FY2000 Investigational Report:

Histological and Hematological Evaluation of Juvenile Lost River Suckers Exposed to Sub-lethal Levels of Ammonia

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Summary: As part of a 1999 water quality experiment, histological specimens of kidney, gill, liver, pancreatic tissue, and intestinal tract from 4 ammonia exposure treatment groups of juvenile Lost River Suckers were evaluated by light microscopy for abnormalities. Differential leukocyte counts were performed on blood smears from the same groups. The proximal convoluted tubules in the kidneys of fish exposed at the medium (0.217 ppm) and highest (0.440 ppm) ammonia levels contained varying amounts of hyaline droplets. The severity of hyaline deposition was correlated with ammonia concentration. Epithelial separation (edema) of the secondary gill lamellae was quite prevalent in the control ammonia group (0.006 ppm NH3-N) but not higher ammonia treatments. Both lesions were considered to be reversible and would not seriously affect the general health of the fish. No statistically significant trend in the Lymphocyte : Granulocyte ratio was detected among the blood smears of the treatment groups.

Introduction: The California - Nevada Fish Health Center (**FHC**) assisted the principal investigator, Elaine Snyder-Conn (Contaminant Specialist, Klamath Basin FWO), in a 1999 cooperative study on the effects of chronic,sub-lethal ammonia exposure on juvenile Lost River Suckers (*Deltistes luxatus*). Adverse water quality in Klamath Lake has been identified as one of several factors affecting this endangered species (Martin and Saiki 1999). Following the ammonia exposures, fish were challenged with the endemic bacterial pathogen *Flavobacterium columnare*. Other cooperators in the study included researchers with the University of Wyoming (H. Lease, J. Morris, J. Meyer, M. Suedkamp, and S. Clearwater – Dept of Zoology and Physiology) and Rich Holt (Oregon Dept. of Fish & Wildlife).

The FHC performed 3 functions for the study: 1) training and equipment for organosomatic analysis, bloodsmear preparation, and histological sampling; 2), differential leukocyte counts on blood smears and 3) histological evaluation of select tissues. Partial funding for supplies and technician time, totaling $5760, was provided to the FHC in FY99 for this study (project code 11450-1261-1N39). . Laboratory analysis and data summary required over 80 hours of staff time.

Methods:

Complete details of the study methods are documented in the principal investigator's final report (Snyder- Conn et al , pending). The five-month old Lost River Suckers (avg. length = 47.2 mm) were obtained from the Klamath Tribes Native Fish Hatchery in Chiloquin, Oregon. After a 3 week acclimation period to basal water quality (22- 23 °C, 50 mg / L hardness, 8.0 mg / L Dissolved Oxygen, pH 9.5) , replicate treatment groups of fish were exposed to 4 levels of ammonia (recorded as NH_3 – N) in flow-through aquaria:

Control	0.006 mg / L
Low	0.115 mg / L
Medium	0.217 mg / L
High	0.440 mg / L

After 62 days of exposure to the 4 treatment levels of ammonia, 6 fish from each aquarium were euthanized in MS-222 and immediately fixed in Davidson's fixative for 24 hours, transferred to 70 % ethanol, later processed for 5 Fm paraffin sections and stained with hematoxylin and eosin (Humason 1979). Specific tissues processed for examination included the liver, pancreatic tissue, intestinal tract, kidney, and gill. All

tissues for a given fish were placed on one slide and identified by a unique code number. Each slide was examined at both low (40X) and high magnification (400X) without knowledge of treatment group.

Specific lesions were given a qualitative rating of:

0	"normal"	< 10 % of the given tissue affected or only minor alteration
1	"mild"	~10 to 30 % of the given tissue affected
2	"severe"	> 30 % of the given tissue affected or significant alteration

Blood smears were prepared by severing the caudal peduncle of 2 additional fish (at 62 days of exposure) from each tank, air drying the smear for 10 minutes, fixing for 15 minutes in absolute methanol, and later staining with Leishman – Giemsa stains (Yasutake and Wales 1983). A differential leukocyte count was performed at 1000x magnification on the first 100 lymphocytes, thrombocytes, neutrophils, monocytes, and eosinophils / basophils observed on the smear. Leukocyte identification criteria was based on Hibiya (1982) and Witten et al. (1998). Because there is poor morphological distinction between the low percentage of rounded thrombocytes (majority are spindle shaped) and the more numerous lymphocytes, the combined number of lymphocytes + thrombocytes divided by the number of granulocytes (neutrophil and eosinophil / basophils) was used to derive a L{T}: G ratio. Higher granulocyte counts result in ***low*** L{T} : G ratio values and can indicate infection, tissue damage, or seasonal blood cell changes (Modra et al. 1998). The use of combine eosinophil / basophil count was based on the observation of Moritomo et al (1999) in that carp blood basophils can become eosinophilic during typical staining procedures.

Results / Discussion:

Histology – Two abnormalities, gill epithelium separation and hyaline droplets in kidney tubule cells, were observed in the study fish (Table 1). No abnormalities were observed in liver, intestine, or pancreatic tissue from any treatment group specimen. Approximately 10 % of each treatment group showed mild inflammation of the adipose tissue adjacent to the intestine. We have observed such inflammatory changes in juvenile chinook during their smolt migration in warm water conditions and speculate that it is related to membrane damage from lipolysis of the fat reserves. Separation of the gill lamellar epithelium was seen in 31 – 86 % of the various sample groups (Fig 1). There appeared to be an inverse relationship between ammonia concentration and severity of this gill lesion (Fig 2). The control (0.006 ppm NH3-N) fish demonstrated both the highest prevalence and severity of all the sample groups followed by the low (0.115 ppm) group. In addition to epithelium separation, epithelial cell swelling (mild hypertrophy?) was seen in 14 % of the affected control gills. Mucus discharge from goblet cells was commonly observed in gills from all the groups and may be a reaction to the initial contact with fixative. No hyperplastic tissue response, filamentous bacteria, parasites, or necrosis was observed in the gill sections.

Table 1. Prevalence and severity of gill and kidney abnormalities observed in histological specimens from suckers exposed to 4 levels of ammonia for 62 days. Recorded as number of samples with specific lesion / total samples (%). Bold format to highlight group most affected by lesion type.

NH_3-N	Gill Epithelium Separation			Kidney Tubule Hyaline Droplet		
Treatment	normal	mild	severe	normal	mild	severe
0.006 ppm	3 / 21 (14)	8 / 21 (36)	**10 / 21 (48)**	17 / 20 (85)	1 / 20 (5)	2 / 20 (10)
0.115 ppm	5 / 10 (50)	4 / 10 (40)	1 / 10 (10)	8 / 9 (89)	1 / 9 (11)	0 / 9 (0)
0.217 ppm	9 / 13 (69)	4 / 13 (31)	0 / 13 (0)	6 / 16 (38)	6 / 16 (38)	4 / 16 (24)
0.440 ppm	11 / 19 (58)	6 / 19 (32)	2 / 19 (10)	0 / 19 (0)	5 / 19 (26)	**14 / 19 (74)**

Gill lamellae are composed of a single epithelial cell layer covering the endothelial cells (pillar cell) separated by a basement membrane. Through this delicate structure, arterial blood exchanges waste products with (i.e. CO_2 , NH_3) and uptakes oxygen from the environmental water. It is reported that freshwater fish excrete the majority of their nitrogenous waste as un-ionized ammonia (NH_3) via diffusion across the gills in a fashion similar for other gases (Heisler 1984, Wilkie and Wood 1996). This exchange requires the presence of a sufficient NH_3 partial pressure difference between the blood (primarily in the NH_4 form) and the boundary layer of water directly next to the gill. Carbon dioxide diffusion from the blood into the boundary water layer becomes hydrated *at neutral pH* resulting in a proton "sink" to trap NH_3 as NH_4. A smaller portion of blood ammonia is also excreted in a Na^+ / NH_4^+ active exchange mechanism. In alkaline waters, ammonia excretion is inhibited as there are fewer protons to create the NH_3 sink and there is shift from NH_4 formation to NH_3 in the boundary water (The pK of the relationship $NH_4 \Leftrightarrow NH_3 + H$ is 9.3 at 23 °C). . Given the pH and temperature of the study's water quality, over a third of the total ammonia concentration would be in the form of NH_3 (Piper et al 1982). This situation could further inhibited diffusion of blood ammonia into the boundary water layer

The observed epithelial separation was likely a reversible condition resulting from localized edema of the gill lamellae. Increased permeability of arterial capillaries in the lamellae would cause an ultrafiltrate to leak into the space between the blood vessel and the overlying epithelium. This lesion has been reported to rapidly occur after exposure to chemical irritants and can also resolve back to a normal state in a matter of days (Roberts 1989, Ferguson 1989).

It is not clear why the low ammonia (control) treatment groups experienced lamellar edema. The relatively soft water used (50 mg / L Hardness) of the challenge water may not have supplied enough calcium ions for adequate regulation of membrane permeability in gills under ion stress (Roberts 1989). Iwama et al. (1997) reported that ammonia excretion was significantly reduced in Lahontan trout (a species adapted to alkaline waters) exposed to alkaline (pH 9.4) water if either Ca^{++} or Mg^{++} ion concentrations were low. As stated above, the high pH (9.5) of the challenge water would result in decreased ammonia excretion from the gills and could possibly affect other ion balances. The "elevated" ammonia treatments appear to have a moderating

effect on this gill response. It would be beneficial to consult with an expert in this field of ammonia / ion regulation in fish from alkaline waters. It should be noted that there was no observation of lamellar hyperplasia that has been reported in salmonids exposed to high ammonia levels of 1.2 – 1.6 ppm NH_3-N (Smith & Piper 1975).

Figure 1. Photomicrograph of control (0.006 ppm NH_3-N) gill section showing separation of epithelium due to presumed edema (100x magnification).

Figure 2. Prevalence and severity of gill epithelial separation from suckers exposed to 4 treatment levels of ammonia.

Hyaline droplets, in the epithelial cells of the proximal convoluted tubule segment, were observed to varying degrees in all treatment groups (Fig 3 and 4). Two fish from the 0.440 ppm treatment showed hydropic degeneration of the affected tubules. No inflammation or glomerular abnormalities were seen in the affected kidneys. This material was probably excess protein from the filtrate due to increase urine production (Cotran et al 1989). Elevated ammonia levels are reported to increase urine output in salmonids through its stimulation of the renin-angiotensin system (Attilio et al. 1981). It is unlikely that this lesion would significantly impair the health of the test fish except for a few of the 0.440 ppm fish. Similar to the gill epithelia lesion, the hyaline droplet occurrence would rapidly resolve to a normal state given better water quality.

Of the 7 moribund fish fixed for histology, successful sagittal sections were prepared from only 3 such fish. One specimen (labeled 24OCT 19 1 Early) showed extensive peritonitis while the other two showed only separation of gill epithelium (labeled T27 F01 11-2-99 15:54 and T14 F02 11-14-99).

Differential leukocyte counts - Extensive microscopic searches were necessary to count 100 leukocytes indicating low numbers in the blood. This occurrence is often seen in salmonid fry and a similar age factor could occur with the sucker fry. The Lymphocyte {thrombocyte} : granulocyte ratios were highly variable (coefficient of variation 69 – 135 %) and failed criteria for normal distribution (Table 2). A Kruskal-Wallis ANOVA on ranks did not detect a significance difference between the 0.06, 0.217, and 0.440 ppm NH_3-N treatment groups (P= 0.315), however, both the control (0.06 ppm) and the high ammonia (0.44ppm) fish tended to have higher numbers of circulating granulocytes (lower L:G ratio) than the 0.217 ppm fish (Fig. 5). Shifts in the L : G ratio have been shown to occur in numerous fish species upon exposure to toxicants (Modra et al 1998). Another explaination may be the host response to tissue damage in the control (gill) and high ammonia treatment (kidney) fish.

Table 2. Mean and median Lymphocyte {thrombocyte} : Granulocyte ratios

	Control 0.006 ppm	*Low 0.217 ppm*	*High 0.440 ppm*
mean	29.8	41.6	24.4
median	11.5	36.5	19.0
no.	9	6	13

Conclusions – No significant tissue lesion indicative of *severe* health impairment was observed in the histological specimens from any treatment group. Control treatment fish experienced gill edema due to a presumed change in membrane permeability. Elevated ammonia levels were associated with hyaline deposition in the proximal convoluted tubules of the kidney (presumably protein). No statistically significant trend was detected in leukocyte counts between the control, low, and high treatment groups. These results correlate with Martin and Saiki (1999) assertion that overt fish mortality was not consistently linked to elevated un-ionized ammonia levels in Klamath Lake but was linked to episodes where dissolved oxygen concentrations were below 1.05 mg / L.

Figure 3. Photomicrograph of hyaline droplets within the proximal convoluted tubule segment of a fish in the 0.440 ppm NH_3-N treatment group (magnification 150X).

Figure 4. Prevalence and severity of hyaline deposition within the proximal convoluted tubules in kidneys from suckers exposed to 4 treatment levels of ammonia.

Figure 5. Lymphocyte : Granulocyte ratio (mean + SEM bar) from blood smears of suckers exposed to 0.006, 0.217, and 0.440 ppm of NH_3-N.

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