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

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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.

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).

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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, 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. 2016). 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 to 62.59 μg/L, Zn from 6.89 to 76.30 μg/L, and pH from 6.07 to 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 2000 mg/L $CuSO_4$ and $ZnSO_4$ stock solutions. The air temperatures in the study room ranged from 18 to 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