
	North Lake Bottom	max	23.44	24.08	22.80	22.56	21.66	21.74
		mean	**20.40**	**20.65**	**20.72**	**20.34**	**19.66**	**19.61**
		min	18.70	18.70	18.56	18.09	17.73	17.73
Dissolved oxygen (mg/L)	**North Lake Surface**	max	17.30	13.38	8.91	8.91	7.74	7.51
		mean	**7.90**	**4.79**	**2.84**	**7.10**	**6.53**	**5.61**
		min	2.22	1.08	0.88	1.72	4.11	3.83
	North Lake Bottom	max	12.78	9.96	6.81	6.81	6.41	7.20
		mean	**7.26**	**4.01**	**2.61**	**3.74**	**4.91**	**5.35**
		min	1.51	0.48	0.48	0.65	1.50	3.82

pH	**North Lake Surface**	max	10.09	9.87	9.35	8.91	7.74	7.78
		mean	**9.51**	**8.83**	**7.79**	**7.24**	**7.32**	**7.42**
		min	8.76	7.27	6.94	6.94	6.95	7.15
	North Lake Bottom	max	10.00	9.65	9.18	8.55	7.82	7.70
		mean	**9.50**	**8.70**	**7.74**	**7.29**	**7.34**	**7.36**
		min	8.57	7.22	6.97	6.97	6.98	7.12

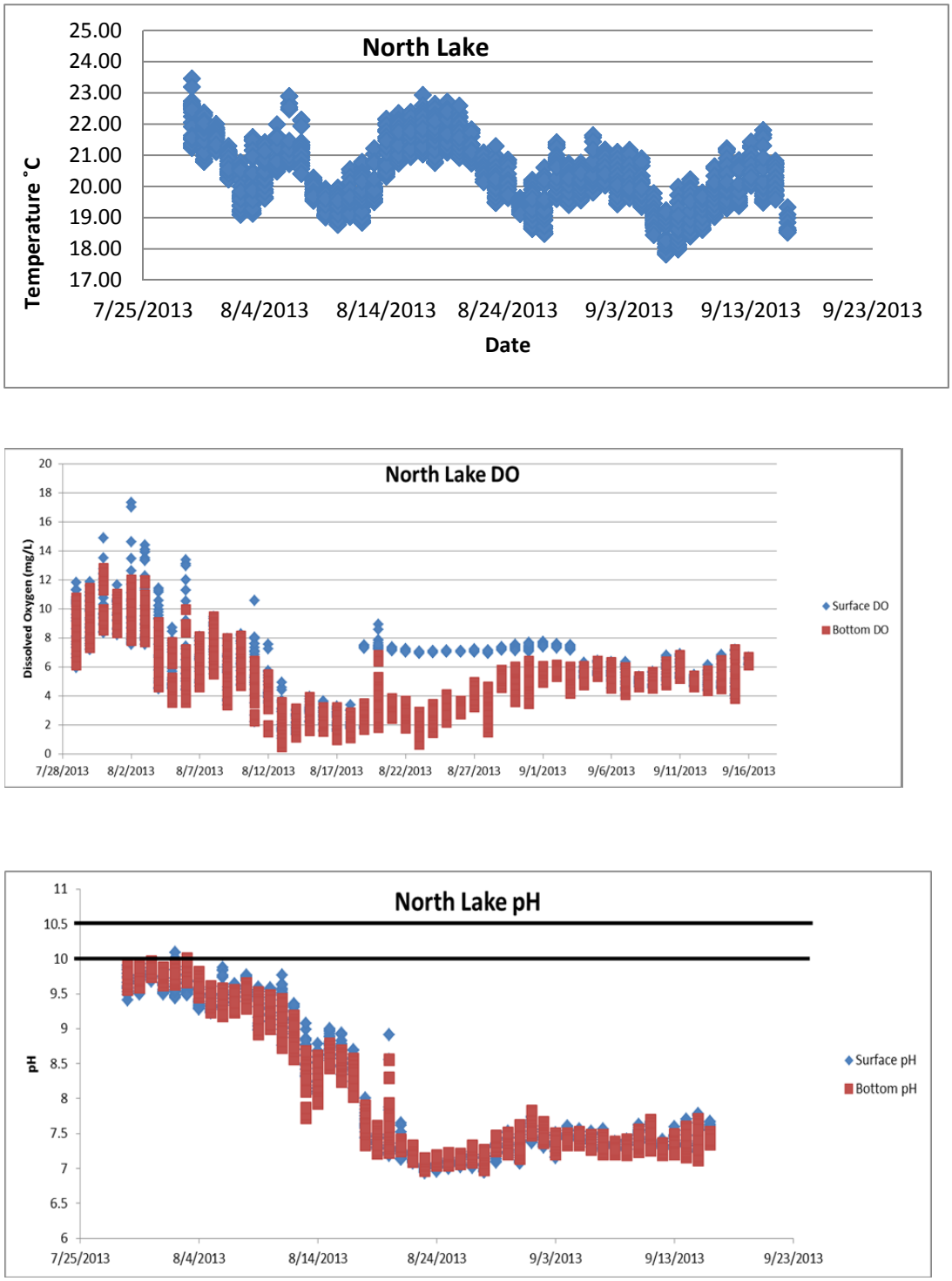


Figure 3. Water temperature, dissolved oxygen concentration and pH at the North Lake site Upper Klamath Lake 29July – 16September 2013. Water quality measurements were obtained hourly using continuous monitors deployed at a depth of 1m (surface) and 1m above bottom (bottom) from a buoy located in the center of the net pen array.

Table 4. Composite point measurements taken at the **North Lake** exposure site of total ammonia NH_4^+ (mg/L), temperature (°C) and pH as well as temperature (°C), pH and percent unionized ammonia NH_3 in aqueous solution values used to calculate unionized ammonia concentrations.

Date	NH_4^+ (mg/L)	Actual Temp (°C)	Actual pH	Temp Used (°C)	pH Used	% NH_3 Used mg/L	**Calculated UIA**
29-Jul	0.02	23.80	9.79	24	10.0	84.100	**0.017**
1-Aug	0.06	20.90	9.54	21	9.5	57.500	**0.035**
5-Aug	0.31	20.60	9.06	21	9.0	29.900	**0.093**
8-Aug	0.10	18.40	9.47	19	9.5	53.900	**0.054**
12-Aug	0.08	18.50	8.93	19	9.0	27.000	**0.022**
15-Aug	0.14	20.00	8.37	20	8.5	11.200	**0.016**
19-Aug*	0.44	20.90	7.27	21	7.0	0.425	**0.002**
22-Aug*	0.22	20.30	7.14	20	7.0	0.396	**0.001**
26-Aug*	0.19	18.60	7.05	19	7.0	0.368	**0.001**
29-Aug*	0.04	19.25	7.35	19	7.0	0.368	**0.000**
3-Sep*	0.02	19.50	7.23	20	7.0	0.396	**0.000**
5-Sep*	0.09	18.10	7.39	18	7.0	0.342	**0.000**
9-Sep*	0.13	18.30	7.27	18	7.0	0.342	**0.000**
12-Sep*	0.11	19.20	7.26	19	7.0	0.368	**0.000**
16-Sep*	0.08	18.10	7.45	18	7.5	1.070	**0.001**

* pH probe on HACH meter broken, used datasonde mean temperature and pH.

Table 5. Median upper lethal concentrations (LC_{50}) for pH, un-ionized ammonia (mg/L, UIA), and temperature (°C), and lower LC_{50} for dissolved oxygen (mg/L, D.O.)for juvenile 0+ Lost River suckers exposed for 48 and 96h as reported by (A) Meyer and Hanson (2002) and (B & C) Saiki et al. 1999.

	(A) 48 h	(B) 48h	(C) 96h
pH	10.39	10.62	10.30
UIA	0.51	0.92	0.78
D.O.	1.58	1.58	1.62
°C	nd	30.8	30.5

nd no data

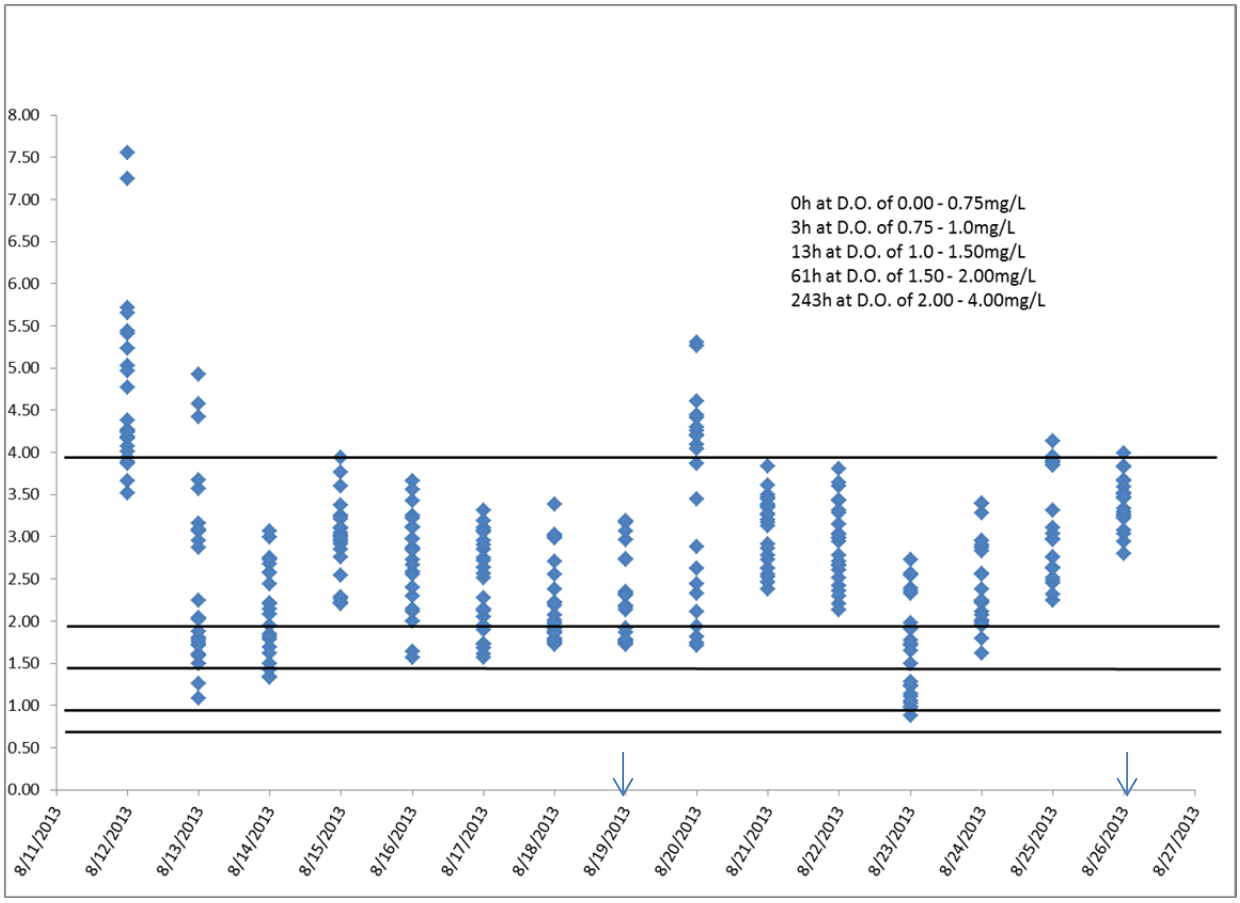


Figure 4. Dissolved oxygen (mg/L) measured by North lake **surface** probe between 11-27August 2013. Lines set at 3.5, 1.5, 1.0, and 0.75 mg/L. Arrows indicate sample dates on which LRS1+ mortality was determined.

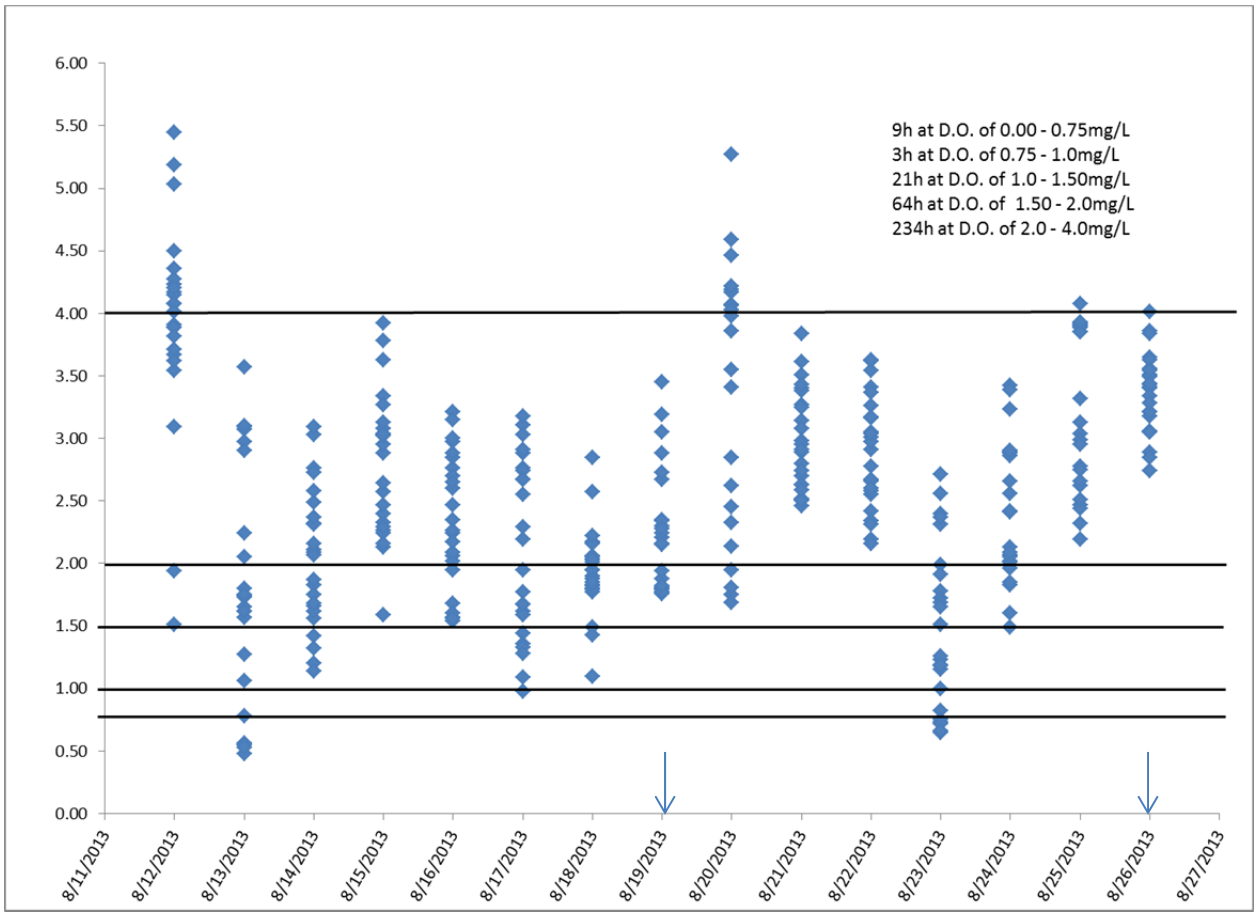


Figure 5. Dissolved oxygen (mg/L) measured by North lake **bottom** probe between 11-27August 2013. Lines set at 3.5, 1.5, 1.0, and 0.75 mg/L. Arrows indicate sample dates on which LRS1+ mortality was determined.

Table 6. Composite point measurements taken at the **Williamson River** exposure site of total ammonia NH_4^+ (mg/L), temperature (°C) and pH as well as temperature (°C), pH and percent unionized ammonia NH_3 in aqueous solution values used to calculate unionized ammonia concentrations (* pH probe on HACH meter broken, used datasonde mean temperature and pH)

Date	NH_4^+ (mg/L)	Actual Temp (°C)	Actual pH	Temp Used (°C)	pH Used	% NH_3 Used mg/L	**Calculated UIA**
29-Jul	0.07	21.50	8.51	22	8.5	12.700	**0.009**
1-Aug	0.05	18.40	8.28	18	8.0	3.310	**0.017**
5-Aug	0.04	19.30	8.30	19	8.5	10.500	**0.004**
8-Aug	0.04	17.20	7.41	17	7.5	0.996	**0.000**
12-Aug	0.04	16.80	8.13	17	8.0	3.080	**0.001**
15-Aug	0.06	17.80	8.10	18	8.0	3.310	**0.002**
19-Aug*	0.04	21.20	8.80	21	9.0	29.900	**0.012**
26-Aug*	0.01	16.50	8.45	17	8.5	9.140	**0.001**
29-Aug*	0.05	17.80	8.57	18	8.5	9.780	**0.005**
3-Sep*	0.03	16.80	8.84	17	9.0	24.100	**0.007**
5-Sep*	0.07	16.20	8.54	16	8.5	8.540	**0.006**
9-Sep*	0.00	16.40	8.51	16	8.5	8.540	**0.000**
12-Sep*	0.02	16.20	8.42	16	8.5	8.540	**0.002**
16-Sep*	0.00	15.60	8.30	16	8.0	2.870	**0.000**

Sentinel triglyceride reserves and condition – Size and triglyceride content (TG) was evaluated in sentinel groups held for 14d at the North lake and ≥ 57d at Williamson River site (Table 7). While condition factor was similar, TG content was higher (P=0.048) in the 26August North Lake LRS 0+ group when compared to the previous two August exposure groups (Figure 3). Elevated metabolic demand and/or inadequate diet appeared to influence sentinel energy reserves during the 29July - 12August period. Control fish held without food (20°C) showed reduced condition factor but high TG values compared to sentinel cohorts.

LRS 0+ fry, held at the Williamson River site from 3 Sept to 30Oct (57d), had low TG content in relationship to both 14d lake sentinels and wetlab controls (Table 7, Figure 6). LRS 0+ held for the entire summer (93d) at the Williamson River site had normal condition factor (mean 1.261), but very low TG content (mean 2.4mg TG/g fish). No TG analysis was performed on bi-weekly Williamson R. sentinels due to time constraints. As mentioned above, overwinter survival was high in both the LRS 0+ and LRS 1+suckers. In March 2014, LRS 0+ (broodyear 2013) were significantly smaller (SL, WT, condition factor, T-test P≤ 0.037) and had less whole body triglyceride content (Mann-Whitney rank sum, P<0.001) than cohorts sampled on the 30Oct planting date (Table 8). Similarly, whole body triglyceride content of LRS 1+ (broodyear 2012) was lower than cohorts sampled on 30October2013 (Mann-Whitney rank sum, P<0.046) however size was similar.

Table 7. Mean (SE) standard length (SL,mm), weight (0.01g), condition factor (KSL=WT/ SL^3x10^5), and whole body triglyceride content (mg TG/g fish) of frozen LRS 0+ and 1+ collected from wetlab control groups after 7d without food, net pen fish after 14d exposures, or fish held at the Williamson River mouth for 57 or 93d.

				LRS 0+					LRS 1+		
Date	Group	no.	SL	WT	KSL	TG	no.	SL	WT	KSL	TG
2-Aug	e1 Control	5	38 (2)	0.69 (0.13)	1.141 (0.053)	21.6 (1.7)	3	108 (5)	16.29 (1.41)	1.278 (0.077)	13.2 (3.7)
12-Aug	e1 14d	5	47 (1)	1.35 (0.02)	1.318 (0.024)	15.0 (2.9)	5	94 (4)	11.12 (1.33)	1.309 (0.013)	11.4 (5.5)
19-Aug	e2 14d	5	50 (2)	1.75 (0.19)	1.415 (0.067)	15.1 (1.5)	5	107(4)	16.39 (2.09)	1.297 (0.027)	20.4 (2.5)
26-Aug	e3 14d	5	55 (1)	2.28 (0.21)	1.349 (0.038	22.1 (1.4)		ND	ND	ND	ND
31-Aug	e5 Control	3	51 (7)	1.88 (0.69)	1.191 (0.133)	23.2 (2.3)	2	117 (6)	17.31 (3.28)	1.066 (0.041)	27.1 (1.9)
30-Oct	e6 WR57d	5	66 (1.3)	3.69 (0.25)	1.279 (0.044)	11.9 (2.6)	5	122 (3)	21.77 (1.64)	1.184 (0.032)	7.9 (1.7)
30-Oct	e1 WR93d	6	43 (2)	1.01 (0.14)	1.261 (0.056)	2.4 (0.2)		ND	ND	ND	ND

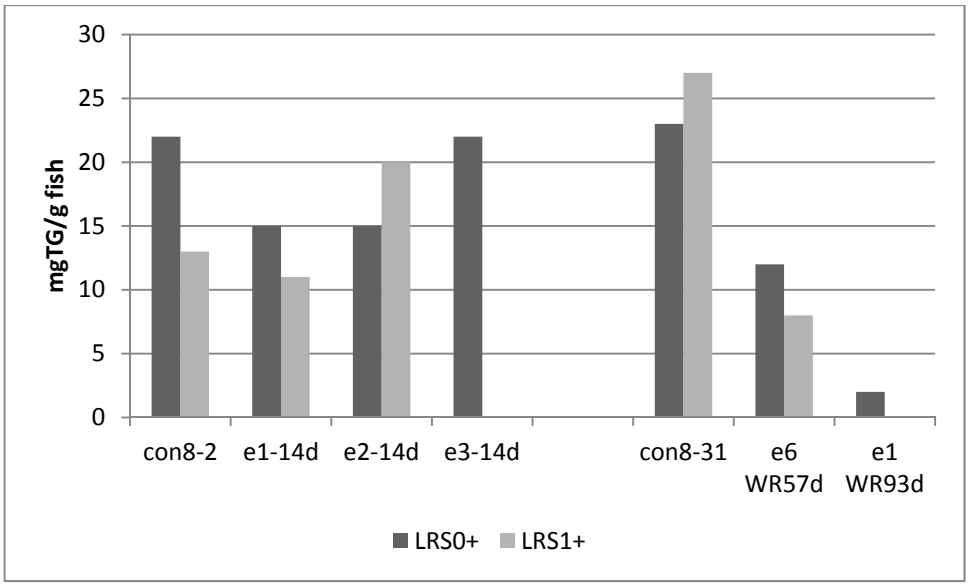


Figure 6. Mean whole body triglyceride content (mg TG/ g fish) of LRS 0+ and 1+ wetlab controls (con), exposure 1 -3 (e) held for 14d , and Williamson River site net pen fish held for 57 and 93d. See Table 7 for standard error data.

Table 8. Mean (SE) standard length (SL), weight (WT), condition factor (KSL), and whole body triglyceride content (TG= mg TG/g fish) of broodyear (BY) 2012 and 2013 LRS placed into overwinter net pens at mouth of Williamson R on 30October2013 and sampled on 10March2014. Number of fish per measurement is listed. Subscribe letters denote significant difference between October and March samples.

	BY13	BY13		BY12	BY12
	30-Oct-13	10-Mar-14		30-Oct-13	10-Mar-14
No.	5	43		5	34
SL	66 (1.3)a	62 (0.6)b		122 (3.0)	122 (1.7)
WT	3.7 (0.2)a	2.6 (0.1)b		21.8 (1.6)	20.6 (1.1)
KSL	1.279 (0.044)a	1.078 (0.020)b		1.1843 (0.0315)	1.142 (0.022)
TG	11.9 (2.6)a	2.6 (0.2)b		7.9 (1.7)a	3.27 (0.45)b
No.	5	24		5	24

Histology- No overt lesions or abnormalities, suggestion of a diseased state, were observed in sections of gill, kidney, liver, and gastro-intestinal tract of both age groups sampled at 7 and 14dpe. Hyaline droplets (presumptive filtrate protein) was seen in the majority of fish sampled from all exposures (Table 9, Figure 7). Many of the affected tubules were highly vacuolated. Four sample groups (including the exposure 6

10dpe group sampled on 12Sept. and not listed in Table 9) did not have these tubule changes. Similarly, wetlab control fish did not show this kidney condition. It is unclear what caused this abnormality as there was no constant association with hypoxic conditions, presence of MCLRa immunoreactivity in the intestine or elevated pH and ammonia. Hyaline droplets within kidney tubule epithelial cells are typically excess protein reabsorbed from the filtrate and do not necessarily indicate dysfunction (Ferguson 1989). Hyaline droplets have been reported in rainbow trout exposed to elevated ammonia levels (Daoust and Ferguson, 1984). We have observed similar hyaline droplets in the tubules of Lost River Suckers reared for 62d in pH 9.5 waters with elevated ammonia content (0.440mg/L NH3-N) and speculate that increase urine output ,stimulated by elevated ammonia, resulted in the observed tubule changes (Foott et al. 2000).

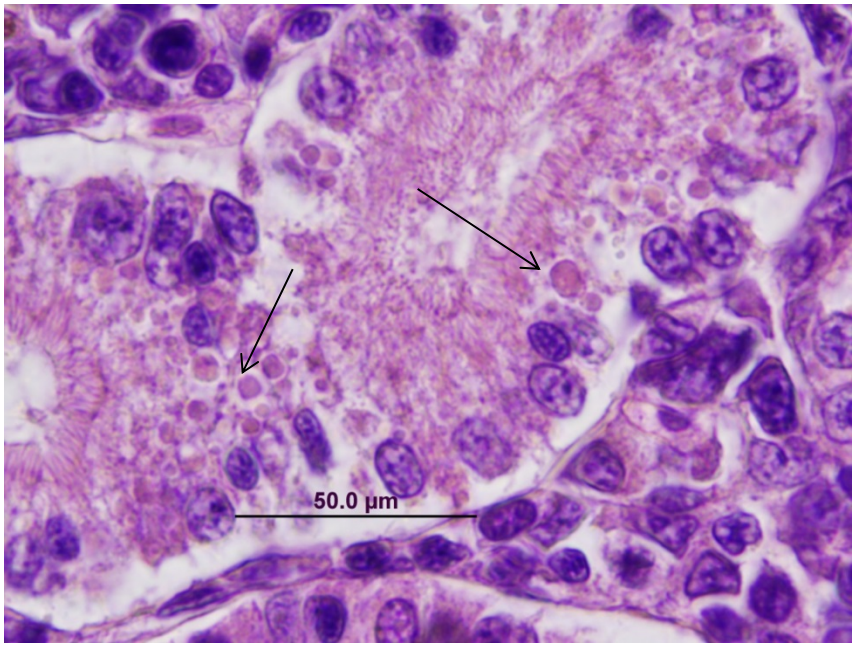


Figure 7. Proximal tubule of LRS 1+ sampled on 9September showing hyaline droplets in cytoplasm (arrows).

Table 9. Prevalence (POI) of kidney tubule hyaline deposits (THD) of LRS 0+ and 1+ sentinels (exposure groups E1-6) sampled at 7 and 14dpe. Data includes number of fish examined per group (No.), date of sample, and THD severity score (0, 1, 2). Exposure groups with no THD changes are highlighted.

			LRS 0+						LRS 1+				
				THD			THD			THD			THD
Date	Group	DPE	No.	0	1	2	Prev.		No.	0	1	2	Prev.
5-Aug	E1	7	8	2	3	3	75%		4	3	1	0	25%
12-Aug	E1	14	5	1	2	2	80%		4	3	0	1	25%
12-Aug	E2	7	4	0	1	2	100%		4	0	3	1	100%
19-Aug	E2	14	5	5	0	0	0%		5	5	0	0	0%
19-Aug	E3	7	3	2	1	0	33%		4	2	1	1	50%
26-Aug	E3 *	14	4	0	1	1	100%		4	2	2	0	50%
26-Aug	E4	7	3	0	1	2	100%		4	0	0	1	25%
3-Sep	E4	15	4	2	2	0	50%		4	0	0	2	100%
3-Sep	E5	8	4	1	2	1	75%		4	3	1	0	33%
9-Sep	E5	14	5	1	3	1	80%		4	1	1	2	75%
9-Sep	E6	6	5	4	0	0	0%		4	2	2	0	50%
16-Sep	E6	13	7	1	4	2	86%		4	0	2	2	100%
	*	Kidney not found in all sections											

No parasites were seen in the summer sentinel tissues. Unlike the 2012 sentinel fry, no hepatocyte or gill edema was observed in the 2013 sentinels. Cross sections of invertebrates with muscle tissue were seen within the intestines of 52% LRS 0+ and 20% LRS 1+ sentinels indicating that feeding occurred within net pens. Similar to 2012 and 2011 sentinels, all exposure groups had MCLR immunoreactive digesta and intestinal epithelium (Table 10). No reactivity was observed in the kidney or liver. It would appear that microcystin was consumed by the fish over the entire summer without inducing liver or kidney damage. Williamson River sentinels sampled on 30October had metacercaria in multiple tissues and were observed with black spot in the dermis. No hyaline deposits were seen in their kidneys however LRS 1+ held for 57 d had pronounced vacuoles in the proximal tubules. Liver, gill, and kidney tissue was examined from 4 yearling (BY12) and under-yearling (BY13) collected on 10March2014 after an overwinter exposure at the mouth of the Williamson R. Asymptomatic infections of presumptive *Myxobolus* sp. parasites in kidney tubules were seen in both age groups. Gill was normal with markedly reduced secondary lamellae and pronounced intra-lamellar cell mass. Liver was normal with 3 of 4 yearling fish having no discernable glycogen or fat vacuoles as well as 2 of 4 under-yearlings.

Table 10. MCLR toxin immunoreactivity in LRS 0+ and 1+ sentinels (exposure groups E1-6) sampled at 14dpe. Data includes number of fish examined per group (No.), date of sample, and occurrence in digesta, distal intestine epithelium (DI epi), small intestine epithelium (SI epi), liver hepatocytes (LV), and kidney (KD).

				LRS 0+							LRS 1+				
					DI	SI						DI	SI		
Date	Group	DPE	No.	digesta	epi	epi	LV	KD		No.	digesta	epi	epi	LV	KD
12-Aug	E1	14	2	2	2	1	0	0		2	2	0	1	0	0
19-Aug	E2	14	2	1	1	0	0	0		2	2	0	1	0	0
26-Aug	E3	14	2	2	2	1	0	0*		2	2	1	2	0	0
3-Sep	E4	15	2	2	1	1	0	0		2	2	0	2	0	0
9-Sep	E5	14	2	2	2	1	0	0*		2	2	2	2	0	0
16-Sep	E6	13	nd	nd	nd	nd	nd	nd		2	1	0	0	0	0
	nd not done														
	* reaction associated with tubule brush border - possible artifact														

PP2A liver activity- In an 18h pilot trial, LRS1+ PP2A activity was depressed by IP injection of both cortisol (0.1µg/g fish) and MCLRa toxin (0.25 and 1.0µg / fish) while gavage MCLRa challenge (1.0µg / fish) stimulated activity when compared to PBS injected controls (Figure 8). Hypoxic challenges did not alter PP2A activities (see hypoxic trial data below). Sentinel fish liver PP2A activity showed similar trends between 7d and 14d fish in both the LRS 0+ fry and LRS 1+ juveniles (Figures 9 and 10). Activity increased in 14d exposure fish compared to their 7d exposure cohorts in 3 of 6 LRS 0+ fry and 2 of 6 LRS 1+ juvenile groups. One exception occurred to the 5th exposure group in which LRS 0+ fry had the trend of 7d<14d while the LRS 1+ juveniles showed the opposite (not statistically significant at P=0.131) pattern. It appears during the week of 19 - 26August both age groups experienced a significant inhibition of PP2A activity. This observation is based on the similar depression in overlapping sample groups (14d exposure 3 and 7d exposure 4). Activities of non-exposure controls (held in the wetlab without food in pH 9.0, 20°C water) was similar between both age groups (Mann-Whitney rank sum test, P=0.529) with five high value outliers occurring in the last two exposures. To conservatively identify individuals with depressed activities, we use the mean values of similar age control fish (LRS 0+ fry mean 6642 ± 581 and LRS 1+ juvenile (mean 6905 ± 520) as a reference in examining the dataset for those fish with < 50% of the control mean (50% control mean LRS 0+ fry = 3321 and LRS 1+ juvenile = 3452 pmoles P/mg protein). The following exposure groups had over half of the fish with PP2A depressed activities (Table 11):

Exposure 1 - 29July -12Aug	LRS**1+**	7 and 14d,
Exposure 3 - 12-26Aug	LRS0+ and LRS**1+**	14d,
Exposure 4 - 19-26Aug	LRS**1+**	7d

This data highlights the sensitivity of the yearling fish to lake conditions and supports the hypothesis that an inhibitory factor (i.e. MCLR toxin) was present during the week of 19 - 26August. All sentinel groups had MCLR immune-reactivity digesta within their intestinal tracts

and a high prevalence of positive intestinal epithelium (Table 9). No necrotic reaction was observed in either the kidney or liver of any sentinel fish. The histological data indicates that suckers are continuously exposed to microcystin toxin via their diet however the concentration does not appear to be sufficient to cause morbidity. Similarly, depressed PP2A activity was not consistently associated with mortality in LRS 1+ (yes for exposure 3 fish but not for exposure 2 fish on 19August).

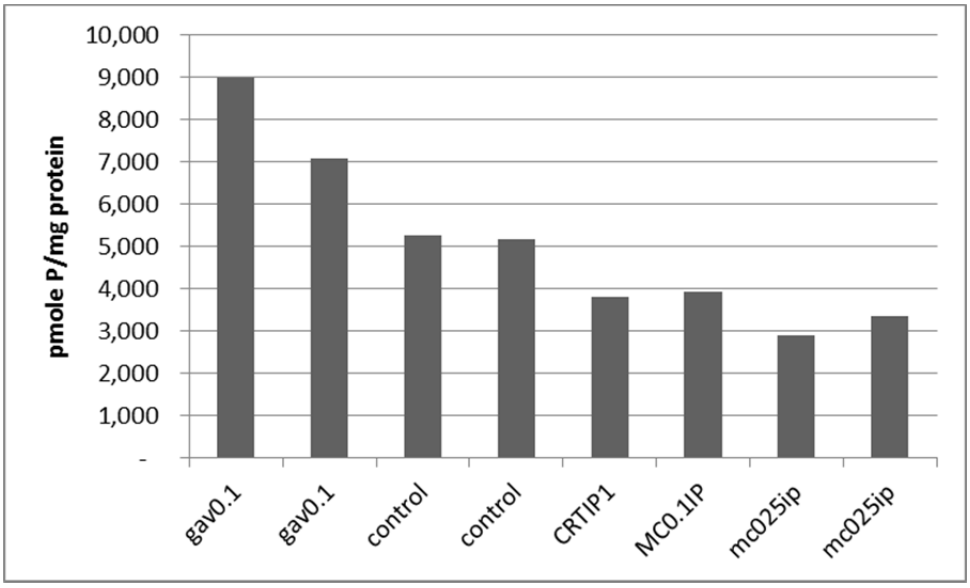


Figure 8. Protein phosphatase 2A activity (pmole P/mg protein) in liver samples from LRS 1+ challenge by gavage (gav0.1) of 0.1µg MCLRa (in 100µL PBS), and intraperitoneal injections (IP) of PBS (control), 80µg cortisol (CRTIP1), 0.1 and 0.025µg MCLRa (MC0.1IP and mc025ip).

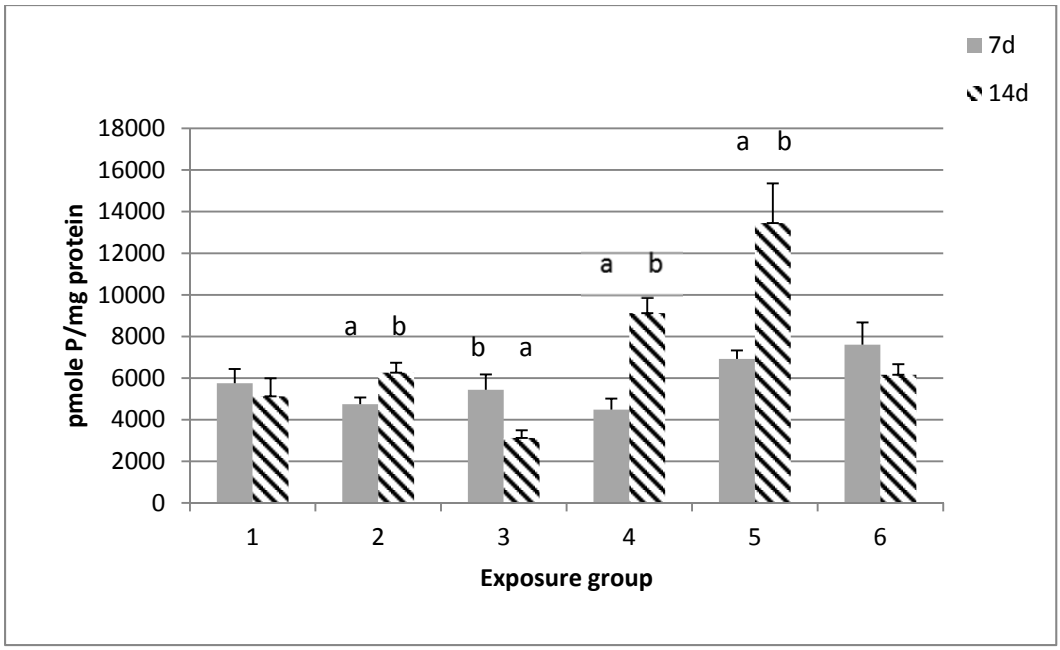


Figure 9. Mean (SE) protein phosphatase 2A activity (pmole P/mg protein) in **LRS 0+** liver samples from both 7 and 14d fish in exposure groups 1 – 6. Different letters above bar indicate significant differences (P<0.05).

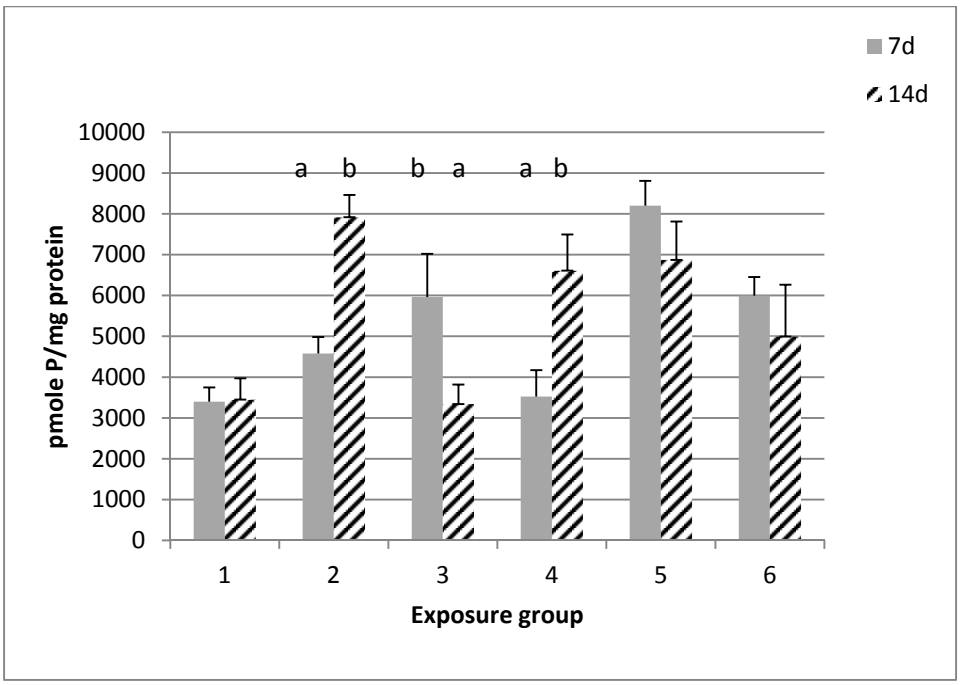


Figure 10. Mean (SE) protein phosphatase 2A activity (pmole P/mg protein) in **LRS 1+** liver samples from both 7 and 14d fish in exposure groups 1 – 6. Different letters above bar indicate significant differences (P<0.05).

Table 11. Mean (SE) protein phosphatase 2A activity (pmole P/mg protein) in LRS 0+ fry and 1+ juvenile liver samples from wetlab controls (7d Control), 7d and 14d exposure (north) fish in groups 1 – 6. Data includes the percent of sample with depressed activity ≤ 50% of mean age-specific control values (bold font indicates prevalence ≥ 50%) and the exposure group dates.

				0+fry			1+Juvenile	
Input	14d	Exposure	7dControl	7dNorth	14dNorth	7dControl	7dNorth	14dNorth
29-Jul	5-Aug	1	5108 (309)	5752 (686)	5123 (2132)	7418 (782)	3397 (351)	3450 (520)
		<50%CON		0	0		**67%**	**50%**
5-Aug	12-Aug	2	3996 (864)	4745 (323)	6263 (473)	4962 (432)	4579 (404)	7917 (547)
		<50%CON		17%	0		0	0
12-Aug	19-Aug	3	6424 (501)	5439 (735)	3125 (366)	6080 (763)	5963 (1057)	3342 (476)
		<50%CON		0	**83%**		17%	**67%**
19-Aug	26-Aug	4	6452 (901)	4487 (530)	9125 (752)	7119 (1361)	3525 (644)	6608 (888)
		<50%CON		17%	0		**67%)**	0
26-Aug	2-Sep	5	7753 (1480)	6924 (406)	13499 (1907)	5771 (741)	8200 (607)	6874 (938)
		<50%CON		0	0		0	0
2-Sep	9-Sep	6	10350 (1888)	7609 (1063)	6162 (510)	10078 (1818)	5996 (455)	4994 (1269)
		<50%CON		0	0		0	33%

Laboratory hypoxia trials- In a pilot experiment, LRS1+ survived for 28h under extreme summer lake conditions of 22°C, 2mg/L D.O., pH 9.24, and UIA of 0.324mg/L (appendix 2). Subsequent trials were run using chloramax to control ammonia levels and continuous base addition for pH control.

Trial 1: No mortality occurred to either LRS 0+, LRS 1+, or 0+ Fathead minnows held at a mean D.O. of 1.98mg/L for 36h as well as another 48h following an 11h reprieve (rose to 8mg/L). The hypoxic system nitrogen bottle ran out during the night on 19November (~36h after the initiation of hypoxia) and hypoxic conditions were re-started at 08:00 on 20November which equates to an eleven hour reprieve. Dissolved oxygen concentrations reach 8.0mg/L during this period (Figure 11). Normoxic fish were exposed to DO concentrations between 8.2 – 9.3 mg/L (Figure 12). Mean temperature in both systems was 22°C and pH of 9.86. Suckers in the hypoxic system tended to become quiescent and lay on the bottom of the aquaria while FHM remain in the water column. We sampled blood and tissues from suckers at the time of initial loading (Time0), 19November after 24h of challenge (H24 & N24), and on 21November (96h). We consider the Time0 samples to represent the fish at their basal level. Test fish size ranged from 20 – 40 mm for 0+ fathead minnows, 75 – 83 mm LRS 0+ and 117 – 148mm LRS 1+ (Tables 12 & 13). Condition factor (KSL) of both LRS 0+ and LRS1+ declined over time but was not related to treatment (T0 > N96 and H96, 1-ANOVA $P \leq 0.04$) at 96h. We speculate that starvation and diuresis was associated with the weight loss (Tables 12 & 13.). Fathead minnow length ranged from 20 – 40mm. Due to their size no other measurements were made on Fathead minnows.

Histological sections of gill and liver of normoxic and hypoxic 0+ and 1+ suckers were normal with hypoxic fish showing marked reduction in the intralamellar cell mass between the secondary lamellae. This adaption to hypoxia would maximize gas exchange at the secondary lamellae. Hyaline deposits (excess protein from filtrate) and vacuolar degeneration were more pronounced in both normoxic and hypoxic system suckers sampled at 24h than cohorts sampled at 96h. The response is likely associated with alkalinity rather than oxygen concentration.

Plasma protein was elevated in the 24h normoxic group (N24) from both 0+ and 1+ LRS in comparison to Time0 and 96h cohorts (1-ANOVA , $P \leq 0.002$, Tables 12 & 13). No difference in plasma protein was observed between normoxic and hypoxic suckers at 96h. Plasma sodium levels in hypoxic fish at both 24 and 96h were greater than their normoxic cohorts (1-ANOVA , $P < 0.01$). Iftikar et al.(2010) report that rainbow trout, under chronic hypoxia, had increased sodium influx into their plasma. Hypoxia did not affect plasma electrolytes (Na^+, CL^-, Ca, K, P and Mg) in tilapia (Choi et al. 2007). Normoxic fish had higher sodium levels than the Time0 group. These data suggest that alkalinity, confinement stress, and oxygen concentration affect ion regulation in yearling suckers. No significant difference was seen between normoxic and hypoxic LRS 0+ at 24 or 96h (Tables 12 & 13). Hypoxia did not influence liver PP2A activity. LRS 1+ liver PP2A activity of normoxic and hypoxic fish at 24hpe were similar to each other and to previous yearling groups (1-ANOVA, $P=0.866$).

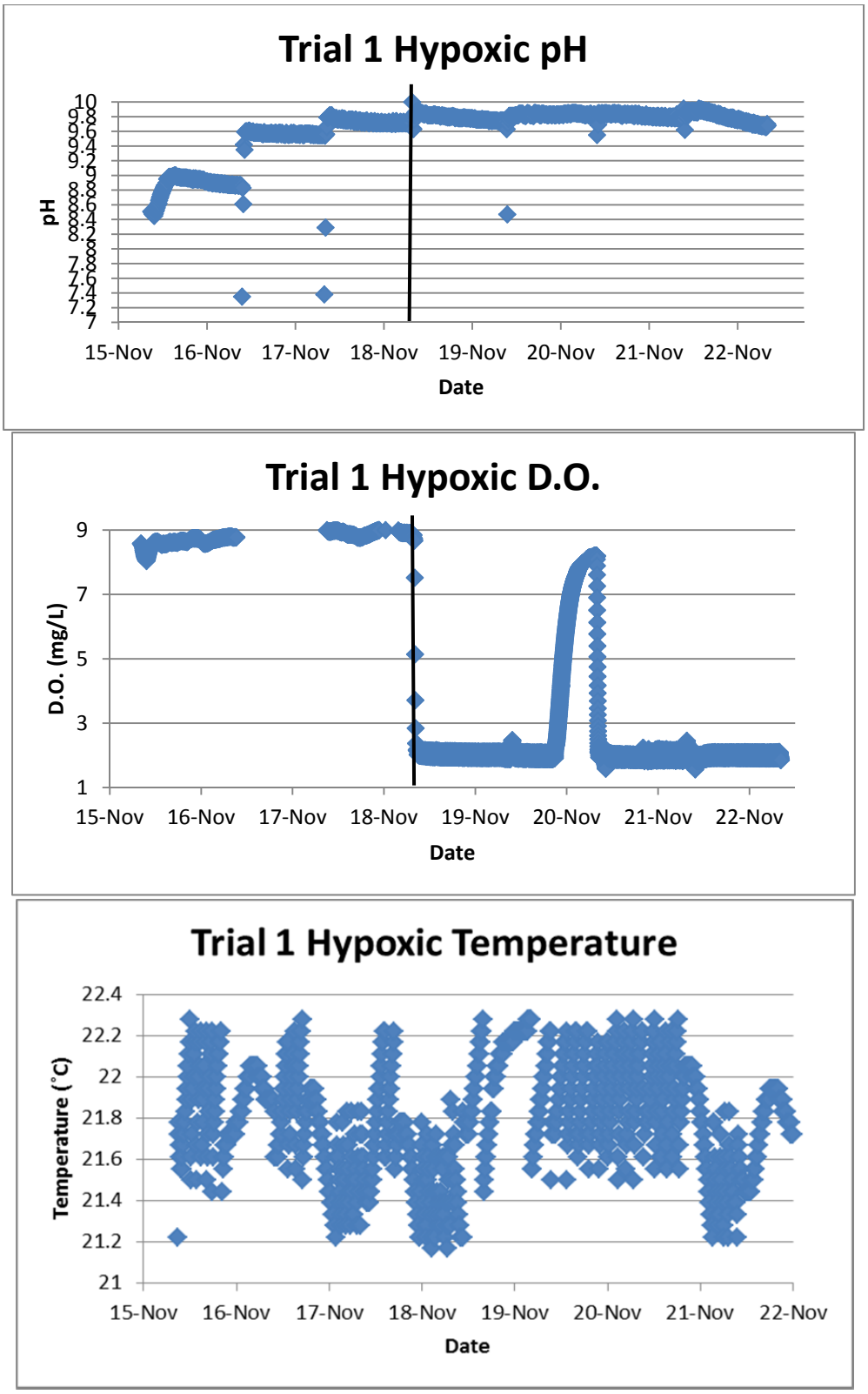


Figure 11. Hourly pH, oxygen, and temperature data for hypoxic system. Outliers are readings taken during a 20 – 30min water exchange. Bar identifies 18Nov start of hypoxic challenge

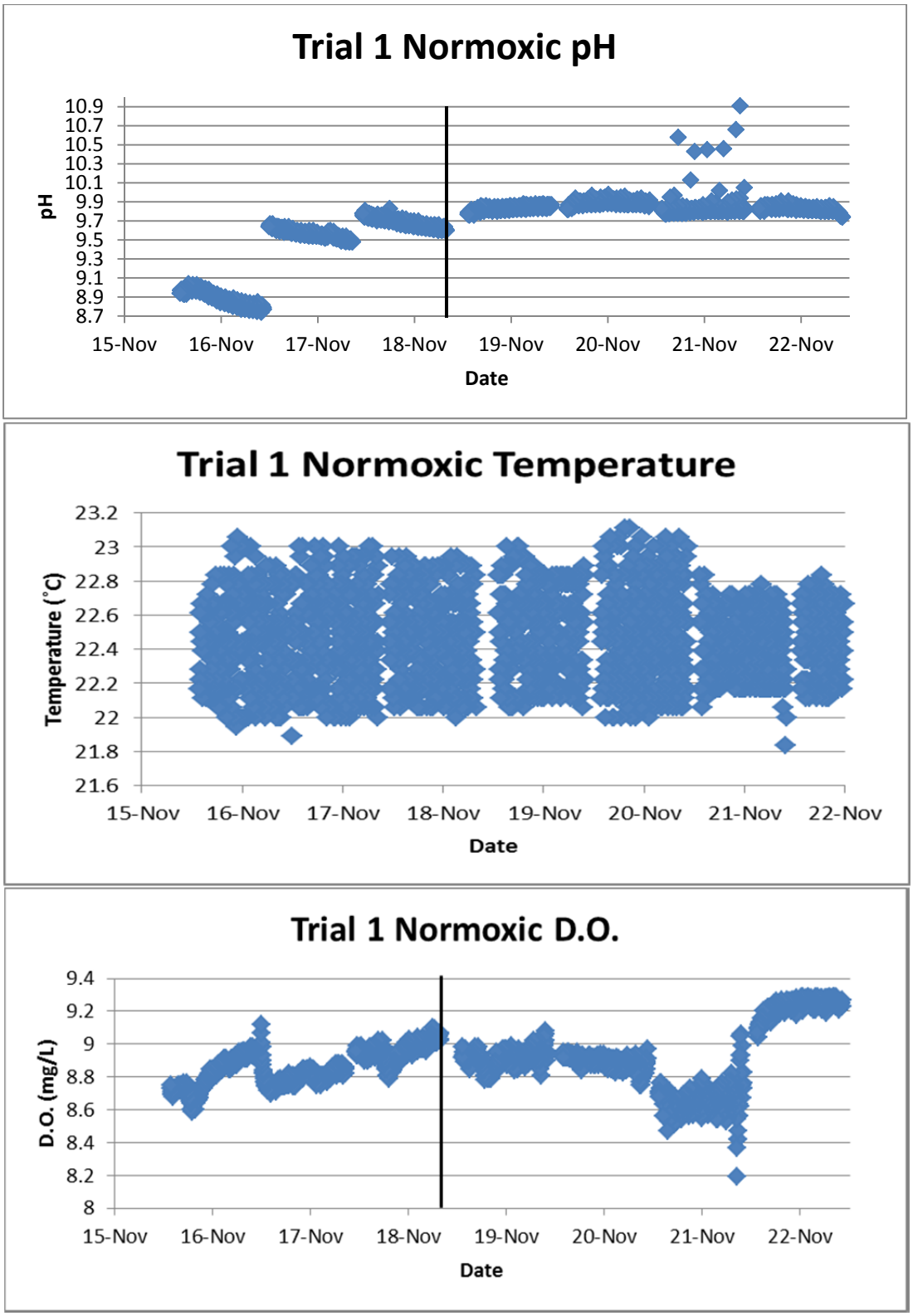


Figure 12. Hourly pH, temperature and oxygen data for normoxic system. Outliers are readings taken during a 20 – 30min water exchange. Bar identifies 18Nov start of hypoxic challenge

Table 12. Somatic, plasma, and liver PP2A measurements of **LRS 1+** held under normoxic (N) or hypoxic (H) conditions and sampled after a common acclimation period (0) or 48 and 96h post treatment. Somatic data includes standard length (mm, SL), weight (0.1g, WT), condition factor (KSL = WT/ SL^3 x 10^5). Plasma data includes plasma protein (mg/mL) and sodium (mmol/L). Liver protein phosphatase 2A activity is reported (PP2A = pmole P/mg protein). Number of fish (No.) for measurement is listed.

	T0	H-24	N-24	H-96	N-96
SL	128 (10)	136 (10)	147 (5)	136 (13)	142 (12)
WT	31.8 (5.3)	33.3 (8.4)	39.8 (5.6)	33.4 (12.8)	37.4 (12.1)
KSL	1.522 (0.132)	1.300 (0.123)	1.260 (0.052)	1.272 (0.101)	1.284 (0.192)
No.	4	4	4	4	4
Plasma Protein	43.4 (6.1)	55.0 (4.3)	61.5 (5.9)	52.7 (8.9)	44.3 (0.1)
No.	4	4	4	3	4
Plasma Sodium	147 (5)	144 (7)	164 (11)	164 (5)	183 (3)
No.	4	4	4	3	3
PP2A	ND	6202 (1193)	5226 (1974)	ND	ND
No.		3	3		

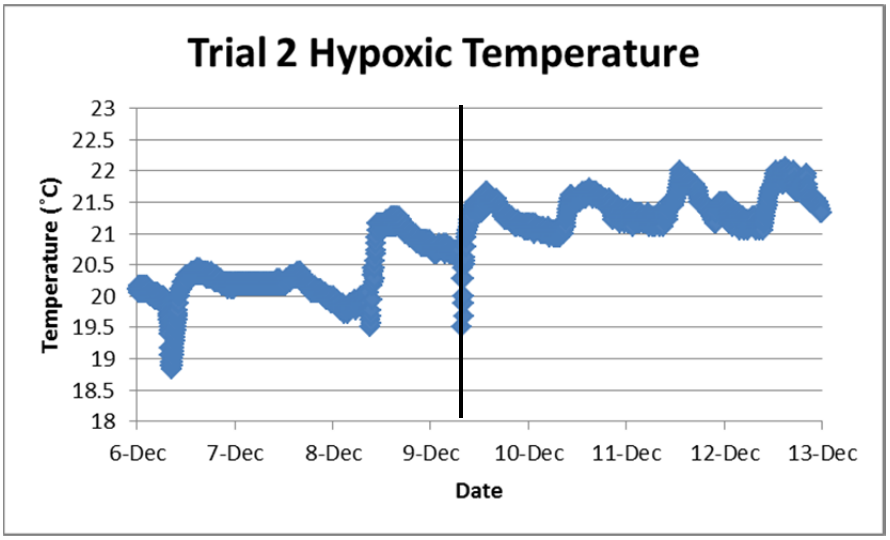
Table 13. Somatic, plasma, and liver PP2A measurements of **LRS 0+** held under normoxic (N) or hypoxic (H) conditions and sampled after a common acclimation period (0) or 48 and 96h post treatment. Somatic data includes standard length (mm, SL), weight (0.1g, WT), condition factor (KSL = WT/ SL^3 x 10^5). Plasma data includes plasma protein (mg/mL) and sodium (mmol/L). Liver protein phosphatase 2A activity is reported (PP2A = pmole P/mg protein). Number of fish (No.) for measurement is listed.

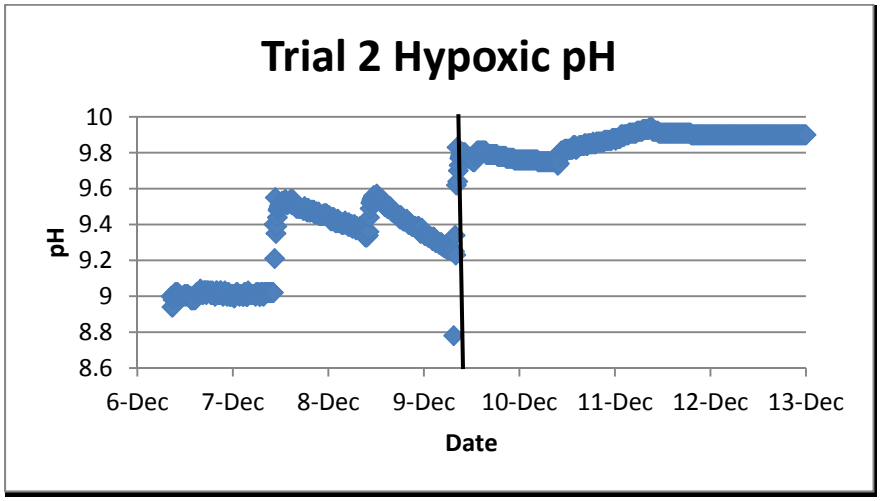
	T0	H-24	N-24	H-96	N-96
SL	79 (7)	79 (3)	85 (9)	86 (13)	81 (7)
WT	7.2 (1.8)	7.0 (0.4)	7.9 (1.7)	8.8	6.8 (1.4)
KSL	1.469 (0.102)	1.409 (0.091)	1.286 (0.129)	1.303 (0.034)	1.276 (0.078)
No.	4	4	4	4	4
Plasma Protein	34.9 (1.7	41.2 (5.0)	51.1 (5.8)	32.7 (5.7)	33.0 (1.6)
No.	4	4	4	2	2
Plasma Sodium	167 (23)	136 (40)	161 (2)	164 (5)	170 (4)
No.	2*	4	4	2	2

* Two fish with extremely high values (267 and 300 mmol/L) excluded as contamination suspected

Trial 2 (06-13December2013): Acclimation pH changes were similar in both systems (9.0 pH day 1, 9.3 to 9.4 pH day 2 and 3) and the target pH of 9.8 was met in both systems over the 96h experimental (mean for both = 9.86 pH, Figures 13 and 14). Temperature was also similar in both systems (21 - 22°C) and ammonia levels remained low (0.00 – 0.05mg/L NH_4-N) over the experiment. Hypoxic system fish experienced a gradual decline in D.O. from a daily mean of 5.66 to 3.25mg/L during acclimation and averaged 1.58mg/L (0.08) during the 96h challenge (Figure 13). Mean dissolved oxygen in the normoxic system was 8.96mg/L (SD =0.11, Figure 14).

One LRS 1+ died within the first 24h of hypoxia (fish had scoliosis and low condition factor). No other mortality occurred over the remaining 72h. Condition factor (KSL) of normoxic LRS 1+ was greater than hypoxic cohorts at 96h (T-test, P=0.0205), however, 3 of 5 normoxic fish had markedly high weights (Table 15). No difference in KSL was observed between treatment groups or over time in LRS 0+ (Tables 14 and 15). Fathead minnows varied in length from 20 – 48mm SL. No hypoxic effect was observed in either LRS 1+ or 0+ liver PP2A activities (Tables 14 and 15). Plasma sodium values were higher at Time0 than at 96h for both LRS 0+ oxygen treatment groups (1-ANOVA, P=0.002). Yearling LRS had similar sodium values regardless of sample time or treatment. Plasma protein was significantly elevated in hypoxic 96h LRS 1+ and LRS 0+ compared to their respective Time0 and N96 cohorts (1-ANOVA ,$P \leq 0.028$). Fish responded to the hypoxia by increasing the number of red blood cells in their blood. Hematocrit (packed RBC volume) was 38 – 42% higher in hypoxic LRS than normoxic cohorts at 96h (Tables 14 and 15). Histological sections of gill and liver of normoxic and hypoxic suckers were normal with hypoxic fish showing marked reduction in the intralamellar cell mass between the secondary lamellae (Figure 15). This adaption to hypoxia would maximize gas exchange at the secondary lamellae with an associated loss of increased of ions. Hyaline deposits (excess protein from filtrate) and vacular degeneration became more pronounced over time in the hypoxic LRS 0+ and 1+ (Figure 16). Tervon et al. (2006) report that rainbow trout increase urine flow and undergo hemoconcentration in response to short-term hypoxia. Fathead minnows showed no abnormalities.





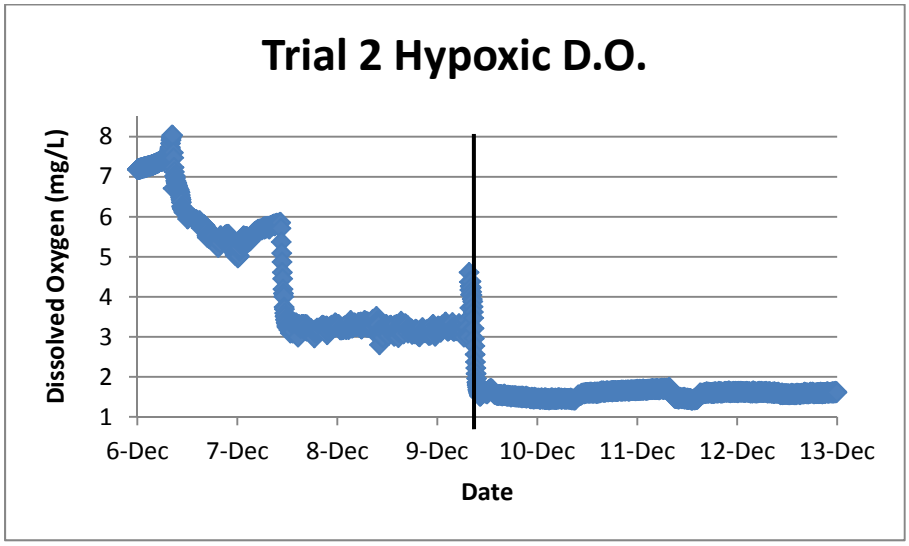


Figure 13. Hourly pH, oxygen, and temperature data for hypoxic system. Bar identifies 9Dec start of hypoxic challenge.

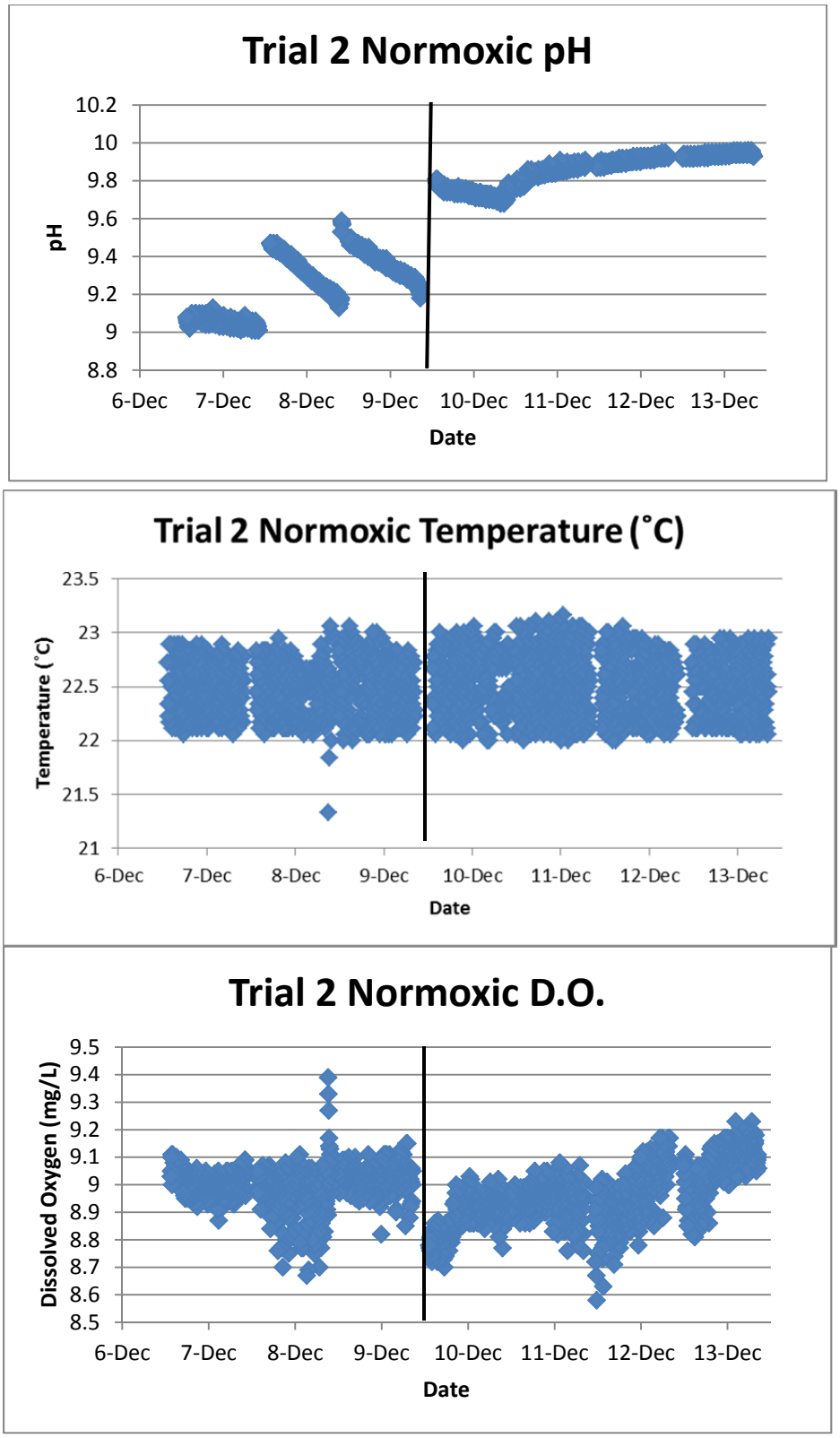


Figure 14.Hourly pH, oxygen, and temperature data for normoxic system. Bar identifies 9Dec start of hypoxic challenge.

Table 14. Somatic, blood, and liver PP2A measurements of **LRS 1+** held under normoxic (N) or hypoxic (H) conditions and sampled after a common acclimation period (0) or 48 and 96h post treatment. Somatic data includes standard length (mm, SL), weight (0.1g, WT), condition factor (KSL = WT/ SL^3 x 10^5). Blood data includes plasma protein (mg/mL), sodium (mmol/L), and hematocrit (HCT%). Protein phosphatase 2A activity (PP2A = pmole P/mg protein). Number of fish in specific measurement is listed (No.).

	N-0	H-0	H-48	N-96	H-96
SL	134 (8)	148 (8)	130 (18)	117 (20)	138 (13)
WT	32.9 (3.7)	40.6 (7.9)	28.2 (11.1)	27.2 (13.2)	34.2 (9.9)
KSL	1.379 (0.126)	1.255 (0.048)	1.241 (0.038)	1.604 (0.249)	1.269 (0.072)
No.	4	2	3	4	4
Plasma Protein	26 (2)	21 (1)	ND	25 (3)	30 (2)
No.	4	2		4	4
Plasma Sodium	135 (12)	119 (8)	ND	138 (10)	133 (3)
No.	4	2		4	4
HCT%	ND	ND	ND	34 (5)	47 (4)
No.				4	4
PP2A	ND	ND	ND	5120 (893)	5737 (1376)
No.				3	4

ND not done

Table 15. Somatic, blood, and liver PP2A measurements of LRS 0+ held under normoxic (N) or hypoxic (H) conditions and sampled after a common acclimation period (0) or 48 and 96h post treatment. Somatic data includes standard length (mm, SL), weight (0.1g, WT), condition factor (KSL = WT/ SL^3 x 10^5). Blood data includes plasma protein (mg/mL), sodium (mmol/L), and hematocrit (HCT%). Protein phosphatase 2A activity (PP2A = pmole P/mg protein). Number of fish in specific measurement is listed (No.).

	N-0	H-0	H-48	N-96	H-96
SL	83 (11)	79 (3)	81 (10)	75 (10)	80 (5)
WT	8.4 (2.8)	6.3 (0.6)	6.8 (2.5)	5.7 (1.7)	6.9 (1.4)
KSL	1.432 (0.127)	1.284 (0.010)	1.244 (0.091)	1.353 (0.103)	1.358 (0.054)
No.	4	2	4	4	4
Plasma Protein	22 (3)	20	ND	24 (2)	27 (2)
No.	4	1		3	4
Plasma Sodium	157 (15)	145 (5)	ND	111 (14)	118 (7)
No.	4	2		3	4
HCT%	ND	ND	ND	28 (3)	40 (3)
No.				3	4
PP2A	ND	ND	ND	5651 (1566)	6160 (941)
No.				3	4

ND not done

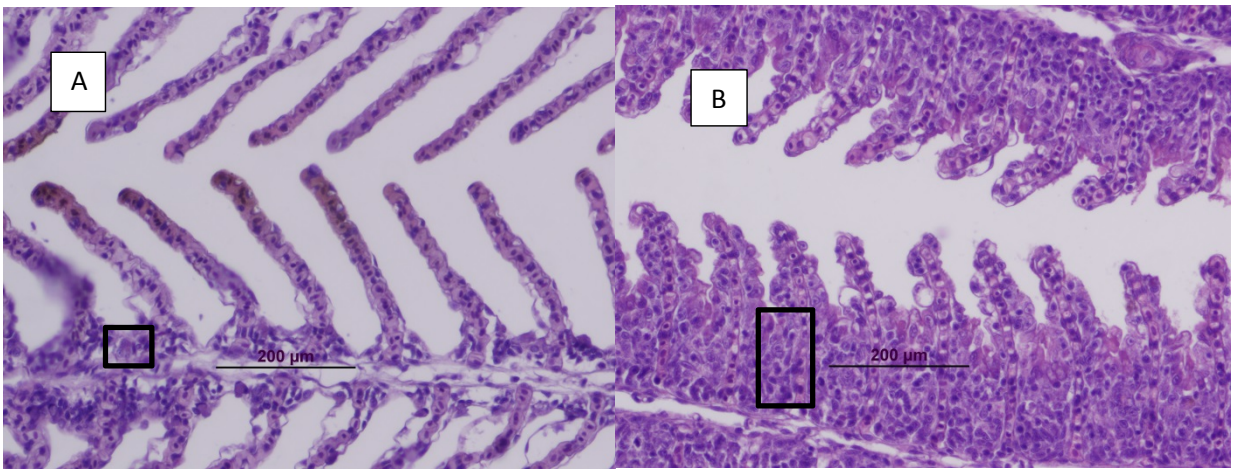


Figure 15. Gill lamellae of LRS after 24h under hypoxia (A) compared to LRS sentinel in lake during September 2013 (pH 7.5, normoxic, low ammonia). Note the lack of intralamellar cell mass (box) in (A) compared to (B) thereby maximizing gas exchange in secondary lamellae.

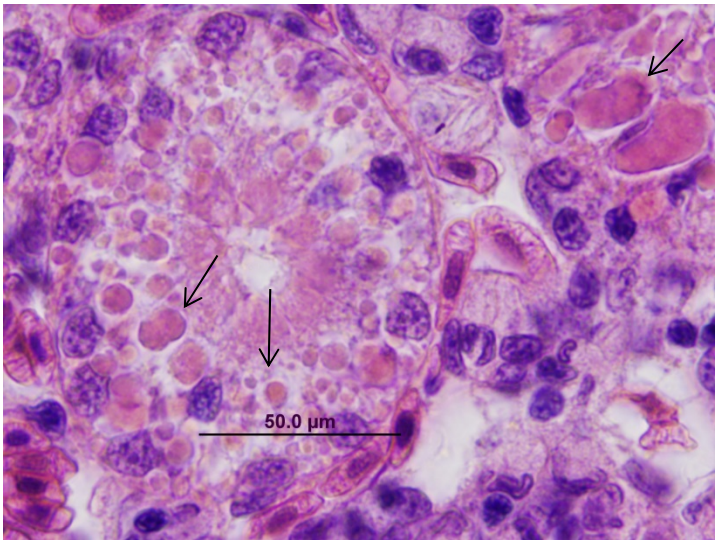


Figure 16. Proximal convoluted tubule within kidney of 96h hypoxia LRS showing hyaline deposits (arrows) and vascular degeneration of cytoplasm.

Trial 3 (10-19January2014): Acclimation pH changes did not occur at the same rate in both systems. Hypoxic pH was increased as scheduled however the normoxic system had a mean pH of 9.07 for the first 3 days of acclimation then increased to a mean pH of 9.8 for the remainder of the trial (Figure 18). Temperatures were similar in both systems ranging between 21.5 - 22.5°C and ammonia levels remained low (0.00 – 0.09mg/L NH_4-N) over the experiment. Hypoxic system fish experienced a gradual decline in D.O. from a daily mean of 4.98mg/L at the start of acclimation to a mean of 0.75mg/L by the end of the trial (Table 16, Figure 17). Mean dissolved oxygen in the normoxic system was 9.25mg/L

One LRS 1+ died during the night of the second acclimation day and no samples were collected. Water quality at that time was pH 8.91, 22.1°C and D.O. of 4.90mg/L. This fish had hemorrhaging along fin bases and slight petechial hemorrhaging on its abdomen. Two LRS1+ died in the hypoxic system on day 7 (between 08:00-10:02, and at 12:11). Both fish lost equilibrium prior to death. One LRS 0+ mortality was also observed on day 7 at 14:37. Fish on

day 7 were exposed to a mean D.O. of 0.78mg/L for ~2 – 8h prior to death. All remaining suckers except one LRS 0+ were dead on the morning of day 8 (06:30). Dissolved oxygen content averaged 0.75 mg/L overnight. No Fathead minnow mortality occurred over the entire trial.

Table 16. Dates, target dissolved oxygen (D.O.) concentrations and mean dissolved oxygen concentrations (D.O.) for hypoxic system.

Date	Target D.O.	Mean D.O.
1/10/2014	5mg/L	4.98
1/11/2014	3-4mg/L	3.82
1/12/2014	2-3mg/L	2.51
1/13/2014	1.5mg/L	1.55
1/14/2014	1.3mg/L	1.36
1/15/2014	1.2mg/L	1.25
1/16/2014	1.0mg/L	1.04
1/17/2014	0.8mg/L	0.75

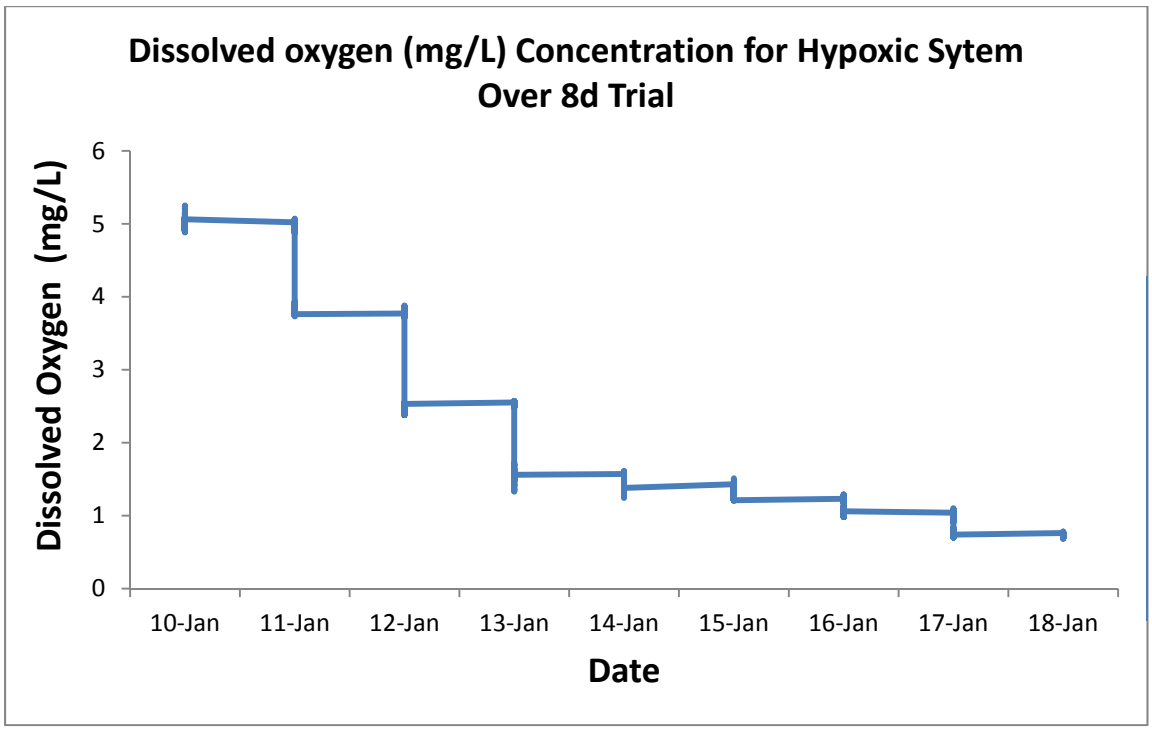
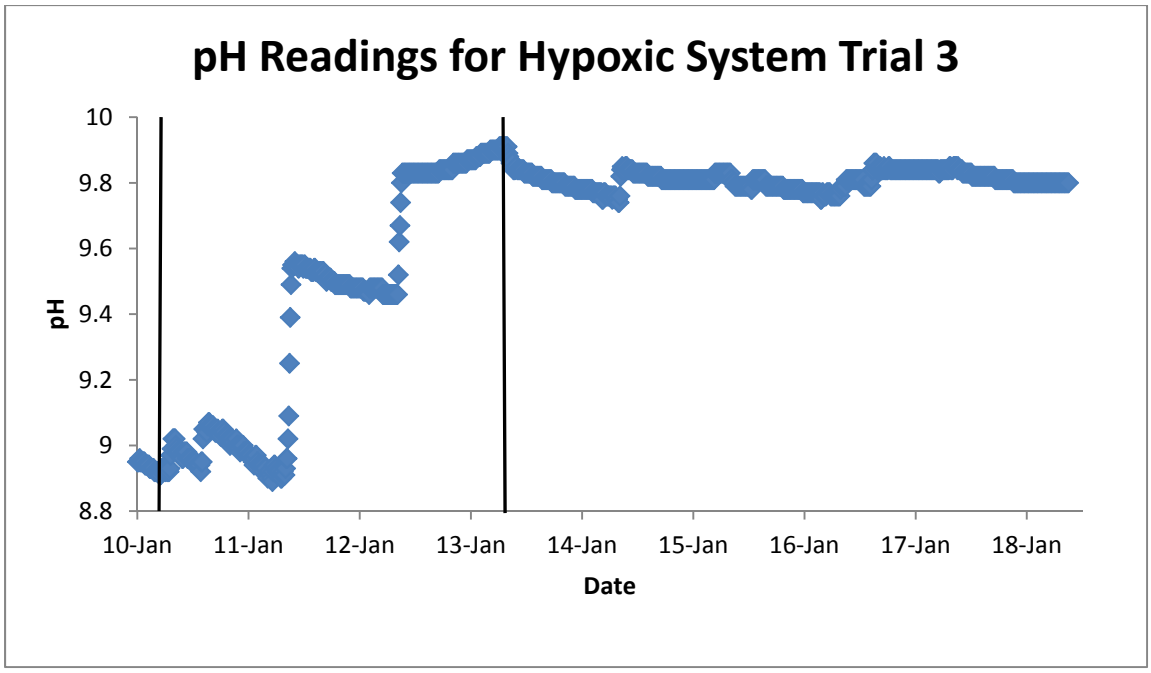


Figure17. Incremental decrease in dissolved oxygen concentration for hypoxic system over the 8d trial.



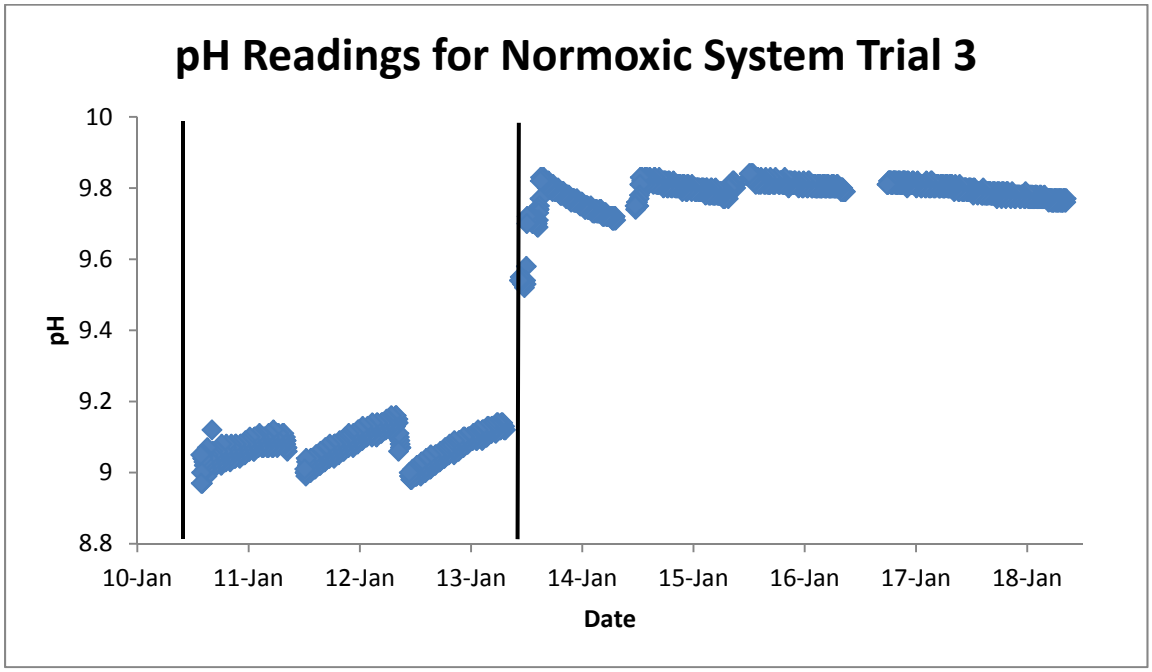


Figure 18. pH for hypoxic and normoxic systems in Trial 3.

Discussion

Water quality was judged to be relatively mild during the 2013 sentinel exposures with high 14dpe survival observed in most sentinel groups. Similar to the 2011 sentinel study, exclusion of avian and teleost predators within full water column net pens, allowed for high survival of LRS fry over the summer. Synergistic effects of adverse water quality, parasitic infection, and ingestion of toxic algae may alter predation rates for juvenile suckers in Upper Klamath Lake. Data on avian predation on sucker fry during the summer could potentially identify increased predator susceptibility associated with adverse water quality.

Despite good water quality, we observed poor survival (11%), heavy metacercaria infection, and little growth in sentinel LRS 0+ held over the summer at the Williamson River mouth. Given the low TG reserves and growth of survivors, we speculate that there could be inadequate food resources for smaller fry at this site (prey size or type?). In 2011, similar poor survival and fish condition occurred to LRS fry held within the Williamson River delta (Goose bay). In contrast, bi-weekly sample groups at this site had high survival over this shorter time period. No energy reserve analysis was performed on these fish to determine if growth occurred. Both yearling and sucker fry held over the winter at this site also had high survival. Advantages of rearing in marsh habitat (e.g. predator avoidance) could be offset by negative health impact of trematode infection. Site selection for net pen culture will need to balance water quality with food resources.

Several observations during the period of 12 - 26August suggest that water quality (and potentially microcystin toxin) was at stressful levels: 1) 21 - 25% mortality of 14dpe LRS 1+, 2) depressed liver PP2A activities, and 3) low whole body TG content in 14dpe sentinels. Low dissolved oxygen concentration (mean [minimum] surface: 2.84 [0.88], bottom: 2.61 [0.48] mg/L) was associated with this stressful period. Benthic DO was $\leq$ 1.50 mg/L during portions of 5 days during late August. Fish respond to hypoxia by first increasing oxygen delivery to the blood (energy intensive increase in ventilation rate and stroke volume, initial splenic contraction to increase erythrocyte concentration in the blood followed by increased erythropoiesis, changes in erythrocyte phosphate concentration and pH to enhance oxygen affinity of hemoglobin), then conserving energy through metabolic depression and reduced activity, and finally enhanced supply of limited anaerobic energy sources such as muscle glycogen (Wu 2002, Pickering and Pottinger 1995, Lai et al 2006). During hypoxic challenges, LRS 0+ and 1+ ventilation rate increased greatly while they remain quiescent on the aquarium bottom. Additionally, circulating erythrocyte numbers (hematocrit) increased in challenged fish as did the respiratory surface area of the secondary lamellae (histological observation). During the low oxygen period of 12 - 26August, yearling sentinels had 21 and 25% mortality (14dpe) in comparison to $\leq$ 3% mortality in 0+ fry. Similarly, LRS 1+ lost equilibrium and died sooner than LRS 0+ during laboratory hypoxia challenges. This sensitivity to hypoxia could be related to higher metabolic demand of yearlings however, Nilsson and Nilsson(2008) report that the general trend is for respiration surface area to be proportional to metabolic need. Laboratory hypoxic challenges demonstrate the critical oxygen minima for LRS is at or below 1.0mg/L and that 130 - 140mm yearling fish are more sensitive than 70 – 80mm LRS 0+. This observation is below the previously reported 48 and 96h LD_{50} value of 1.58 mg/L. Fathead minnow fry survived 24h exposures to dissolved oxygen levels of 0.75mg/L which highlights this species success in Upper Klamath Lake. Low oxygen concentrations during the summer are likely a major driver in recruitment failure but may be acting indirectly.

Physiological exhaustion may play a role in the chronic mortality responses of sentinel LRS observed in 2011, 2012, and 2013. Jensen et al. (1993) describes that metabolic rate of fish typically increase by a factor of 2-3x for every 10°C rise in water temperature. Solubility of oxygen drops with increased temperature (example 0.7mg/L difference between 20°C and 25°C). Low oxygen alkaline waters of the lake are likely to require constant feeding to meet the high energy demands of this environment. Additional energy would be needed for sucker fry forced to move from the anoxic bottom and swim near the surface for days or to search for better oxygen conditions. Immune function is also likely to be impaired by hypoxia thereby exposing sucker fry to opportunistic bacterial infections in this eutrophic lake (Choi et al. 2007).

Our in-situ and laboratory studies to date have not conclusively linked recruitment failure with **direct** mortality due to adverse water quality in Upper Klamath Lake. Relevant to this statement, we have conducted 4 previous studies with juvenile suckers that demonstrate their adaptation to adverse conditions (Foott et al. 2007, 2009, 2012, and 2013). In August 2007, sucker fry held in semi-static aquaria of Upper Klamath Lake water survived short periods of hypoxia (< 2mg/L) and elevated UIA (0.102mg/L). Over the 22d trial, fry were observed near the surface interface and experienced only 7% mortality. In a 2006 controlled laboratory experiment, alkaline summer conditions did not impair survival, growth, or energy reserves of LRS fry reared for 27d at a mean pH of 9.58 and fed an adequate diet. In a July – September 2011 sentinel study, LRS fry in Upper Klamath Lake had ≥ 48% survival at 21dpe and over 38% after 76d. Fry, held throughout the summer, had normal growth and reached 70mm by October. Net pen loading density was a likely influence in survival and highlights the importance of food resources in this energy intensive environment. In 2012, we observed a delayed mortality pattern in 30-35mm sentinel fry held within benthic net pens. Between 7 and 14dpe sentinel groups experienced ≥ 77% mortality. No mortality occurred for the first 7d. The early August exposure group experienced both high pH (>10.0) and ammonia while low dissolved oxygen concentrations (2 - 4mg/L) were associated with last two affected groups. Physiological exhaustion, due to the above stressors, could have been the primary driver in benthic net pen sentinel mortality. Conversely, wild fry would have access to surface waters and the ability to move from hypoxic regions. Infectious disease has not been associated with sentinel fry held in the lake. Despite histological examination of hundreds of survivors, no definitive diagnosis for sentinel mortality has been made in the 2011 – 2013 studies. Our currently working hypothesis is that synergistic effects of adverse water quality, parasitic infection, and ingestion of toxic algae may substantially increase predation rates for juvenile suckers in Upper Klamath Lake during the summer.

In the current study, we assayed LRS liver for PP2A activity as a sub-lethal indicator of microcystin toxin exposure. Depression of liver PP2A activity during 12 - 26August period was likely associated with ingestion of a threshold concentration of microcystin toxin. The concentration of toxin absorbed by the fish appears to be below a threshold associated with overt liver damage. Fischer et al. (2000) describe the limitation of IHC assay in trout as necrosis was seen prior to positive IHC staining of the affected hepatocyte. They speculate that MCLR must be covalently bound to hepatocyte proteins for positive IHC staining. We also observed increased PP2A activity in three 14dpe groups in comparison to their 7dpe cohorts. Li et al. (2011) report that low concentrations (2μM) of MCLR stimulate PP2A activity of cultured

HEK293 cells possibly by re-activation of a regulatory subunit of the enzyme. At higher concentrations (10μM), PP2A was inhibited and the cellular cytoskeleton dissociation. We speculate that suckers compensate for low level microcystin intake by increasing liver enzyme concentration. Microcystins are cyclic hepatotoxins, with two characteristic amino acids, N-methyldehydroalalnie (Mdha) and 3-amino-9-methoxy-2-6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), that are transported from the intestine to liver by a bile acid carrier. *Microcystin aeruginosa*, via its hepatotoxin microcystin-LR, has been identified as human health threat in Upper Klamath Lake by Oregon Department of Agriculture (Gilroy et al. 2000.). The Adda moiety of microcystin toxin inactives protein phosphatase 1 and 2a in multiple taxa, including fish. Phosphatase 2A cleaves phosphate from phosphorylated serine and threonine proteins which act in the regulation of various cell signaling processes include apoptosis, as well as RNA transcription, DNA repair, cell proliferation, and cytoskeleton assemble (Jannssens and Goris 2001).

Lake mud appears to moderately beneficial for sucker fry during early rearing. We observed significantly higher weight gain in larvae reared over lake sediment and provided dry diet than cohorts without mud. Effect on fry mortality was complicated when we mixed populations. In a substrate trial, there was little difference between fry size of “no substrate” controls and mud substrate fish however the 1%BW/d feeding rate was deemed inadequate (Appendix 1). This data supports LRS culture using fertilized earthen ponds with adequate water quality and supplemental feeding.

In conclusion, sentinel survival of LRS 0+ was high throughout the summer of 2013. Stressful periods, associated with low oxygen occurred in August, invoked several sub-lethal responses in the sentinel suckers. The PP2A assay appeared to document both compensatory increases and sub-lethal depression in enzyme activity of fish exposed to microcystin toxin. Our data from several studies suggest that recruitment failure of 0+fry in Upper Klamath Lake is indirectly tied to adverse water quality and that the role of avian predation during such periods needs to be evaluated.

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Appendix 1. **Pilot Experiment: Effect of lake mud on 0+ fry growth and gill hyperplasia**

(J. Jacobs)

Summary: Autoclaved substrates (mud and sand) were associated with the highest mortality and prevalence of gill hyperplasia. Mortality and gill changes increased beginning after 2 weeks into the trial. Despite the low feed rate (1%), all groups demonstrated positive growth over the 6 week trial. Substrates did not appear to have an effect on mean length, weight, and KSL until the end of week 6. Autoclaved Mud treated suckers had significantly greater weight and length than Raw Mud treated fish, but did not weigh significantly more and were not significantly longer than the Control and Sand Control treated fish. Autoclaved Mud treated suckers also had a significantly higher condition factor than the Control treated suckers but were not significantly higher in condition factor when compared to the Raw Mud and Sand Control treated suckers. Histological examination of liver showed that Sand Control and Control tank fish had low hepatocyte fat and glycogen stores (vacuoles). No definitive conclusion of the benefits of rearing LRS fry over substrate can be made with the available data from this trial.

Methods: Lost River sucker fry were placed in a trial to determine if substrate had any effect on fry health. The primary objective of the trial was to differentiate organic (bacteria, fungi, protozoan, etc.) components of mud from inorganic (micro nutrient, physical gastric aid (grit), environment-behavioral only) components on the observed growth and condition of the fry.

Mud was collected from the Upper Klamath in mid-July. A total of 6 aquariums were used in the trial; 2 aquariums were filled with 6L each of autoclaved mud from the Upper Klamath, 2 with 6L each of raw mud from the Upper Klamath, 1 with 6L of autoclaved sand (non-mud substrate control), and 1 with no substrate (no substrate control). Sample populations of 100 suckers, all collected from the same group of suckers being housed in the wet lab, were added to each aquarium after substrates were allowed to settle for 24h. A mean weight and mean standard length was collected and condition factor calculated ($KSL = WT/SL^3 \times 10^5$) for the group of sample fry the day before the trail was started. These numbers were used as Week 0 data. Suckers were fed a diet of Otohime™ B2 and C1 with the addition of Crisco® vegetable oil (Ingredient: soybean oil) for increased palatability.

Table 1. Nutritional information for Otohime™ B2 and C1 diets.

Shape	Size	Crude Protein % MIN	Crude Fat % MIN	Crude Fiber % MAX	Crude Ash % MAX	Crude Calcium % MIN	Phosphorus % MIN	Moisture % MAX
B2	Granule	360 – 650 µm	51.0	11.0	3.0	15.0	2.3	1.5
C1	Granule	580 – 840 µm	51.0	11.0	3.5	15.0	2.3	1.5

Otohime™ B2 and C1 ingredients are krill meal, fish meal, squid meal, potato starch, wheat flour, fish oil, brewers yeast, calcium phosphate, guar gum, soy lechithin, Betaine, licorice plant, apple extract, and wheat germ. Suckers were fed at 1% body weight per day equating to 1g feed per tank per day and held within similar water quality conditions. The trial was run for 41 days with bi-weekly measurements of standard length and weights on twelve random fish from each aquarium. Histological samples were collected randomly from two fish from each standard length and weight sampling group for observation of gill and liver condition.

Analysis was performed with IBM SPSS Statistics Grad Pack 21.0 PREMIUM software on raw data. Normality was tested by the Welch's t-test method at the $P = 0.05$ level. One-way ANOVA used in conjunction with Tukey HSD method (alpha ≤ 0.05) to compare groups. The repeated measures ANOVA was used to observe if treatments caused changes in mean weight, mean standard length, and mean condition factor over time.

Mortality of suckers was highest in the Autoclaved Mud and Sand Control tanks throughout the study with the majority of deaths occurring after 18 days into the experiment. The Raw Mud tanks and No Substrate Control had lowest mortality both with 10% loss at the end of the study (Fig 1).

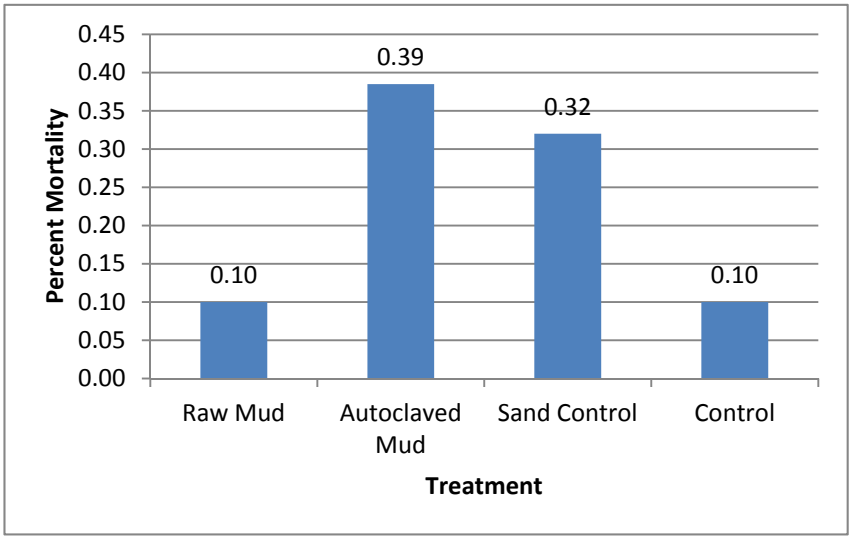

Figure 1. Bar Graph of percent Mortality per treatment group of Lost Rivers Suckers at the end of the six week trial.

Histological analysis of sagittal sections of fry demonstrated that gill hyperplasia occurred in all groups except the no substrate controls. Fry in the Autoclaved Mud or Sand Control treatment had the highest prevalence (50%) of this lesion (Table 2). This gill abnormality became apparent in the groups from week2 on indicating that it develops after exposure to treatment conditions. While no causality was determined, the development of gill hyperplasia was associated with the increase in mortality during the trial. We have observed this gill change during previoius mortality events with other LRS juvenile populations reared in the wet lab. Hepatocyte vacuoles were rarely observed indicating low glycogen and fat energy reserves on the restricted diet. Other liver abnormalities were judged to be mild and did not always correspond to gill hyperplasia.

Table 2. Prevalence of gill hyperplasia and hepatocyte abnormalities (mild cell swelling, focal coagulative necrosis, cytoplasmic droplets) in LRS 0+ fry reared in lake mud, autoclaved mud (Auto mud), autoclaved sand (Auto sand), and no substrate (Control) aquaria and sampled at 2,4, and 6 weeks.

		week2	week4	week6		Prevalence
Mud	gill HP	1 / 4	0 / 4	2 / 4		3/ 12 (25%)
	Liver abn*	0 / 4	2 / 4	1 / 3		3/ 11 (27%)
AutoMud	gill HP	0 / 4	3/ 4	5 / 8		8 / 16 (50%)
	Liver abn*	1 / 4	0 / 1	5 / 8		6 / 13 (46%)
AutoSand	gill HP	0 / 2	2 / 2	1 / 2		3 / 6 (50%)
	Liver abn*	0 / 2	1 / 1	1 / 2		2 / 5 (40%)
Control	gill HP	0/1	0 / 2	0 / 2		0 / 5 (0%)
	Liver abn*	0/1	0 / 2	0 / 2		0 / 5 (0%)

There was no significant difference among treatments in mean standard length at the end of week two and four (Figure 2). There was significant difference between treatments in mean standard length at the end of week six. The Raw Mud treatment suckers were significantly shorter than the Sand Control and Autoclaved Mud suckers but not significantly different from the Control treatment suckers. The Control, Sand Control, and Autoclaved Control treatments were not significantly different from one another in mean standard length.

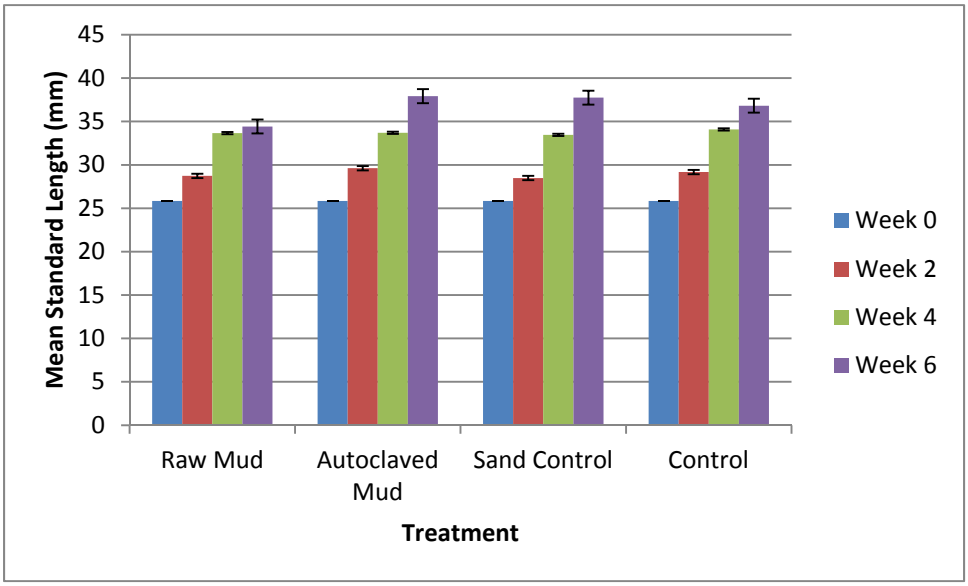

Figure 2. Mean Standard Length (mm) at Time 0 and for each treatment from sample groups at the end of Weeks 2, 4, and 6.

There was no significant difference among treatments in mean weight at the end of week two and week four (Figure 3). There was significant difference between treatments in mean weight at the end of week six. The Raw Mud treatment suckers weighed significantly less than the

Autoclaved Mud suckers but did not weigh significantly less than the Control and Sand Control treatment suckers. The Control, Sand Control, and Autoclaved Mud treatments were not significantly different from one another in mean weight.

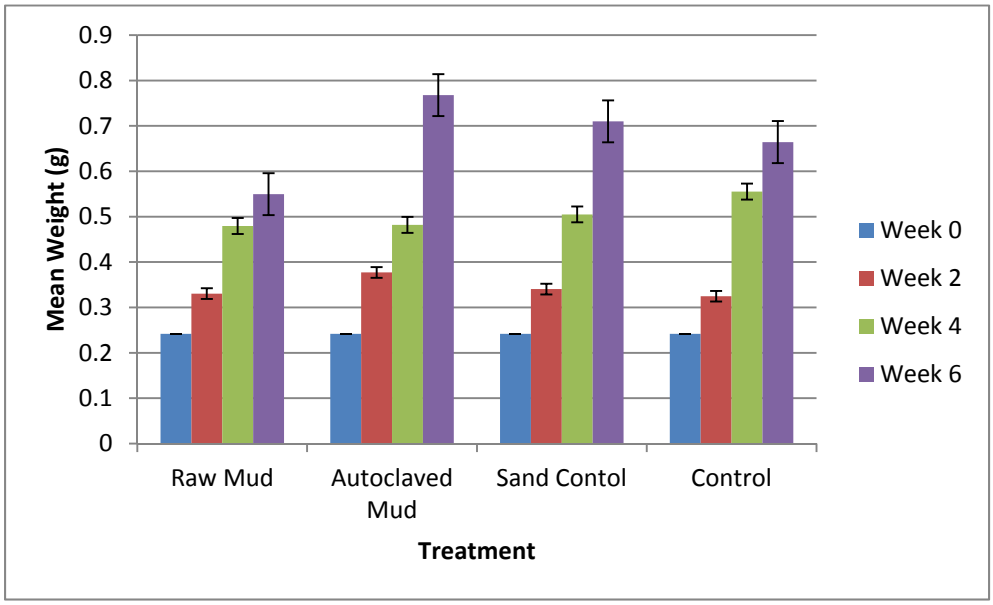

Figure 3. Mean Weight (g) at Time 0 and for each treatment from sample groups at the end of Weeks 2, 4, and 6.

There was significant difference between treatments in mean condition factor for weeks two, four, and six (Figure 4). The mean condition factor for the Control treatment group was significantly less than that of the Sand Control treatment group at the end of week two. The Raw Mud, Autoclaved Mud, and Sand Control were not significantly different from each other in mean condition factors at the end of week 2. The mean condition factor for the Raw Mud treatment and Autoclaved Mud treatment groups was significantly less than that of the Control treatment group at the end of week four. There was no significant difference in mean condition factor for the Sand Control and Control treatment groups at the end of week four. The mean condition factor for the Control treatment group was significantly less than that of the Autoclaved Mud treatment group at the end of week 6. There was no significant difference in mean condition factor between the Sand Control, Raw Mud, and Autoclaved Mud treatment groups at the end of week six.

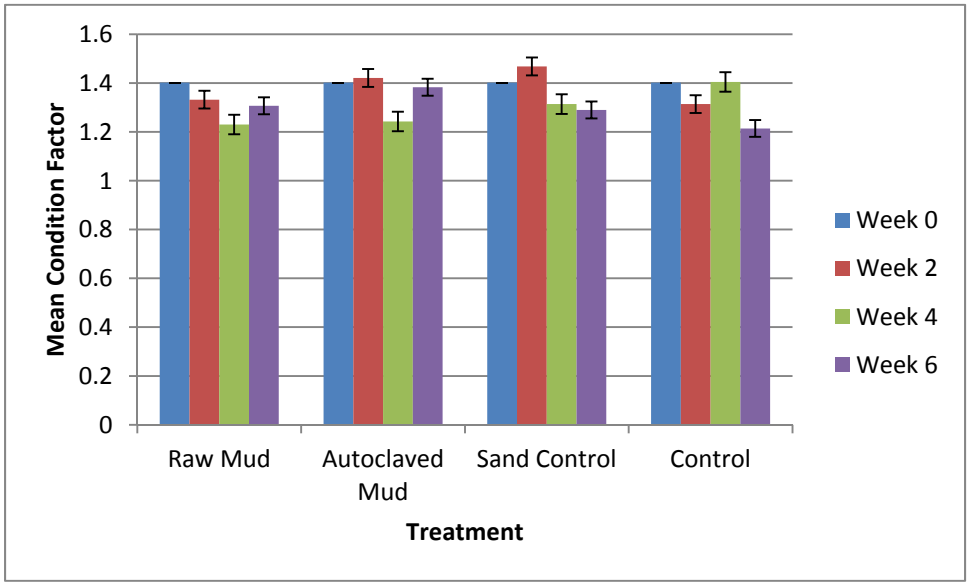

Figure 4. Mean Condition Factor at Time 0 and for each treatment from sample groups at the end of each sample week.

Appendix 2. **Pilot trial Static system daily water exhanges 27September2013 (S Foott)**

Summary: Test system did not perform satisfactory (poor alkalinity, ammonia, and oxygen control) as static aquaria with daily water exchange. Complete mortality occurred to both 0+ and 1+ LRS exposed to dissolved oxygen levels ≤ 1.26mg/L for over 24h. Fathead minnows survived and were at the surface. **Suckers survived for 28h under extreme summer lake conditions of 22°C, 2mg/L D.O., pH 9.24, and UIA of 0.324mg/L.** These same fish were sampled live the next day following a water exchange and increased aeration. Water quality at the time of sampling was 21°C, pH 9.22, 4mg/L D.O., and **UIA of 0.547mg/L** (TAN= 1.3mg/L). All fish showed edema in gill secondary lamellae epithelium, severe hyaline degeneration of the kidney proximal but normal hepatocyte structure. Gill and kidney changes are often seen in response to alkalinity but appear to enhanced by the experimental conditions of elevated ammonia and hypoxia. *Lost river suckers are able to survive limited exposure to high ammonia (0.3 and 0.5mg/L) and dissolved oxygen of 2.0mg/L.* Imperfect water quality data limits our evaluation of short-term LRS 0+ and 1+ survival under severe conditions. Future test system will require continuous recirculation, ChlorAm-X® ammonia control, and continuous NaOH and nitrogen input to maintain specific water quality..

Methods: Three 55L aquaria with air stones (minimal input) were immersed in a 22°C water bath along with 3 companion 60L water buckets. The buckets were used for preparation of isothermic alkaline pH water to perform one daily water exchange in each aquaria. Four each LRS 0+, LRS 1+, and FHM 0+ were held in each aquaria. Fathead minnows were captured at the A-canal facility in July and reared at the FHC wetlab. No continuous water quality measurements performed. Timeline is listed below:

27September 14:00 Fish loaded into aquaria at ambient tank conditions (19.3°C, pH 7.40 – 8.02, normoxic). Increased pH to 9.03. No dissolved oxygen (D.O.) or total ammonia nitrogen (TAN) measured.

28September 07:30 Water at 21.7˚C, pH 7.82 -8.79 (decline overnight)
No mortality, FHM and LRS 0+ near surface, LRS 1+ holding still on the bottom
Increase pH to 9.52 – 9.56, no D.O. or TAN measured

29September 11:50
Aquarium 1 fish alive, 22.2˚C, pH 9.24, D.O. = 2.04mg/L, TAN = 0.77 (UIA=.324)
Water exchange done, increased bubble rate
Aquarium 2 and 3 – 28h after last check all suckers dead, FHM alive
pH 9.5 and 9.95, D.O. = 0.94 and 1.26mg/L, no TAN measured

30September 10:13 (~68h total time)
Aquarium 1 fish alive, 21˚C, pH 9.22, D.O. = 4.05mg/L, TAN = 1.3 (UIA=0.547)
Fish sampled for histology

Histology:
FHM (4) gill secondary lamellae epithelium with edema , kidney tubules normal, hepatocytes with distinct eosinophilic cytoplasmic droplets without any necrosis

LRS 1+: Three of 4 gill secondary lamellae epithelium with edema, proximal tubules with hyaline droplets (#3 score) with vacuolar degeneration (4 of 4), hepatocyte normal

LRS 0+: four of 4 gill secondary lamellae epithelium with protein-filled edema, proximal tubules with hyaline droplets (#2-3 score) with vacuolar degeneration (4 of 4), hepatocyte normal

Appendix 3. Reviewer comments:

R1 and 2 had no specific comments and agreed with conclusions.

> I think the conclusion is fine. Actually, pretty good to synthesize some of the earlier efforts in conjunction with the current studies.
>
> The discussion appears to be on solid Foott-ing, and I think enough data has been collected over the last three years to support your conclusions. I don't think it's a big shock that synergistic WQ-parasites-toxins may be accounting for low survival. However, assuming anybody reads it, they should be surprised that the Williamson River Delta is not a good place for young LRS looking for a meal. Who would have guessed?

R3: "don't know if you are still receiving comments on your end of year report, but I have read through it and made a very few comments. Overall, I was left with a lot of questions through the results and therefore the discussion as well. Mainly I would suggest more details about the analysis in the results. Which test was actually performed in each case, and what were the inputs and results (coefficients etc)? I realize that there is a ton of information here to present and that makes it difficult to present in the first place and subsequently revise with such generic comments given."

Reply: Agreed. Statistical tests were indicated for each statement using P-values.

R4 Review of Foott et al. 2014, Juvenile Lost River Sucker sentinel survival in Upper Klamath Lake mesocosom net pens (July-October 2013) and laboratory hypoxic challenge. FY2013 Technical Report, California Nevada Fish Health Center, Anderson , CA.

In this study, several interesting hypotheses about the cause of juvenile sucker mortality were explored. The feasibility of a variety of techniques for study of juvenile sucker health and rearing were demonstrated . The most convincing conclusion from the study is that within Upper Klamath Lake the period from August 12-26, 2013 was a particular challenge to age-1+ suckers. Tying this result to low dissolved oxygen is appropriate. Other conclusions stated throughout the paper, however, were based on small sample sizes and in some cases inconsistent results. These conclusions should be presented in such a way that their preliminary nature is very clear. Given the inconclusive results of most trials, the present study should be followed up with full studies committed to just one or two of the most promising hypotheses.

There are several conclusions stated throughout this report that are not supported by the data. These should be expressed with associated caveats and cautions. For example:

1) I don't think the data support the conclusion that the Williamson River Delta was a poor place to rear fish because of a lack of food. Fish held in the delta had lower triglycerides (based on a small sample size) than fish held at the North Lake site and in the hatchery. However, Williamson fish were held for 57 days compared to 14 days, and despite the evidence that fish in the enclosure were able to consume some food, I have a hard time believing that 1.6 mm mesh

didn't limit access to food. To make this conclusion I think you need to conduct multiple trials where you hold fish at both sites the same number of days and you need larger sample sizes at each site during each trial.
No definitive statement on food resources at this site can be ascribed to the observation of poor growth, low survival and body triglyceride content, and metacercarial infection in fry held from 29July to 3Sept. The intent of sentinel groups at this site was to evaluate whether if could support netpen culture. As survival of bi-weekly Williamson R. groups and overwinter fish was high, the site is certainly sufficient for the rearing of large fry or juveniles particularly if supplemental feeding occurs. Statements on Williamson R. were revised in the report.

2) I don't think you can make the statement "It would appear that microcystin was consumed by the fish over the entire summer without inducing liver or kidney damage". There is no data presented on microcysitn concentrations during exposures. Histology identified kidney damage in large portions of sentinel fish (although you have small sample sizes). There is no histological comparison to unexposed or exposed control fish. The statement appears to be made based on Table 9, where 2 fish in each exposure group were tested for immunoreactivity and no activity was detected in the liver or kidney. This is an interesting pilot level result but it is not conclusive.
The trend of MCLRa immunoreactivity in the intestine and not the liver or kidney has been seen in the last 3 years. In addition, liver abnormalities were not seen in the much larger histological sample (H&E stain) set of the north lake sentinels. Hyaline deposits observed in kidney tubules are the result of diuresis invoked largely by the alkaline conditions and common to experimental and lake samples.

3) The claim that PP2A was depressed in sentinel fish in mid to late August due to MC exposure appears to be based on comparison with 2 control fish and 2 fish gavaged with a low dose of MC. The gavaged fish appear to have been tested once 18 h after exposure and had increased PP2A levels not decreased levels. Therefore I am not sure what to make of this result. The criteria for depressed PP2A levels of a 50% reduction below control fish levels is conservative. The fact that this conservatively low level of PP2A was detected is interesting but it needs to be followed up with more research.
The statement of PP2A depression was based on the number of fish with 50% reduction levels. As we do not have an analytical method to determine MC concentration in sentinel liver, the data is presented as an observation of a sub-lethal response only.
4) The jump between the present study and bird predation seems unjustified.
Our sentinel studies have not demonstrated an overt and direct mortality to lake water quality in fry held in full-water column netpens. We hypothesize that predation during poor water quality periods could be an important factor in sucker recruitment failure.
5) The magnitude of the difference in mean weight between control fish and fish reared over sediment is interesting (Appendix 1). However several major problems prevent this from being a convincing result: 1) fish were mixed after the second week of the study, 2) you only had one control, one treatment, and one trial (no replicates).

The use of mud was performed to increase our chances of success in growing fry to sufficient size and health for a July deployment. It was not meant as a study. In appendix 1 we describe a substrate trial to address this question..

A few other comments:

The report appears to be written by multiple authors and there are inconstancies in some of the numbers (e.g. concentration of toxin in gavage), formatting, and writing style. Some editing could be done to make the report flow better among sections. The quality of writing throughout the report should match that of the first few paragraphs of the discussion.

Agreed – one author has performed the edits and moved sections into the main body of the report.

It would be helpful to have some background about previous studies in the introduction. Specifically, the logical progression that lead to the development of present hypotheses. It would help to know why you are doing each part of the study. For example, you could explain that PP2A is an indicator of MC exposure but is also elevated by cortisol. It would help to state the specific hypotheses here. The objectives do not do this, rather they are stated as a list of tasks. So for example, you might say that you hypothesize that fish exposed to microcystin by oral gavage in lab trials will have depressed PP2A, detectable immunoreactivity, and cell necrosis in digestive tract, kidney, and liver. You could cite previous studies that lead you to have that expectation.

Agreed - done

Sample sizes and error bars need to be included on all plots of means.

Addressed where applicable.

Appendix 2: I was disappointed by the lack of discussion about the trial described in appendix 2. The idea explored here is interesting and deserves more attention. It would be nice to see an introduction with some cited literature that explained what you were expecting to find with this study. While it appears that fish reared over Raw Mud grew less but survived better than fish reared over Autoclaved Mud or Sand, a different result might be obtained if more data were collected. More replicates in each treatment group and more fish per replicate would help this study. I am curious about the variation in mortality, weight, length, and condition factor among the replicates that you did have. Was there much difference between the two Autoclaved Mud replicates? Or the two Raw Mud replicates? I also wonder if it is worth running trials with and without feeding. Appendices 1 and 2 make a good pilot study that should be expanded on.

Our wetlab tank numbers in May limited the number of replicates. Our primary goal was to document the effect of substrate on the occurrence of gill hyperplasia and mortality however the mechanism of the gill abnormality is uncertain.

Appendices 3-5 read like notes and are difficult to follow. It is unclear to me what the goals were in each of these sub-studies. **Hypoxia trial 1- 3 were moved into the body of the report and edited.**