10 January 2008

Tami Gingrich
Geauga park District
9160 Robinson Road
Chardon, OH 44024

Dear Tami,

Enclosed is the final report for "Metal Toxicity and Population Declines in *Rana sylvatica* and the *Ambystoma jeffersonianum* Complex in NE Ohio" now under a different title "Effects of Metals and High Water Hardness at Higher pHs on *Rana sylvatica* and *Ambystoma jeffersonianum*." This report will be modified by the end of spring (I hope) to include some additional literature citation information to get it ready for submission for publication. You requested a hard copy and a digital copy, both are enclosed. The statistical analyses proved to much more challenging than anticipated; consequently the delays. If you have questions please contact me at the Museum. Thanks for the funding for the project. Without the funding from the Geauga Park District and a Cleveland foundation the project would not have been undertaken. As you work your way through the report you will find information that can/should be applied to some parks areas for management and/or future development if amphibians and especially the Jefferson Salamander complex is considered.

Sincerely,

Timothy O. Matson, Ph.D.
Curator of Vertebrate Zoology

FIELD STUDY METHODS AND RESULTS: In addition to collecting egg masses of both Wood Frogs and Jefferson Salamanders for the laboratory study, Pond 2 (low elevation pond off White Oak Trail near Big Creek) was trapped for salamanders. This pond was used as one of 11 transplant ponds during 2006. Egg masses of both Wood Frogs and Jefferson Salamanders were transplanted in separate containers to test survivorship in the ponds and to correlate survivorship with metal concentrations in the pond. During 2006 there was 100% mortality of embryos or early stage larvae of both species. No eggs masses of Wood frogs were observed in the pond; however, and adult was observed in the pond in early March. Numerous egg masses of Jefferson Salamanders were observed but there was very high early mortality or high infertility observed. No Spotted Salamander eggs were noted in the pond. In June however, both Wood Frog tadpoles and Jefferson Salamander larvae were observed and presumably some of each species transformed later in the summer. Since this pond was intended to be used, but was not, as a source pond for eggs in 2007 and had very high mortality within the egg, it was necessary to determine if some of the Jefferson Salamanders using the pond were polyploids; consequently, the trapping study during 2007.

Results of the trapping conducted 21-23 March 2007:

21 March	Set 4 traps, some *A. jeffersonianum* egg masses werepresent	
	Ice covered except for the margin	
22 March	Trapped 7 *A. jeffersonianum*	5 Female, 2 Male [2 F were triploid]
	All were typical and blue-black	
	Took blood samples for microscopy; also took tail tissue sample for future genetics study	
	A. jeffersonianum egg masses abundant; collected some eggs for laboratory study	
23 March	Trapped 2 *A. jeffersonianum*	1 Female, 1 Male [F was triploid]
	Female was blue-black w/brown tail	
	Trapped *A. maculatum*	1 Male

Results of Pond 1 (near parking lot for White Oak Trail) and Pond 3 (off Hemlock Trail) searches; trapping was not conducted.

Pond1 21 March	Many *A. jeffersonianum* egg masses
	Observed several *Rana clamitans* tadpoles
	No *Rana sylvatica* eggs
23 March	5 *Rana sylvatica* calling
	Collected some *A. jeffersonianum* eggs for laboratory study
	Collected water sample for metals testing
Pond 3 23 March	Saw 3 *A. jeffersonianum* , including 1 gravid female
	A. jeffersonianum eggs masses were abundant; collected some for laboratory study
	Water clear; pond remained one fourth ice covered.

Collected 8 *Rana sylvatica* egg masses and numerous *A. jeffersonianum* n 26 March from the powerline cut west of the Robinson Road maintenance complex.

Repeat of 2006 transplant study at Pone 2.

Six stakes were placed in the pond on 21 March as eff container attachment sites
Placed eggs of Wood Frogs and Jefferson Salamanders into the pone on 31 March.
On 31 March there were about 20 naturally laid Wood Frog egg masses in the pond; I noted 4 small Spotted Salamander egg masses; numerous Jefferson Salamander egg masses, many were dead or had numerous dead eggs within the mass.
Water levels had already dropped at least 75 mm over 10 days

22 April—Many transplant eggs had hatched and were transferred to 5 gallon containers.

Mortality was very high but not 100%
Many of the Wood Frog eggs laid in the pond had hatched, but the number of hatchlings seemed greatly lower than expected based upon the number of egg masses laid. Spotted Salamander eggs were developing normally. Jefferson Salamander egg masses with apparently viable eggs showed some deformities but many normal embryos.

21 May—Some containers showed no evidence of Wood Frog tadpoles; others had only dead tadpoles; I assume all died and deteriorated since 22 April. Transplant Jefferson Salamander larvae died in their containers; some containers were only half submerged due to drought. Some (few) naturally occurring Wood Frog tadpoles and numerous Jefferson Salamander larvae were free swimming in the pond; the volume was probably reduced by 60% at this time.

14 June—Wood Frog and Jefferson Salamander larvae were common and very concentrated in the remaining small pool of water. No Spotted Salamander larvae were observed (probably due to extremely high rate of predation). Jefferson Salamanders were present in several very distinct size cohorts. I have observed great size differences several times previously in NE Ohio Jefferson Salamander breeding ponds. Normal sized larvae were common but a small percentage of the larvae were larger, had huge heads, and had a body shape similar to that of small bullheads. This “size morph” is probably not genetically based as a distinct morph, but some individuals are probably genetically predisposed or have the plasticity to develop the large head and body when sensing that their natal pond is drying very quickly. I am unaware of other accounts describing these large, large-haded larvae. Several larvae of both size cohorts were collected and preserved (1 large headed larva and 2 normal larvae were retained alive to watch their growth and development). Blood samples were taken from 3 large headed larvae and 4 normal larvae; all were diploids. One large headed larva was observed in the pond off Hemlock Trail in 2006.

26 June—Pond dry (during 2006 this pond had nearly 2 feet of water in it at this time). Several Wood Frog metamorphs were in the pond basin; no salamander metamorphs were detected.

Effects of Metals and High Water Hardness at Higher pHs
on *Rana sylvatica* and *Ambystoma jeffersonianum*

The purpose of this project was to investigate the extent and severity of the problem of metal toxicity in NE Ohio on two taxa of amphibians, the Jefferson Salamander and the Wood Frog. The effects of low pH on eggs and larvae of certain species of amphibians have been documented (Albers & Prouty 1987; Clark 1986; Clark and LaZerte 1987; Clark and Hall 1985; Freda and Dunson 1986). However, the effects of low pH are often confounded by the toxicity and ontogenetic ameliorative effect of metals in pond water in which embryos and larvae develop (Freda 1991; Flemming and Trevors 1989; Sadinski and Dunson 1992).

Horne and Dunson (1994, 1995a, 1995b) collected egg masses of Jefferson Salamanders from breeding ponds in central Pennsylvania and tested metal toxicity on embryos and larvae in both the field and laboratory. Most of their field testing was conducted at pH < 6 and their extensive laboratory testing was conducted in microcosms at pH 4.5 and 5.5. On the Erie Lake Plain Section and Portage Escarpment many seasonal ponds or semi-permanent wetlands used by or potentially used by Jefferson Salamanders and Wood Frogs vary between pH 6 and 7.5. At low pH [metals], especially those in free soluble states, are higher while at higher pH more cations are complexed through chelation and may no longer be in toxic form (Fred 1991; Horne and Dunson 1994 and 1995b). However, Al and Cu were found to be extremely toxic to larvae of both taxa in individual metal toxicity tests at pH 4.5 and 5.5 in acute (7 day) and chronic (28 day) exposures. Jefferson Salamanders and Wood Frogs experienced higher larval mortality at higher pH (5.5) with low hardness when subjected to [Al] = 525 μg/L. There was complete mortality of Jefferson Salamanders at both pH 4.5 and 5.5 in high and low hardness (4300 and 2200 μg/L) in chronic tests with [Cu] = 15 μg/L whereas Wood Frog mortality was highest at pH 5.5 with high hardness. They suggest that Cu and Al in breeding ponds may be more toxic at higher pH. If their hypothesis is correct, then the chemical environment in some seasonal and semi-permanent wetlands on the Lake Erie Plain and Portage Escarpment could exclude Jefferson Salamanders and Wood Frogs from using them for recruitment. A toxic environment could be present even though the soluble [Cu] and [Al] may be lower because of complexed ions due to chelation by organic acids.

In a 10 year amphibian drift-fence study conducted at a seasonal pond in the Portage Escarpment, the transitional belt between the Erie Lake Plain Section and the Glaciated Allegheny Plateaus Section (Brockman 2002), Matson and Quinn (to be submitted for publication in 2008) found a steep downward trend in population size of the Jefferson Salamander. The apparent trend was substantiated by Jolly-Seber population estimates. The Jefferson Salamander population declined to the level where during the final two years of the study only two and one males, respectively, migrated to the breeding pond. We consider the local population reproductively extirpated. We believe there to be three causes for the population decline (which also occurred in the Spotted Salamander, *A. maculatum,* but not to the same degree); drought, predation from the Marbled Salamander, *A. opacum*, and possibly most importantly toxic levels of total Cu, [Cu] = 20 μg/L at pH 7.0 and hardness 307,000 μg/L.

Wood Frogs were planted in upland mitigation ponds created in 2001 at Mentor Marsh State Nature Preserve which is positioned near Lake Erie on the Lake Erie Plain. Reintroduction of Wood Frogs began in 2002 with translocation of egg masses and has occurred each of the four years 2002-2005. A calling male population was established by 2003, it increased in size in 2004, but seemed to have declined slightly in 2005. Of many 1000s of eggs planted into 10 ponds in 2005 no tadpoles were netted during monthly monitoring after early May. During 2002-2003 mature tadpoles were very numerous and apparent in June and metamorphs were abundant in July, but an apparent decline in tadpole survival was noted during monitoring in 2004. Water aliquots collected from three mitigation ponds on 29 March 2005 revealed total [Al] from 100-400 μg/L and [Cu] from 30-80 μg/L with pH from 6.66-6.87 and hardness from

61,000-100,000 μg/L. Although no published studies address the toxicity of Cu at these pH and hardness levels our hypothesis was that highly toxic levels of Cu and/or Al had accumulated to cause mortality of either embryos or larvae of the Wood Frog.

Aliquots of pond water from five other seasonal or semi-permanent ponds were tested for metals in 2005 to determine if the problems with metals at the drift-fence site and at Mentor Marsh were more widespread in NE Ohio. One seasonal pond on the Lake Erie Plain, one on the Portage Escarpment, and three on the terraces of the ancient Grand River Lake in the Glaciated Allegheny Plateaus were included in the testing. The pond on the Erie Lake Plain lies within the Ashtabula River drainage system and is the only known drainage basin site for the occurrence of the Jefferson Salamander (Matson and Quinn, nine year survey). Few larvae were detected in 2000 when the population was first located; 12 larvae were netted in 2003; no adults were trapped, no egg masses were oviposited, and no larvae were netted in 2005. Water chemistry tests on water withdrawn on 12 April revealed total [Cu] = 20 μg/L with pH 6.88 and hardness 170,00 μg/L. Although more trapping and monitoring are required this local population appears on the verge of extirpation and the biodiversity of the Ashtabula River watershed will be diminished.

The Portage Escarpment pond, located within a Cleveland Museum of Natural History natural area, supports a small Jefferson Salamander Complex population, the only known location for the taxon within the Conneaut Creek drainage system (Matson et al., 2004). Only two egg masses were observed in 2005 along with about 60 of *A. maculatum*. Water chemistry testing on 14 April 2005 revealed total [Cu] =30 μg/L with pH 7.21 and hardness 255,000 μg/L.

Although not conclusive, these examples indicate that a problem affecting Jefferson Salamanders and Wood Frogs, which also have ceased recruiting from the ponds, exists and the suspected agent may be toxic levels of Cu, Al and/or other cations. The apparent problem and extent of the problem needed to be addressed through expanded study.

Previous cation toxicity research conducted in Central Pennsylvania, in other eastern states, and by the Cleveland Museum of Natural History in NE Ohio prompted the Museum to conduct an extensive toxic metal/amphibian project during 2006. Two pond water aliquots were collected three times from mid-March through mid-June from 30 ponds in NE Ohio. Eleven ponds were selected for egg transplant studies, included were several of the ponds described above, three ponds at Mentor Marsh State Nature Preserve, one pond at Big Creek Park, the Indian Point Metropark drift-fence pond, and ponds in each the Ashtabula River and Conneaut Creek watersheds where the Jefferson Salamander complex occurs. Results of the pond water analyses were used to establish low and high concentrations for the cations studied. Mortality data are being analyzed and will be correlated with water chemistry data; however, mortality data for both the Wood Frog and Jefferson Salamander complex were very high. Mortality was complete (100%) in some ponds and very high in nearly all other transplant ponds. Metal concentrations, pH, and other water quality variables studied will be compared between transplant ponds (11 ponds) and others in the study (19), between ponds in the study known to support or were found to support Wood Frogs and/or Jefferson Salamanders during the study, and those that were not found to support these taxa. The need for additional research during 2007 involving metal cations, pH, and hardness became apparent to help explain the results of the 2006 study.

Objectives included 1) collection of egg masses of the Jefferson Salamander and Wood Frog from source ponds; 2) rearing known quantities of eggs and larvae of each taxon in treatments of known metal concentrations in the laboratory; 3) temporal collection of mortality/survivorship data; 4) chemical analyses of [metal] in treatment water prior to planting of eggs and at the end of the experiment; 5) statistical comparison of mortality/survivorship between treatments and within treatments.

Methods: All treatments were conducted in artificial soft water (ASW) which included 1 mg/L of each of the following cations: Na, K, Ca, and Mg (Freda and Dunson 1986; Horne and Dunson 1994, 1995a).

Egg masses of each taxon were obtained from 2-3 source ponds in northeastern Ohio soon after oviposition, placed in ASW and transported to the laboratory. Ponds from which egg masses were collected were located in Grand River Terraces Preserve, Ashtabula County; the Ravenna Arsenal, Portage County; and Big Creek Park, Geauga County. Source ponds for Jefferson Salamander eggs included the Ravenna Arsenal and Big Creek Park, whereas source ponds for Wood Frog egg masses were located in the Grand River Terraces Preserve and Big Creek Park. Conspecific egg masses from several source ponds were mixed and randomly assigned to treatments. The stage of embryonic development was determined prior to placement (for *Ambystoma*, Rugh 1962; for *Rana*, Gosner 1960). Wood Frog eggs were all less than 24 h old in Gosner stages 2-4, whereas Jefferson Salamanders were several days old and in Rugh stages 5-9. Entire Jefferson Salamander egg masses or partial masses of 20 eggs or less were assigned to each treatment replicate. Wood Frog egg masses because of their size and large number of eggs were divided into subsets of less than 20 per replicate. Egg masses were incubated in 3 L glass jars containing 2 L of treatment water. Four replicates of each treatment were used in each of 47 treatments for a total of 188 replicates. The project was conducted in the Case Western Reserve University greenhouse at Squire Valleevue Farm. The experiment was conducted without shade screen during the first half of the study, but it was added later to help reduce the room temperature. Greenhouse ridge vents were kept open much of the time to cool the room. Treatment water temperatures varied between 11_C and 21.4_C and were reflective of higher than normal April temperatures with above average clear skies. The photoperiod was natural and increased over the course of the project.

Embryonic development was inspected daily after transplanting and mortality was quantified when all embryos had either hatched or died. Survivorship was quantified by counting the number of embryos successfully hatching. When eggs of many Wood Frog and Jefferson Salamander treatments had hatched the contents of the 2 L jars were transferred to larger 12 L polypropylene rearing containers having the same treatment. Consequently, the larger container housed dead eggs, eggs with slower developing deformed embryos, and hatchlings. Hatching of Wood Frogs occurred very synchronously within about 24 h; contrastingly, Jefferson Salamander embryos developed at very different rates, even within replicates of the same treatment. Salamander embryos that developed slowly often expressed deformities. Tadpoles were fed algae while frozen brine shrimp, frozen *Daphnia*, and live *Daphnia* were fed to salamander larvae every day. Larval mortality at 7 days (acute) and 28 days (chronic) post hatch was determined by counting surviving larvae. Larvae surviving to the end of the experiment were sacrificed, weighed, placed in a drying oven at 60_C for 24 hours, and then weighed immediately to obtain dry mass. The dry mass was then divided by the number of surviving tadpoles to obtain the mean dry mass. Surviving Jefferson Salamander larvae were dried weighed but were not used for analysis because many replicates had complete mortality and some treatments could not be compared.

Table 1. Concentration of cations used in laboratory treatments. Combination treatments were additive of either low or high concentrations of all cations (μg/L).

Level	Hardness	Al	Cu	Fe	Zn	
Low	25,000	100	10	300	20	
High	120,000		400	20	1200	60

Aliquots of treatment water were collected in plastic bottles containing no preservatives and were transported to a commercial lab for chemical analyses to assure that treatment concentrations were as specified. Metals tested include; Al, Ca, Cu, Fe, Mg, and Zn. Hardness was determined through addition of Ca and Mg, i.e. ([$CaCl_2$] + [$MgCl_2$]) in a ratio of 3:1, respectively. Water samples were placed in a chilled cooler and transported to the laboratory within 12 hours where they were filtered through 0.45 μm filter, preserved with nitric acid, and stored until analyzed. Cation concentrations reflected values the 2006 study of 30 ponds in NE Ohio. Treatments were prepared by adding all metals as chlorides.

Mortality data percentages were square root transformed and were tested for normality using the Shapiro-Wilk test. Several other data transformations were made to normalize the data without success. Square-root transformation of mortality data sets was used for all comparisons except for dry mass. Results were transformed back to percentages for presentation. Kruskal-Wallis nonparametric analysis of variance was used to detect differences between distributions. The Comparison of Mean Ranks (CMR) was used to control experiment-wise error. Dry mass data were not transformed; the data violated the assumption of normality and Kruskal-Wallis nonparametric analysis of variance with CMR was used for analyses.

Results

Placing Jefferson Salamanders into ASW resulted in high mortality (69.0%) with low variance within repetitions, whereas Wood Frogs mortality was much lower 7.7% and the variance was large (Table 2). Jefferson Salamanders suffered high mortality in 46 of 47 treatments. Many eggs failed to hatch and were either not fertilized, died in early developmental stages, or developed but could not hatch. Hatching was highly asynchronous and extended over several weeks. Some embryos were deformed and died within the egg whereas others hatched and the larvae were deformed and/or often stunted in growth. Apparently unable to forage effectively, they often died or were consumed by larvae or tadpoles.

Table 1. Percent mortality of *Ambystoma jeffersonianum* and *Rana sylvatica* in embryonic and larval stage through 28 days post-hatch in ASW controls and ASW adjusted to two levels of pH and two levels of hardness. Mean mortality and one standard deviation are presented. Dry mass (g) per tadpole of *R. sylvatica* tadpoles 28 days post-hatch is presented.

Nearly all Wood Frog eggs hatched and little abnormal embryonic development was observed (mean hatching success over 47 treatments was 96.3%). Hatching was synchronized within 24h. Growth rates of tadpoles varied between treatments; tadpoles in many treatments grew rapidly, but in some treatments, the growth rates were lower. In several treatments (e.g., high concentrations of all cations combined at pH 7.5 with high hardness) the growth rate was low initially, but after 10-14 days the rate increased substantially so that by the end of the experiment their dry mass per tadpole was similar to that of those that seemed to exhibit normal growth. A low percentage of tadpoles developed a distinct bend or kink near the proximal end of the tail while others developed a sigmoid curvature.

Ambystoma jeffersonianum Adjusting the pH of ASW to 6.5 or 7.5 had no effect on mortality ($p = 0.874$; Table 2). Mortality was high in all three treatments. Adding low hardness increased mortality at both levels of pH. The addition of low hardness to pH 6.5 water produced a 30% increase in mortality ($p = 0.020$) but the comparison of mean ranks indicated no significant difference. Addition of low hardness to pH 7.5 water produced a small increase in mortality over similar water without hardness ($p > 0.05$). Water at pH 6.5 with high hardness caused 100% mortality in each of the four replicates, significantly above ASW without added hardness ($p = 0.014$) but not different from the addition of low hardness to pH 6.5 water ($p = 0.131$). The mortality of pH 7.5 ASW with high hardness was higher but not significantly different from that with low hardness or from ASW without added hardness ($p = 0.832$) or from water at pH
6.5 with high hardness ($p = 0.047$; however, CMR indicated no difference between means). Apparently adjusting the pH of ASW to 6.5 or 7.5 did not alter mortality of Jefferson Salamanders, however addition of hardness elevated mortality at the both levels tested. The addition of low hardness caused greater mortality than did the addition of higher hardness suggesting hardness above a specific level does not elevate mortality but may instead cause it to level off or decline slightly.

Kruskal-Wallis analyses of all metals in separate treatments and in combination treatments at pH 6.5 with low hardness indicated significant differences ($p = 0.045$; Table 3). Pair-wise comparisons found that low [Al] caused a decrease in mortality (96.3 to 66.1%, p = 0.020), high [Al] caused a decrease (96.3 to 57.8%; p = 0.020), and low [Fe] caused a decrease (96.3 to 75.0%; p = 0.020); however, CMR of these

Table 2. Percent mortality of *Ambystoma jeffersonianum* and *Rana sylvatica* in embryonic and larval stage through 28 days post-hatch in ASW controls and ASW adjusted to two levels of pH and two levels of hardness. Mean mortality and one standard deviation are presented. Dry mass (g) per tadpole of *R. sylvatica* tadpoles 28 days post-hatch is presented.

	Ambystoma jeffersonianum		*Rana sylvatica* (mortality)		*Rana sylvatica* (dry mass)	
Treatment	Mean	SD	Mean	SD	Mean	SD
ASW	69.0	0.339	7.7	4.053	0.0156	0.0021
ASW @pH 6.5	66.1	2.044	1.1	4.412	0.0109	0.0021
ASW @pH 7.5	69.0	4.685	3.2	5.121	0.0134	0.0023
ASW @pH 6.5 w/Low Hardness	96.3	0.479	6.3	0.041	0.0108	0.0029
ASW @pH 7.5 w/Low Hardness	76.8	0.700	2.1	3.025	0.0156	0.0025
ASW @pH 6.5 w/High Hardness	100.0	0.000	15.2	3.222	0.0149	0.0043
ASW @pH 7.5 w/High Hardness	84.9	0.506	2.4	3.336	0.0134	0.0090

cations were similar and the differences were not significant. Both low and high concentrations of combined cation treatments caused declines in mortality, but the declines were not significant (p = 0.196). In general, high Al ameliorated toxic effects of low hardness (96.3%) more than the lower concentration (57.8 vs 66.1% mortality, respectively); the [Cu] had no effect on mortality, and low [Fe] and [Zn] decreased mortality more than higher concentrations. Low [Zn] lowered mortality more than any other treatment at low pH with low hardness but the within treatment variance was very large.

No significant differences in mortality were detected in either concentration of any cations in treatments conducted at pH 7.5 with low hardness (p = 0.101). Low [Al] caused the greatest increase in mortality (76.8 to 97.7%) while other cation concentrations had minimal effects.

At pH 6.5 and high hardness only the low concentration combined cation treatment significantly lowered mortality. The cations reduced mortality from 100 to 48.9% (p = 0.013). The only other cation to reduce mortality below 80% was the high concentration of combined cations (p = 0.047), but the reduction was not significant (CMR were similar).

At pH 7.5 and high hardness only the high [Zn] had a significant moderating effect upon mortality by reducing it from 84.9 to 44.9% (p = 0.021). Both combined cation treatments reduced the mean mortality to levels similar to those of Zn, but they both had high variance between treatments whereas the variance found in high Zn treatments was a factor of 10 lower. Low [Al] elevated mortality to 100%, but the increase was not statistically significant (p = 0.047; CMR were similar); however since mortality was complete in all four replicates there may be some biological significance attributable to Al.

Rana sylvatica Adjusting the pH of ASW to 6.5 and 7.5 had no significant effects on Wood Frog mortality (p = 0.515; Table 2). The addition of hardness to pH adjusted ASW produced variable results. Mortality varied directly with hardness at pH 6.5, but the difference was not significant (p = 0.170). In contrast, addition of hardness to pH 7.5 water had no apparent effect upon Wood Frog mortality (p = 0.962). Mortality was greater at pH 6.5 than at pH 7.5 at both levels of hardness (p = 0.559 and p = 0.245, respectively), but the differences were not significant.

Analyses of single metals and combined cation treatment at pH 6.5 with low hardness (Table 4) detected significant increases in mortality at low [Zn] (p = 0.019), and high Fe (p = 0.020); however CMR of both cations indicated similar means. In comparisons of cations at pH 7.5 and low hardness only the mortality at low concentrations of combined cations was significant (p = 0.042); however CMR indicated similar mean ranks.

None of the treatments with high hardness at either pH 6.5 and 7.5 had significant differences in mortality.

Although *A. jeffersonianum* alive at the end of the experiment were dried and weighed, comparisons of mean mass per larva were not made because of low to no survivorship between treatments; consequently, only dry mass data for Wood Frogs are presented. Adjusting the pH of ASW caused a decline in mass (p = 0.037), but only the decline in the pH 6.5 treatment was significant (0.0156 to 0.0109; p = 0.021; Table 2). The addition of low hardness to pH 6.5 and pH 7.5 water produced no change in mass (p = 0.058); although tadpole mass was greater at pH 7.5 (0.0108 to 0.0156). Addition of high hardness to pH adjusted ASW had no effect on tadpole mass (p = 0.967). Comparisons of dry mass between hardness levels at pH 6.5 (p = 0.234) and pH 7.5 (p= 0.285) were not significant.

All cations added to pH 6.5 low hardness treatments increased dry tadpole mass (Table 5). Significant increases in mass occurred in low [Fe] (p = 0.043), high [Cu] (p = 0.020), and high [Fe] (p = 0.021), and low all cations combination (p = 0.043).

Cations added singly or in combination to low hardness pH 7.5 treatments (p = 0.263), high hardness pH 6.5 treatments (p = 0.069), or high hardness pH 7.5 treatments (p = 0.066) did not yield significant differences in tadpole dry mass.

Comparison of mass between all cations added singly or in combination over 10 treatments at low hardness at both pH 6.5 and pH 7.5 were not significant (p = 0.679). Similar comparisons at high hardness indicated that cations caused significant differences in mass between the two pHs (p = 0.050) with that at pH 7.5 being larger (0.020 > 0.0180). In paired treatment comparisons tadpoles at pH 7.5 in high hardness were larger than those at pH 6.5 in high [Cu] (0.0153 > 0.0116; p = 0.043).

Comparisons of treatments between hardness levels at pH 6.5 were not significant (p = 0.799). Similar comparisons at pH 7.5 were significant (p = 0.003). Tadpoles in Low levels of Zn indicated that tadpoles were much larger in high hardness (p = 0.027). Tadpoles in high levels of Al also grew larger in high hardness water (p = 0.029).

Discussion

The degree of mortality in Jefferson Salamanders in ASW and in pH adjusted ASW was above expectations. The disparity in the degree of mortality within these treatments between Jefferson Salamanders and Wood Frogs is great and may reflect taxonomic distance or biological incompatibilities of Jefferson Salamanders to adjust to the medium, even though it has been successfully used by Freda and Dunson(1986) and Horne and Dunson (1994, 1995a, 1995b). All Jefferson Salamanders egg masses were collected in Ohio and the possibility of obtaining eggs from polyploids is present. Egg masses were obtained from multiple pools, some of which are now known to support diploid and triploid populations.

Many Jefferson Salamanders egg masses were collected from ponds on the Ravenna Arsenal which is not known to support polyploids. Mixing and random selection of masses for treatment repetitions would tend to minimize and distribute the effects between treatments and may contribute to the observed variance within some treatments.

In field studies of 30 ponds in northeastern Ohio conducted during 2006 (to be reported elsewhere) wide ranges of all of the chemical parameters studied here were detected. Transplant studies with Jefferson Salamander and Wood Frog eggs in a subset of 11 ponds often suffered high to complete mortality. Local populations of these two taxa occupy habitats in northeastern Ohio with diverse environmental chemical composition; consequently they have adapted to those local environments. Placement of eggs adapted to very different chemical environments into ASW may account, at least in part, for the high mortality evident in Jefferson Salamander eggs placed in treatments of ASW, pH adjusted ASW, and presumably accounts for 66-69 of the mortality observed in all replicates of each treatment.

Hardness elevated mortality in Jefferson Salamanders; addition of most single cations or in combination ameliorated mortality to varying degrees. The addition of low hardness to pH 6.5 water resulted in nearly complete mortality which was far higher than in other studies (Freda 1991; Horne and Dunson 1994, 1995a, 1995b). At higher pH (7.5) the addition of low hardness caused lower mortality than at pH 6.5. High hardness at higher pH also increased mortality but less so than at lower pH. Jefferson Salamanders apparently are very sensitive to Ca and/or Mg ions at high non-lethal pHs. Low hardness here is much harder than that used or reported in most other studies.

The effects of hardness on Wood Frogs were similar to those of Jefferson Salamanders, but the level of mortality was much lower. Adding low hardness at pH 6.5 decreased mortality compared to that in ASW; high hardness also decreased mortality but to a lesser degree. High hardness at pH 6.5 elevated mortality in Wood Frogs more than any single cation. In contrast, adding low or high hardness to pH 7.5 had no effect on mortality. Freda and Dunson (1985) found Wood Frogs embryos to be very sensitive to Ca ions at non-lethal low pHs. At higher pH Wood Frogs appeared less sensitive to increased hardness, especially at pH 7.5.

Water hardness was found to ameliorate the effects of low pH in *A. maculatum* and *R. pipiens* but to increase acid toxicity in Wood Frogs (Freda, 1991). Calcium elevates the degree of curling at low pHs in these species but Wood Frogs cannot escape the vitelline membrane in order to hatch and died whereas *A. maculatum* and *R. pipiens* managed to hatch (Freda, 1991). Curling did not appear to be a problem with Wood Frogs in this study at higher pH; however, some curling, scoliosis, and other deformities were common even at higher pHs in Jefferson Salamanders Based upon these results Freda (1991) suggested that acidic ponds with higher hardness may support more successful populations of some species of amphibians than similar ponds with low hardness. Beattie and Tyler-Jones (1992) found that adding lime to ponds increased fertilization and survival of *R. temporaria* embryos; they suggest that at the pH levels (4.5 to 6.0) and hardness levels (627—28,600 μg/L) they tested that liming can effectively increase survival of amphibian embryos in some acidic ponds. They also indicated that liming could have negative effects on other life stages or upon other species. Adding Ca and Mg in the current study had minimal negative effects upon Wood Frogs embryonic or larval development except at pH 6.5 with high hardness (120,000 μg/L), a factor of four greater that added by Beattie and Tyler-Jones (1992). However, Jefferson Salamanders were adversely effected by adding hardness, especially at pH 6.5 with high hardness (complete mortality). As a management protocol liming ponds may have serious adverse consequences upon some species in local amphibian communities.

Aluminum moderated the effects of hardness at pH 6.5 in Jefferson Salamanders High Al decreased mortality more than low Al. The ameliorative effects of Al were greatest at high concentrations and pH 6.5. At pH 7.5 and high hardness low [Al] caused complete mortality whereas at pH 6.5 it caused a slight moderation in mortality. In contrast to the results of Horne and Dunson (1995b) where Jefferson

Salamanders larvae subjected to much lower hardness levels than in this study and at lower pHs (4.5 and 5.5). Al produced high larval mortality after chronic exposure. Initially Horne and Dunson found elevated hardness and/or lower pH resulted in lower mortality of young larvae, but chronic exposure produced high mortality at both hardness and pH levels. Their results are similar to those found here in that both [Al]s ameliorated mortality at both hardness levels at pH 6.5 and high [Al] had little effect at pH 6.5, however, at pH 7.5 total mortality (embryos and larvae) increased. It appears that the toxic effects of low Al are not moderated at pH 7.5 at either level of hardness. Al ions that are free in solution or some of the Al complexes formed are toxic to Jefferson Salamanders at these higher pHs and levels off hardness even at concentrations as low as 100 μg/L.

Aluminum did not produce significantly elevated mortality in Wood Frogs at the pHs and hardness levels tested. Wood Frogs suffered high chronic mortality at he lower pHs and hardness levels compared by Horne and Dunson (1995b).

Copper had minimal effects upon Jefferson Salamanders and Wood Frogs at the pHs, hardness levels, and concentrations studied. Horne and Dunson (1994) found Cu to be acutely toxic to Jefferson Salamanders embryos at pH 4.5 and early stage larvae (1995a). When larval Jefferson Salamanders and Wood Frogs tadpole were reared in microcosms at pH 4.5 and 5.5 with [Cu] at 15 μg/L there was complete mortality of Jefferson Salamanders and high mortality of Wood Frogs after 28 days (Horne and Dunson, 1995b). They found Cu to induce mortality more rapidly in both Jefferson Salamanders and Wood Frogs at higher pH 5.5) and hypothesized that Cu may be more toxic in higher pH environments. In the microcosms used here the potential toxic effects may have been greatly reduced by high hardness resulting in competitive inhibition (Freda, 1991; Horne and Dunson, 1995b). Chelation may have increased over the term of the experiment due to the production of waste products by larvae, decomposition of dead individuals, and the decomposition of food resources.

Both levels of Cu reduced Jefferson Salamanders mortality at pH 6.5 in both levels of hardness. At pH 7.5 both levels of Cu increased mortality at both levels of hardness except high [Al] at high hardness which ameliorated the effect of high hardness. Low [Cu] had little effect on Wood Frogs mortality at either pH; however, at it moderated the effects of high hardness at pH 6.5 and elevated it at pH 7.5 High [Cu] at low hardness elevated mortality at both pHs; at high hardness it decreased mortality at 6.5 but increased it at pH 7.5.

Zinc moderated the effects of hardness upon Jefferson Salamanders. Only high [Zn] at low hardness at pH 7.5 caused an increase in mortality, whereas it greatly ameliorated the effects of high hardness. Zn had negative effects in both concentrations and at both pHs and hardness levels on Wood Frogs Horne and Dunson (1995a) found [Zn] to increase hatching of Jefferson Salamanders at pH 4.5, but at pH 5.5. [Zn] had produced no effects on percent hatch. They did find a toxic effect on early stage larvae at pH 4.5 but not at pH 5.5.

Both [Fe] at low hardness moderated mortality at pH 6.5 and elevated it at pH 7.5 in Jefferson Salamanders. At high hardness both [Fe] decreased mortality at both levels of pH. [Fe] effected mortality of Wood Frogs only slightly; the greatest effect was an increase in mortality at high [Fe] at low hardness at pH 6.5.

In cation combination treatments only the low [cation] and high [cation] at low hardness at pH 7.5 slightly elevated mortality. All other cation, pH, and hardness combinations reduced mortality and those at high hardness reduced the hardness effects more than 50%. Horne and Dunson (1995a) found Fe to be toxic to Jefferson Salamanders embryos at low pH (4.5), but there were no toxic effects at pH 5.5.

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Table 2. Percent mortality of *Ambystoma jeffersonianum* and *Rana sylvatica* in embryonic and larval stage through 28 days post-hatch in ASW controls and ASW adjusted to two levels of pH and two levels of hardness. Mean mortality and one standard deviation are presented. Dry mass (g) per tadpole of *R. sylvatica* tadpoles 28 days post-hatch is presented.

	Ambystoma jeffersonianum		*Rana sylvatica* (mortality)		*Rana sylvatica* (dry mass)	
Treatment	Mean	SD	Mean	SD	Mean	SD
ASW	69.0	0.339	7.7	4.053	0.0156	0.0021
ASW @pH 6.5	66.1	2.044	1.1	4.412	0.0109	0.0021
ASW @pH 7.5	69.0	4.685	3.2	5.121	0.0134	0.0023
ASW @pH 6.5 w/Low Hardness	96.3	0.479	6.3	0.041	0.0108	0.0029
ASW @pH 7.5 w/Low Hardness	76.8	0.700	2.1	3.025	0.0156	0.0025
ASW @pH 6.5 w/High Hardness	100.0	0.000	15.2	3.222	0.0149	0.0043
ASW @pH 7.5 w/High Hardness	84.9	0.506	2.4	3.336	0.0134	0.0090

Table 3. Percent mortality of *Ambystoma jeffersonianum* in embryonic and larval stages through 28 days post-hatch at two levels of pH and two levels of hardness. Mean mortality in bold font; one standard deviation below mean.

	No Cations		Low Al		High Al		Low Cu		High Cu		Low Zn		High Zn	
	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5
Low Hardness	**96.3** 0.479	**76.8** 0.700	**66.1** 1.327	**97.7** 0.541	**57.8** 0.555	**78.7** 1.104	**88.0** 0.237	**88.0** 0.921	**89.5** 0.260	**87.0** 0.204	**51.4** 10.246	**75.4** 0.133	**95.6** 0.664	**82.2** 0.772
High Hardness	**100.0** 0.000	**84.9** 0.506	**86.9** 0.765	**100.0** 0.000	**89.8** 0.631	**76.6** 1.110	**93.5** 0.203	**97.7** 0.054	**100.0** 0.000	**60.7** 5.742	**93.5** 0.049	**73.9** 0.958	**92.3** 0.254	**44.9** 0.587

	Low Fe		High Fe		Low All Cations		High All Cation	
	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5
Low Hardness	**75.0** 0.585	**78.2** 0.046	**85.4** 1.569	**93.2** 0.205	**77.8** 1.160	**78.6** 0.228	**71.7** 2.375	**78.7** 0.207
High Hardness	**93.8** 0.153	**79.6** 0.813	**97.0** 0.092	**76.4** 2.431	**48.9** 3.157	**42.4** 8.602	**75.6** 1.775	**46.6** 10.144

Table 4. Percent mortality of *Rana sylvatica* in embryonic and larval stages through 28 days post-hatch at two levels of pH and two levels of hardness. Mean mortality in bold font; one standard deviation below mean.

	No Cations		Low Al		High Al		Low Cu		High Cu		Low Zn		High Zn	
	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5
Low Hardness	**6.3**	**2.1**	**9.5**	**8.9**	**2.1**	**3.3**	**6.0**	**3.7**	**10.0**	**13.1**	**13.8**	**3.1**	**9.9**	**3.90**
	0.041	3.025	4.679	4.344	2.983	4.469	9.173	1.649	4.788	7.470	0.175	4.167	1.289	5.261
High Hardness	**15.2**	**2.4**	**4.5**	**12.1**	**16.5**	**10.4**	**3.7**	**6.1**	**5.0**	**5.1**	**15.9**	**6.7**	**11.2**	**19.3**
	3.222	3.336	2.131	0.728	0.325	6.111	5.061	0.398	2.951	2.461	1.614	9.030	5.615	10.989

	Low Fe		High Fe		Low All Cations		High All Cation	
	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5
Low Hardness	**4.0**	**5.4**	**19.7**	**8.8**	**6.1**	**15.9**	**29.3**	**4.3**
	5.563	3.342	0.663	5.004	2.891	1.091	16.556	7.490
High Hardness	**15.9**	**6.6**	**9.7**	**5.8**	**13.8**	**26.4**	**12.9**	**11.7**
	1.614	9.030	0.396	3.372	0.873	9.114	5.740	2.821

Table 5. Dry mass of *Rana sylvatica* larval stages after 28 days post-hatch at two levels of pH and two levels of hardness. Mean mass in bold font; one standard deviation below mean.

	No Cations		Low Al		High Al		Low Cu		High Cu		Low Zn		High Zn	
	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5
Low Hardness	**0.0108** 0.0029	**0.0156** 0.0025	**0.0145** 0.0019	**0.0164** 0.0012	**0.0129** 0.0022	**0.0156** 0.0016	**0.0140** 0.0057	**0.0140** 0.0047	**0.0176** 0.0015	**0.0148** 0.0034	**0.0144** 0.0016	**0.0136** 0.0013	**0.0141** 0.0038	**0.0138** 0.0023
High Hardness	**0.0149** 0.0043	**0.0134** 0.0090	**0.0167** 0.0057	**0.0201** 0.0012	**0.0164** 0.0029	**0.0200** 0.0028	**0.0143** 0.0033	**0.0195** 0.0098	**0.0116** 0.0015	**0.0153** 0.0020	**0.0132** 0.0022	**0.0189** 0.0062	**0.0154** 0.0026	**0.0188** 0.0058

	Low Fe		High Fe		Low All Cations		High All Cation	
	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5
Low Hardness	**0.0164** 0.0282	**0.0144** 0.0031	**0.0182** 0.0029	**0.0156** 0.0011	**0.0183** 0.0033	**0.0169** 0.0037	**0.0185** 0.0073	**0.0200** 0.0052
High Hardness	0.0266 0.0282	0.0161 0.0031	**0.0204** 0.0045	**0.0167** 0.0014	**0.0171** 0.0033	**0.0249** 0.0086	**0.0246** 0.0115	**0.0271** 0.0034