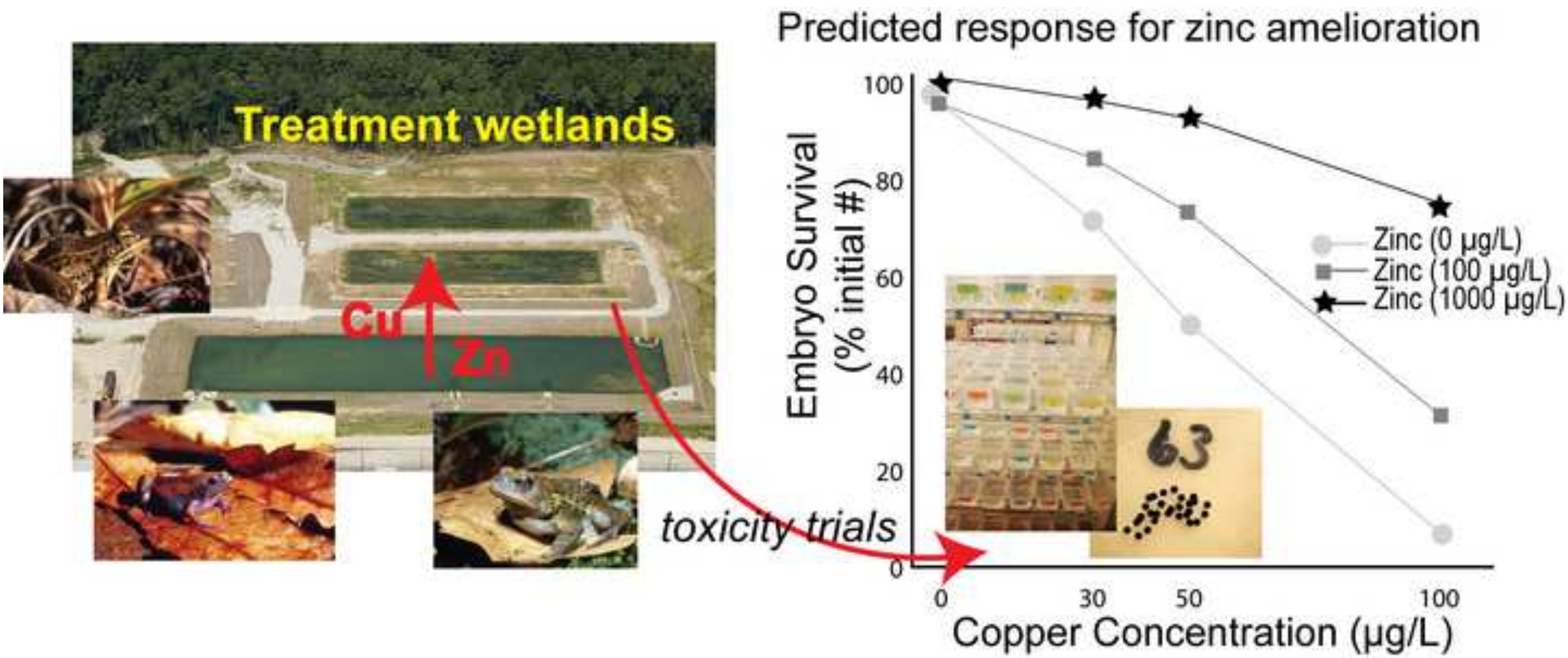
*Graphical Abstract



Title: Environmental levels of Zn do not protect embryos from Cu toxicity in three species of amphibians

Scott M. Weir[1], R. Wesley Flynn, David E. Scott, Shuangying Yu[2], Stacey L. Lance[*]

University of Georgia, Savannah River Ecology Laboratory, Aiken, SC, USA 29803

[*]Corresponding Author

Dr. Stacey L. Lance

University of Georgia

Savannah River Ecology Laboratory

Aiken, SC, USA 29803

lance@srel.uga.edu

Present Address:

[1]Queens University of Charlotte

Biology Department

Charlotte, NC, USA 28274

weirs@queens.edu

[2]Queens University of Charlotte

Chemistry and Environmental Science Department

Charlotte, NC, USA 28274

yus@queens.edu

ABSTRACT

Contaminants often occur as mixtures in the environment, but investigations into toxicity usually employ a single chemical. Metal contaminant mixtures from anthropogenic activities such as mining and coal combustion energy are widespread, yet relatively little research has been performed on effects of these mixtures on amphibians. Considering that amphibians tend to be highly sensitive to copper (Cu) and that metal contaminants often occur as mixtures in the environment, it is important to understand the interactive effects that may result from multiple metals. Interactive effects of Cu and zinc (Zn) on amphibians have been reported as antagonistic and, conversely, synergistic. The goal of our study was to investigate the role of Zn in Cu toxicity to amphibians throughout the embryonic developmental period. We also considered maternal effects and population differences by collecting multiple egg masses from contaminated and reference areas for use in four experiments across three species. We performed acute toxicity experiments with Cu concentrations that cause toxicity (10 – 200 µg/L) in the absence of other contaminants combined with sublethal concentrations of Zn (100 and 1000 µg/L). Our results suggest very few effects of Zn on Cu toxicity at these concentrations of Zn. As has been previously reported, we found that maternal effects and population history had significant influence on Cu toxicity. The explanation for a lack of interaction between Cu and Zn in this experiment is unknown but may be due to the use of sublethal Zn concentrations when previous experiments have used Zn concentrations associated with acute toxicity. Understanding the inconsistency of amphibian Cu/Zn mixture toxicity studies is an important research direction in order to create generalities that can be used to understand risk of contaminant mixtures in the environment.

Capsule: For three species of amphibians, environmentally relevant Zn concentrations consistently had no significant effects on acute toxicity of Cu.

Keywords: metals, anurans, mixtures, maternal effects, populations

1. INTRODUCTION

Chemical contamination is widespread in aquatic systems and organisms are often exposed to a mixture of contaminants (Kolpin et al. 2002). Contaminant mixtures can result in effects that are not predicted from simple additive models, and non-additive effects (i.e., synergism, antagonism, and potentiation) are common (e.g., metal and metalloid mixtures, Norwood et al. 2003). Nonetheless, most toxicity tests consider chemicals individually. While this is useful for elucidating mechanisms of toxicity, or assigning cause-and-effect relationships, it is not as useful for understanding the consequences of realistic exposures (Sibly 1999).

Metals and metalloids often occur as mixtures due to anthropogenic activities such as mining, coal combustion, and other industrial activities. As an example, coal combustion wastes (CCWs) are a complex mixture of trace metals and previous research into CCWs suggests strong effects on aquatic organisms inhabiting contaminated wetlands and ponds (reviewed by Rowe et al. 2002). Often, wetlands are created for the purpose of mitigating contaminants from industrial wastewater, drainage from mines, or stormwater runoff (Vymazal 2011). The use of constructed wetlands for such mitigation has increased (IWA, 2000; Vymazal 2011) and occurs globally (Whitney et al. 2003; Chen et al. 2006; Lesage et al. 2007; Vymazal et al. 2007). These created wetlands can sequester high concentrations of a variety of pollutants (Bishop et al. 2000) and it is common for them to have mixtures of metals (e.g. Khan et al. 2009; Terzakis et al. 2008).

Amphibian species use constructed wetlands (e.g., Lance et al. 2012;2013), and negative effects of contaminants at these sites on amphibian fitness have been reported previously (Rowe et al. 2001; Snodgrass et al. 2004; Snodgrass et al. 2005; Roe et al. 2006; Metts et al. 2012). In particular, industrial effluent wetlands can contain elevated concentrations of specific metals (e.g., copper (Cu) and zinc (Zn), Knox et al, 2006; Lance et al. 2013; Flynn et al. 2015). Importantly, natural wetlands may also contain mixtures of contaminants such as metals. For example, a failure in a CCW impoundment may release contaminants into natural wetlands (Lemly and Skorupa 2012). Mining and smelting activities can contaminate riverine systems and their associated floodplain wetlands (Gomez-Parra et al. 2000, Leduc et al. In Press). For the purpose of estimating risk, it is important to understand the role of mixtures on the negative effects of contaminants on amphibians, especially given the increasing use of constructed wetlands for mitigation.

Amphibians remain one of the least studied vertebrate taxa in ecotoxicology (Sparling et al. 2010). Despite recent increases in toxicity studies that focus on amphibians, mixture-toxicity data remain scant, especially for specific combinations of metal contaminants. We know of only two previous reports examining toxicity of Cu and Zn mixtures on amphibians and results are contradictory. Using similar ranges of Cu and Zn concentrations, Herkovits and Helguero (1998) suggest an antagonistic interaction while Gottschalk (1995) suggests synergism. Toxicity data for fish may provide some insight into the potential interaction between Cu and Zn, but available literature provides conflicting results. Copper concentrations in the 1-3 mg/L range combined with Zn concentrations in the 3-12 mg/L range have a synergistic interaction (Eisler and Gardner 1973). However, Cu concentrations more similar to those that cause toxicity to amphibian embryos (10-100 μg/L, see Lance et al. 2012; Lance et al. 2013) are less toxic when high

concentrations of Zn are present (Finlayson and Verrue 1982). Other data suggest simple additive toxicity (i.e., no interaction: Lloyd 1961; Sprague and Ramsay 1965; Brown and Dalton 1970). The nature of the interaction also depends on additional factors (e.g., water hardness, Lloyd 1961). A review of the effects of Cu/Zn mixtures on aquatic biota found that strictly additive effects were observed in only 5% of experiments (1 out of 21), whereas synergistic (9 out of 21) and antagonistic (11 out of 21) effects occur at higher frequencies, suggesting that non-additive effects may be common (Norwood et al. 2003). Given the high likelihood of non-additive effects of Cu and Zn, further experimentation with amphibians is warranted. The mechanism describing antagonistic effects may be due to Zn reducing uptake of Cu (Rossowska et al. 1995), reducing the creation of reactive oxygen species (Stohs and Bagchi 1995), or inducing metallothionein expression (Irato et al. 1996). Mechanisms related to synergistic effects are not as well understood. Importantly, previous research on Cu/Zn mixtures and amphibians used very high Zn concentrations (> 1 mg/L) that may not represent environmentally relevant scenarios. We were interested in determining possible interactions using more moderate Zn concentrations.

The purpose of our experiments was to investigate the effect of zinc on copper toxicity to amphibian embryos in three species known to differ in their responses to elevated Cu concentrations. We compared three species of anurans for which we have previous toxicity data confirming differences in sensitivity to copper: southern leopard frogs (*Lithobates sphenocephalus*, Lance et al. 2012), southern toads (*Anaxyrus terrestris*, Lance et al. 2013), and eastern narrowmouth toads (*Gastrophryne carolinensis*, Flynn et al. 2015). We chose sites with a recent history of copper contamination to compare to multiple reference sites. We further performed experiments on the offspring of separate females to understand maternal effects. Our

results provide insight into the relative importance of multiple external factors on the toxicity of metal mixtures to amphibians.

2. MATERIALS AND METHODS

2.1 Study Species

The three species used in our studies (*G. carolinensis, A. terrestris, and L. sphenocephalus*) encompass three different genera and a range of life history strategies and known tolerances to metals (Birge et al., 2000; Duellman and Trueb, 1986). All three species are locally abundant throughout the southeastern United States, though they differ in feeding strategy and larval period, and potential means and duration of exposure to contaminants. As larvae, *A. terrestris* and *L. sphenocephalus* feed by scraping biofilms from vegetation and other submerged structure, while *G. carolinensis* filter feed plankton from the water column; *L. sphenocephalus* tadpoles are in the aquatic environment for ≥3 mo, versus shorter larval periods for the other two species.

2.2 Study Sites

We collected all amphibian species from study sites located on the U.S. Department of Energy's Savannah River Site (SRS), in Aiken and Barnwell Counties, South Carolina. The metal-contaminated site is the H-02 constructed wetland complex, a surface-flow wetland constructed in 2006-2007 to remediate wastewater elevated in Cu, Zn, and pH (see Lance et al., 2012 for detailed account). At the H-02 site concentrations of aquatic Cu range from 1.42 – 62.59 µg/L, Zn from 6.89 – 76.30 µg/L, and pH from 6.07 – 9.87 (Flynn et al., 2015). The four reference sites were all natural wetlands, with no known history of contamination, that vary in size and hydroperiod; from smallest to largest these sites are: Rainbow Bay (RB), Craig's Pond (CP),

Flamingo Bay (FB), and Ellenton Bay (EB). Ellenton Bay and RB are considered to have temporary hydroperiods, while CP and FB are considered semi-permanent. In statistical models described below, “Source” is the wetland site from which we collected pairs of males and females, and “Clutch” is the batch of eggs acquired each two-parent breeding event.

2.3 Experimental Design

We conducted a total of four separate studies to test for the effects of Zn and it’s interaction with Cu, on the embryonic stages of three amphibian species; two trials were conducted on *L. sphenocephalus*, the second of which used a lower Cu concentration (60 µg/L). In most cases, we collected adults from representative populations using pitfall traps and bred them in the laboratory to obtain full-sib families; however for the 2012 *L. sphenocephalus* study, we collected egg masses from pools where they were known to be deposited and fertilized the previous night. In this case, only egg masses that were at least 1-m apart were chosen to ensure all masses were from unique females. We conducted all trials at the University of Georgia Savannah River Ecology Laboratory’s Animal Care Facility, where environmental variables were precisely controlled. We controlled water chemistry variables by using a standard synthetic dilution soft water for toxicity tests using freshwater organisms (US Environmental Protection Agency, 2002) consisting of 48 mg/L $NaHCO_2$, 30 mg/L $CaSO_4$, 30 mg/L $MgSO_4$, and 2 mg/L KCl added to 50-L nanopure MILLI-Q® water, plus appropriate volumes of a 2,000 mg/L $CuSO_4$ and $ZnSO_4$ stock solutions. The air temperatures in the study room ranged from 18-21 °C. In all cases, we placed a subset of early stage embryos in 0.5-L containers containing 400 mL of synthetic soft water and the appropriate concentrations of Cu and Zn (see Table 1 for trial specific starting stages, range of Cu and Zn treatments, and embryos per replicate); among

species variation in egg mass structure resulted in greater variability in initial egg numbers in the *Lithobates* and *Anaxyrus* experiments than in the *Gastrophryne* experiment, which was accounted for in our statistical models. Concentrations of Cu were chosen to reflect environmentally relevant levels, as well as species-specific toxicity profiles based on pilot data. Corresponding concentrations of Zn were also chosen to encompass environmentally relevant concentrations (i.e. 100 µg/L), as well as higher concentrations (1000 µg/L) that may be necessary to exert a beneficial effect of Zn against Cu toxicity (Zn:Cu ratio of 10-15:1 (Herkovits and Helguero 1998); Zn alone has a relatively low toxicity to many amphibians (Birge et al., 2000). We randomly distributed containers across shelves and checked embryos daily for survival, at which time any dead embryos were removed. Trials were concluded when all surviving embryos had reached GS 25-26, when they are free-swimming and feeding larvae (Gosner, 1960).

2.4 Verification of Nominal Concentrations

We analyzed water samples (15 mL) from a random set of containers across different treatments in the 2012 *G. carolinensis* study to quantify actual Cu and Zn concentrations present in the nominal treatments at the end of the trial (Table S1). The 15-mL water samples were acidified to 1% HNO3 using trace metal grade nitric acid and analyzed using optical emission spectroscopy.

2.5 Prediction of Joint Effects Using the Independent Action Model

To provide an additional line of evidence to investigate interactions between Cu and Zn, we determined the joint effect of Cu and Zn using the independent action model described in Payne et al. (2001) and Coors and De Meester (2008):

(1) $$E_{mix} = 1 - \prod_{i=1}^{n}(1 - E_i)$$

Whereas, Ei is expressed as:

(2) $$E_{\mathrm{i}} = \frac{(e_{\mathrm{i}} - e_{\mathrm{control}})}{(e_{\mathrm{max}} - e_{\mathrm{control}})}$$

E_{mix} denotes the joint effect of two stressors, and E_i the effect of each single stressor. To apply the IA model (Equation 1), E_i must be transformed to a proportional response using Equation 2. e_i represents the observed effect in absolute units (e.g., mortality, incidence of malformations, or body length) and $e_{control}$ is the response of controls without either stressor. In the present study, e_{max} was set to 100% for mortality. E_{mix} was then rescaled to absolute units (E'_{mix}) using Equation 3 for comparisons with observed effects (Coors, personal communication):

(3) $$E'_{\mathrm{mix'}} = E_{\mathrm{mix}} \times (e_{\mathrm{max}} - e_{\mathrm{control}}) + e_{\mathrm{control}}$$

We evaluated if effects of Cu/Zn mixtures were synergistic or antagonistic by comparing the predicted E'_{mix} to the 95% confidence interval around the observed E'_{mix}. If any overlap occurred with the predicted E'_{mix} and the confidence interval, we defined the interaction as additive. If the predicted E'_{mix} fell below the confidence interval, we defined the interaction as synergistic, and as antagonistic if the predicted value fell above the range of the confidence interval.

2.6 Statistical Analysis

Our experiments made use of both continuous and categorical factors as well as fixed and random factors. Therefore, we used a generalized linear mixed effects model (GLM) to

investigate the effects of Cu, Zn, Clutch, and Source on mortality of embryos. We did not compare across trials as differential tolerance required using different Cu concentrations across species. In one trial (i.e., *Lithobates* in 2012) only one source population was used. In the other three trials, because females came from specific source populations, we defined source as a random effect and estimated a separate intercept for each source population; we also considered clutch as a random factor. We have previously reported that the effect of initial number of eggs in replicates can affect mortality (Lance et al. 2012; 2013), so we used initial egg number as a covariate in all models to account for this potential effect.

We performed all analyses in SAS (version 9.3). We created GLMs using Proc GLIMMIX. We used the “events/trials” syntax in which the final number of surviving embryos was divided by the initial number of eggs. The “events/trials” syntax uses a binomial distribution and logit link function. We assessed model fit by the ratio of the generalized χ^2 to the error degrees of freedom. This value should be near 1, with large values suggesting overdispersion of the data. All values in our models fell between 0.81 and 1.06.

3. RESULTS

Verified concentrations of Cu and Zn were very similar to nominal concentrations (Table S1). In most cases the mean verified concentration was within 20% of nominal. The only exception was 1000 µg/L Zn in which the mean verified concentration was 1210 µg/L.

Concentrations of Cu provided good dose response curves in most cases and always resulted in a significant effect on mortality (for each trial, $p < 0.001$). For all three species, the Zn concentrations chosen did not result in any significant mortality in the absence of Cu (all $p \geq 0.11$; Table 2), even at concentrations up to 1000 µg/L. Southern leopard frogs were much more

tolerant of Cu than southern toads and narrow-mouthed toads. Though we did not calculate LC_{50}s, it took much greater concentrations of Cu to elicit toxicity in southern leopard frogs.

For the southern toad 2011 trial, there was a significant effect of source ($F_{1,172} = 83.2$, $p < 0.001$; Figure 1), but there was only a marginally significant effect of clutch within source ($F_{3,172} = 2.3$, $p = 0.08$, see Figure S1). There was no significant Cu x Zn interaction ($F_{6,172} = 0.4$, $p = 0.86$; Figure 1).

For the southern leopard frog 2011 trial, there was no significant effect of source ($F_{1,147} = 1.5$, $p = 0.22$), but there was a significant effect of clutch within source ($F_{4,147} = 3.1$, $p = 0.018$, see Figure S2). There was no significant Cu x Zn interaction ($F_{4,147} = 0.8$, $p = 0.55$; Figure 2). For the southern leopard frog 2012 trial, results were similar to the 2011 trial. We once again found a significant effect of clutch on embryo survival ($F_{3,95} = 9.7$, $p < 0.001$, see Figure S2). Only one source population was used in this trial so we could not test the effect of source. This trial was the only trial to have a near significant Cu x Zn interaction ($F_{4,95} = 2.4$, $p = 0.058$; Figure 2). However, the data show very little obvious interaction; therefore this marginally significant interaction does not appear to be biologically significant (Figure 2).
In the narrow-mouthed toad 2012 trial we found a marginally significant source effect ($F_{1,196} = 3.5$, $p = 0.062$; Figure 3), apparently driven by a strong Cu x Source interaction ($F_{3,196} = 7.2$, $p = 0.0001$; Figure S3; see Flynn et al. 2015). We did not detect any significant Cu x Zn interaction ($F_{6,196} = 0.9$, $p = 0.52$; Figure 3).

Our IA models were generally in agreement with our statistical analysis. The only interactions that appeared non-additive were synergistic interactions in the 2011 and 2012 study with southern leopard frogs (Table 3). The 2012 trial had a marginally significant interaction from our GLM, but the 2011 southern leopard frog trial did not, which represents the only

disagreement between the IA models and the statistical models. It is worth noting that the statistical models assess the Cu/Zn interaction across all concentrations, while the IA model looks at each combination individually. Perhaps a single interaction found in one combination would not be found with the GLM models across all combinations.

4. DISCUSSION

We found little evidence of an effect of Zn on toxicity of Cu to amphibian embryos across multiple species and, for southern leopard frogs, across multiple trials within the same species. At the concentrations of Zn tested, no significant mortality occurred due to Zn alone. We also found no significant interactions between Zn and Cu in any of our experiments, despite using a range of Zn concentrations (100 and 1000 µg/L). Our IA model results generally agree with this finding with only two significant synergistic interactions found in the southern leopard frog experiments out of a total of 20 possible significant interactions (Table 3). The weight of evidence suggests very little interaction between Cu and Zn at these concentrations. Because we chose concentrations to represent a dose-response for Cu, Cu was always a very significant effect on mortality, and effects of source populations and clutch nested within source occurred in some experiments (Table 2). Overall, our results suggest little effect of Zn on the range of Cu concentrations that cause toxicity to amphibians.

Our results differ from many previous investigations that report Zn has non-additive effects on Cu toxicity in aquatic systems (see Eisler 1998; Norwood et al. 2003). There are numerous potential explanations for why our results disagree with previous investigations. It is possible for amphibian physiology to differ significantly from fish which have been the subject of the majority of previous research into Cu/Zn interactions. Experiments investigating the

differences in sensitivity of fish and amphibians are relatively rare and tend to focus on LC50s (e.g., Birge et al. 1979; Bridges et al. 2002). Perhaps the fact that we exposed embryos rather than fully developed larvae may account for discrepancies between our results and previous studies. For example, McKim et al. (1978) reported that embryos were less sensitive to Cu than larvae/young adults of 8 fish species. The physiological mechanisms of toxicity or interactions may not have completely developed in embryos during these exposures. If the physiological pathway by which the interactions occur has not yet developed, we might not expect to see an interaction. However, Herkovits and Helguero (1998) and Gottschalk (1995) report significant interactions between Cu and Zn toxicity on amphibian embryos.

Understanding why previous experiments have found non-additive effects of Zn/Cu while we found no interactions should be considered in light of the Cu and Zn mechanism of action. Transition metals may have multiple mechanisms of toxicity, but many (including Cu) contribute to the creation of reactive oxygen species (Stohs and Bagchi 1995). Interestingly, Zn is an unusual cation as it seems to provide protection against reactive oxygen species caused by transition metals (Stohs and Bagchi 1995). This potential interaction would likely result in antagonistic interactions, which seem fairly common between Cu and Zn (Norwood et al. 2003), but this mechanism is unlikely to provide insight into the lack of interaction seen in the current study. Further, there is evidence that Zn may protect against absorption of Cu (Rossowska et al. 1995). Again, this mechanism provides an explanation for antagonistic effects, but does not provide insight into our current results. Mechanisms of interactions would likely be concentration dependent (Herkovits and Helguero 1998, Sharma et al. 1999), and the protective effect of Zn has been suggested to occur at very high concentrations of Zn (reviewed in Eisler 1993), but no interaction was seen in the current study despite a range of Zn concentrations used

(100 to 1000 µg/L). However, all the concentrations of Zn tested in this experiment were not in the toxic range, as no significant toxicity due to Zn was seen during our experiments. It seems that previous investigations of Cu/Zn effects on fish and amphibians found interactions at much higher concentrations of Zn (Table 4), but concentrations well above 1 mg/L are less likely to be environmentally relevant than <1 mg/L (reviewed in Eisler 1993). For instance, Herkovits and Helguero (1998) found antagonistic effects of Zn on Cu toxicity in toads at very high Zn concentrations (e.g., 8-30 mg/L). Within the same range (1-20 mg/L) Gottschalk (1995) found a synergistic effect of Zn on Cu toxicity in leopard frogs. Clearly more research is needed to understand the circumstances in which Cu and Zn interact when occurring as a mixture, especially at levels of Zn that are environmentally relevant.

The purpose of this experiment was to investigate the effect of Zn on Cu toxicity and the concentrations of Zn used in this experiment (≤1 mg/L) are unlikely to cause acute toxicity to amphibian embryos. Khangarot and Ray (1987) report the 96-hr LC_{50} of Zn to *Bufo melanostictus* larvae as 19.86 mg/L. Lefcort et al. (1998) report a 96-hour LC_{50} of 28.38 mg/L to *Rana luteiventris* larvae. Interestingly, Birge et al. (1979) report the 7-day LC_{50} for *Gastrophryne carolinensis* embryos as 0.01 mg/L. We exposed eastern narrow-mouthed toad embryos to concentrations of 0.1 and 1.0 mg/L Zn without any significant mortality (see Figure 3). It is unknown why such large differences were found between our study and that of Birge et al. (1979). Birge et al. (1979) use a continuous flow-through system for exposure; however, over 24 to 96 hours it is unlikely that there would be large differences between a static exposure and a flow-through system (e.g., Bailey et al. 1985).

It is becoming well established that amphibian populations and families can vary in sensitivity to contaminants. Bridges and Semlitsch (2000) reported significant variation in

pesticide tolerance both among and within populations of leopard frogs. There are also significant differences between populations for sensitivity to inorganic compounds (Johansson et al. 2001 [NO_3]; Pierce and Harvey 1987 [pH]; Gomez-Mestre and Tejedo 2003 [salinity]). We found some differences among source populations, and fairly consistent differences among clutches, in Cu toxicity, which conforms with our previous experiments with amphibian embryos (Lance et al. 2012; 2013; Flynn et al. 2015). Southern leopard frog embryos from two different sources had significant differences in toxicity between clutches (Lance et al. 2012), but we were unable to test for differences in sources due to study design differences. Southern toads from different populations had significantly different sensitivity to Cu, as well as significant clutch differences (Lance et al. 2013). Finally, we reported significant effects of population and clutch on Cu toxicity to eastern narrow-mouthed toads (Flynn et al. 2015). Taken together, these consistent results suggest that within- and among-population variability in tolerance is an important consideration for contaminant toxicity. Understanding the genetic and/or plastic mechanisms behind clutch- and population-level differences is an important step to understanding if amphibians will be able to adapt to contaminated environments or if those environments will act as sinks in the local landscape.

Our study represents a simple first step in understanding the effects of Cu toxicity on amphibians when it occurs with a mixture of Zn at environmentally relevant concentrations. Across the country, remediation wetlands may be used by amphibians (Rowe et al. 2001; Lance et al. 2012; Lance et al. 2013), and it is important to understand the effects of the contamination of the mitigation wetlands on amphibians. Mitigation wetlands are used to reduce the concentration of some metals in outflow prior to introduction to the watershed (Rowe et al. 2002; Knox et al. 2006). Beyond remediation wetlands, natural wetlands can also be contaminated by

mixtures of metals from various sources (Gomez-Parra et al. 2000; Lemly and Skorupa 2012). Understanding the risks of exposure to a mixture of chemicals can be difficult, especially when trying to predict impacts from contamination events in the future. It is important to find generalities that can be used to predict the effects of current or future mixture exposures on population persistence (e.g., Backhaus and Faust 2012). It is also important to investigate patterns that do not fit current generalities. Contrary to much of the previous research on fish and amphibians, we found no significant effects of Zn on Cu toxicity in embryos of three anuran species. Understanding the circumstances that lead to interactions or a lack of interactions is an important future research direction to provide generalities that can be used for risk assessment in the future.

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Table 1. Summary of experimental design details for four copper (Cu)/zinc (Zn) trials conducted on three amphibian species from five wetland source populations. Tx refers to treatment. In all cases there were three replicates and the temperature was maintained between 18 and 21°C

Species	***L. sphenocephalus***	***L. sphenocephalus***	***A. terrestris***	***G. carolinensis***
Year	2011	2012	2011	2012
Wetland(s)	EB and H-02	H-02	CP and FP	RB and H-02
# Clutches	3 per wetland	4	3 per wetland	4 and 2 respectively
Start Date	2/19/2011	2/23/2012	3/3/2011	5/25/2012
End Date	2/25/2011	2/28/2012	3/7/2011	5/31/2012
Time in Tx	6	5	4	6
Stage entering Tx (GS)	10-17	10-17	4-7	2-4
Cu (µg/L)	0, 100, 200	0, 60, 200	0, 10, 30, 50	0, 10, 30, 50
Zn (µg/L)	0, 100, 1000	0, 100, 1000	0, 100, 1000	0, 100, 1000
# Tx in analysis	9	9	12	12
# Experimental units	162	108	216	234
Embryos/container	18-62	16-63	15-58	2-11

[a]EB = Ellenton Bay, CP = Craig's Pond, FB = Flamingo Bay, RB = Rainbow Bay

[b]Gosner (1960)

Table 2. Summary of statistical analysis for each amphibian species embryo trial. Source represents the wetland sites from which adults were collected to breed and females deposit egg masses. Female is nested within source to test for among-clutch variation. N refers to the numerator degrees of freedom (for each factor) while D refers to the denominator (error) degrees of freedom.

Year	Species	Factor	N (df)	D (df)	F ratio	P value
2011	*Lithobates sphenocephalus*	Cu	2	148	256.5	<0.0001
		Zn	2	148	2.2	0.111
		Cu*Zn	4	148	0.8	0.552
		Source	1	148	1.5	0.220
		Fem(Source)	4	148	3.1	0.018
2012	*Lithobates sphenocephalus*	Cu	2	95	280.3	<0.0001
		Zn	2	95	1.2	0.314
		Cu*Zn	4	95	2.4	0.058
		Fem	3	95	9.7	<0.0001
2011	*Anaxyrus terrestris*	Cu	4	172	118.9	<0.0001
		Zn	2	172	0.7	0.500
		Cu*Zn	6	172	0.4	0.855
		Source	1	172	83.2	<0.0001
		Fem(Source)	3	172	2.3	0.080
2012	*Gastrophryne carolinensis*	Cu	3	196	108.2	<0.0001
		Zn	2	196	0.9	0.402
		Cu*Zn	6	196	0.8	0.602
		Source	1	196	3.5	0.062
		Fem(Source)	3	196	7.2	0.0001

Table 3 Summary of results of independent action models to investigate potential non-additive interactions between Cu and Zn. We found only one instance of a synergistic interaction, which agreed with statistical results in which only one marginally significant interaction between Cu and Zn occurred for the same experiment.

Species	Cu (μg/L)	Zn (μg/L)	Observed E_{mix}	95% CI of Observed E_{mix}	Predicted E_{mix}	Interaction
Anaxyrus - 2011	10	100	0.482	0.266 - 0.698	0.471	Additive
	30	100	0.874	0.632 - 1.116	0.918	Additive
	50	100	0.991	0.968 - 1.015	0.994	Additive
	10	1000	0.548	0.280 - 0.816	0.519	Additive
	30	1000	0.948	0.874 - 1.021	0.925	Additive
	50	1000	0.995	0.987 - 1.004	0.994	Additive
Gastrophryne - 2012	10	100	0.152	-0.037 - 0.341	0.032	Additive
	30	100	0.607	0.370 - 0.843	0.745	Additive
	50	100	0.927	0.846 - 1.008	0.956	Additive
	10	1000	0.162	0.018 - 0.307	0.049	Additive
	30	1000	0.603	0.414 - 0.792	0.750	Additive
	50	1000	0.987	0.957 - 1.016	0.957	Additive
Lithobates - 2011	100	100	0.266	0.169 - 0.362	0.311	Additive
	200	100	0.811	0.607 - 1.015	0.819	Additive
	100	1000	0.355	0.142 - 0.567	0.312	Additive
	200	1000	0.920	0.857 - 0.984	0.820	Synergistic
Lithobates - 2012	60	100	0.085	0.064 - 0.107	0.070	Additive
	200	100	0.899	0.709 - 1.088	0.875	Additive
	60	1000	0.102	-0.158 - 0.363	0.069	Additive
	200	1000	0.913	0.877 - 0.949	0.874	Synergistic

Table 4 Summary of fish and amphibian investigations into Cu/Zn toxicity interactions. Species names match those used by the authors of the provided sources.

Species	Life Stage	Time (hr)	Alkalinity (mg/L $CaCO_3$)	Salinity	Cu LC50 (mg/L)	Zn LC50 (mg/L)	Range of Cu (mg/L)	Range of Zn (mg/L)	Zn interaction?	Ref.[a]
Salmo gairdneri	1 yr old	48	240	-	0.75	4	0.15-0.60	0.8-3.2	No	1
Salmo gairdneri	Not Reported	72	15-20	-	0.044[b]	0.56[b]	1X[c]	6X	Synergistic	2
Salmo gairdneri	Not Reported	168	320	-	1.1[b]	3.5[b]	1X	6X	No	2
Salmo salar	Yearling	168	14	-	-	-	0.014 - 0.22	0.3 - 4.2	Synergistic	3
Fundulus heteroclitus	Not Reported	96	-	20	-	-	1.0-3.0	3.0-12.0	Synergistic	4
Oncorhynchus tshawytscha	Juvenile	96	18-19	-	0.32	0.84	1X	3X-12X	Additive/ Antagonistic	5
Rana pipiens	Larvae	96	45	-	0.0761	10.48	LC0 - LC50 (by 10)	LC0 to LC50 (by 10)	Synergistic	6
Bufo arenarum	Larvae	168	-	-	0.085	-	LC10, LC50, 2x LC100	0.05 - 180	Antagonistic	7

[a]References: [1] Brown and Dalton 1970, [2] Lloyd 1961, [3] Sprague and Ramsay 1965, [4] Eisler and Gardner 1973, [5] Finlayson and Verrue 1982, [6] Gottschalk 1995, [7] Herkovits and Helguero 1998.
[b]Reported as median survival times for the given duration
[c]Actual concentrations not reported in text, simply described as a ratio of Cu:Zn concentrations

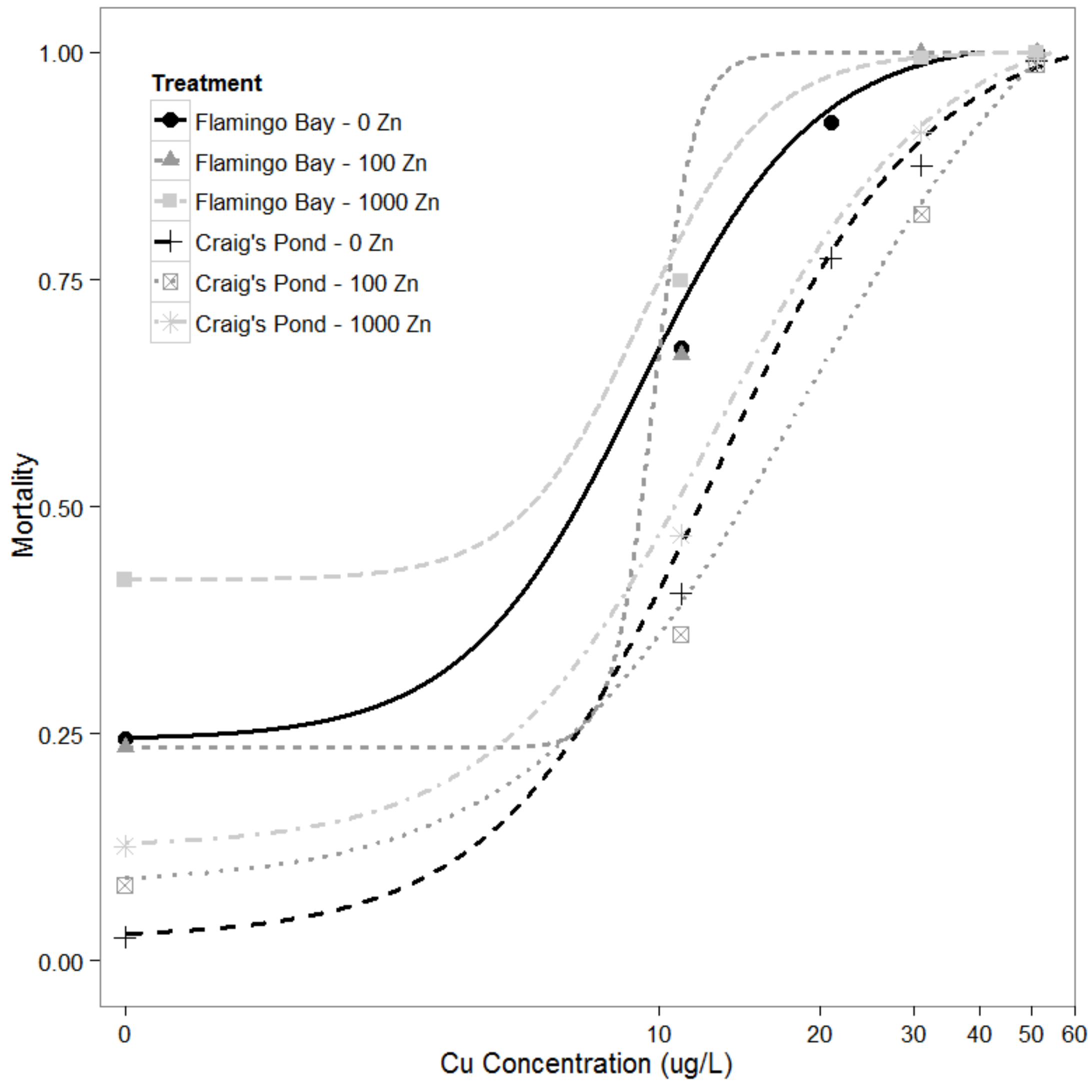

Figure 1 Mortality of southern toad embryos (2011 trial) as a function of copper concentration. There is a significant source effect ($F_{1,172}$ = 83.2, p < 0.001) but no significant effect of Zn ($F_{2,172}$ = 0.7, p = 0.5). There is no significant interaction between Cu and Zn ($F_{6,172}$ = 0.4, p = 0.85).

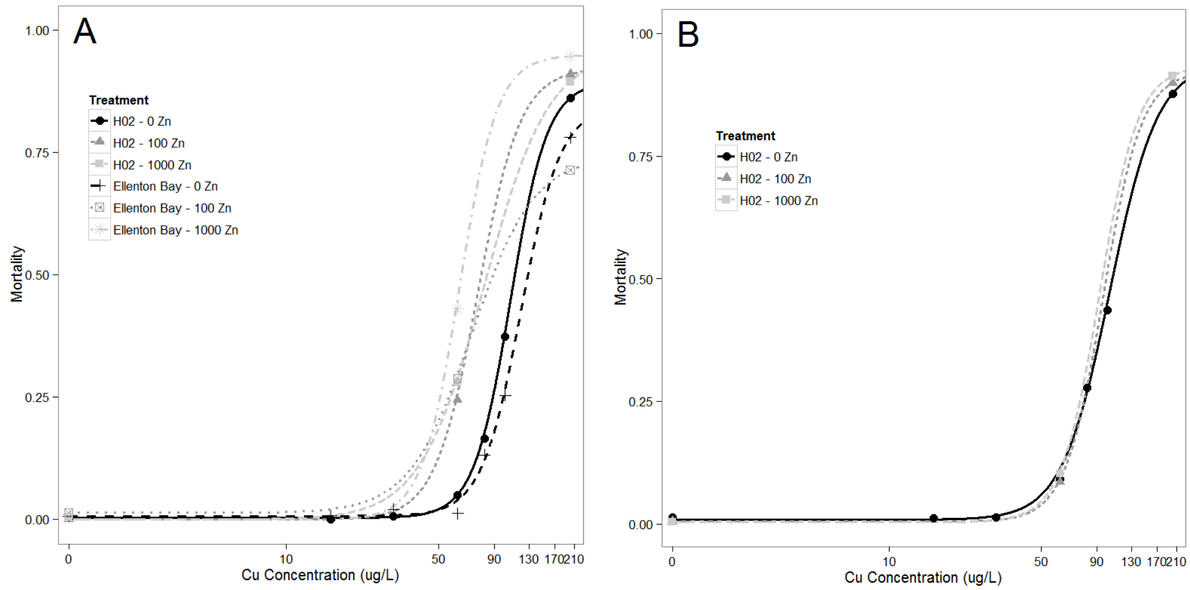

Figure 2 Mortality of southern leopard frog embryos (2011 and 2012 trials) as a function of copper concentration. In 2011 (panel A), there is no significant source effect ($F_{1,148}$ = 1.5, p = 0.22) or Zn effect ($F_{2,148}$ = 2.2, p = 0.11). There is also no significant interaction between Cu and Zn ($F_{4,148}$ = 0.8, p = 0.55). In 2012 (panel B), there is no significant Zn effect ($F_{2,95}$ = 1.2, p = 0.31). There is a "marginally" significant interaction between Cu and Zn ($F_{4,95}$ = 2.4, p = 0.058), but this does not appear to be biologically significant.

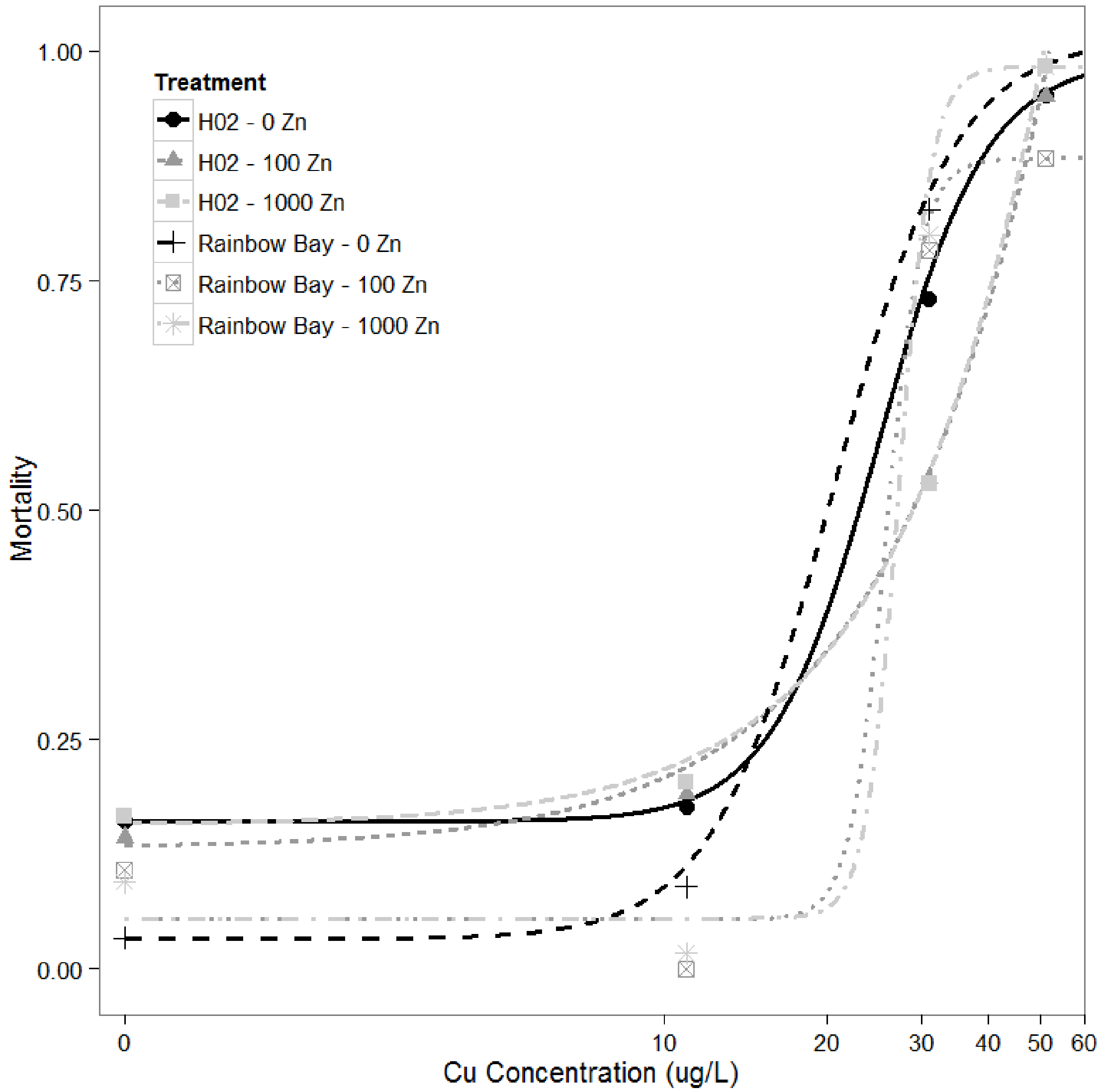

Figure 3 Mortality of eastern narrowmouth toad embryos (2012 trial) as a function of copper concentration. There is a marginally significant source effect ($F_{1,196} = 3.5$, $p = 0.062$) but no effect of Zn ($F_{2,196} = 0.9$, $p = 0.4$). There is also no significant interaction between Cu and Zn ($F_{6,196} = 0.8$, $p = 0.6$).