LARVAL AND POST-METAMORPHIC CONSEQUENCES OF AQUATIC STRESSOR EXPOSURE IN SOUTHERN TOADS (ANAXYRUS TERRESTRIS)

by

CAITLIN TERESA RUMRILL

(Under the Direction of Stacey Lance)

ABSTRACT

The complex life histories of amphibians make them susceptible to both aquatic and terrestrial stressors, but we are only just piecing together how exposure in one life stage may affect another. Additionally, exposure to one stressor is almost always coupled with exposure to another, whether it be anthropogenic or natural, and the effects of one on an individual can be altered by interactions. Furthermore, parental exposure to one or many stressors can carry through to offspring, affecting their ability to cope with the same or novel stressors. In Chapter 2 we showed that both parental exposure history and larval exposure to predator cue and copper stressors affect physiological performance, growth, and survival of southern toads (*Anaxyrus terrestris*) after metamorphosis. Our work highlights the need to incorporate the impacts of environmental stressors together, across life stages and generations.

INDEX WORDS: Amphibian; Ecotoxicology; Multiple stressors; Maternal effects; Latent effects; Copper; Predator cue

LARVAL AND POST-METAMORPHIC CONSEQUENCES OF AQUATIC STRESSOR EXPOSURE IN SOUTHERN TOADS (ANAXYRUS TERRESTRIS)

by

CAITLIN TERESA RUMRILL

B.A., Colby College, 2008

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2015

LARVAL AND POST-METAMORPHIC CONSEQUENCES OF AQUATIC STRESSOR EXPOSURE IN SOUTHERN TOADS (ANAXYRUS TERRESTRIS)

by

CAITLIN TERESA RUMRILL

Major Professor:	Stacey Lance
Committee:	Robert Bringolf
	Andrew Davis

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
August 2015

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Stacey Lance for her support and trust, which have been invaluable during my undergraduate and graduate careers. Secondly, I would especially like to thank David Scott who has given an extraordinary amount of time and effort to help me set up this experiment and ensure its proper design. His continual efforts from assisting with and teaching statistical analyses to manuscript edits have been instrumental, as have been his unending support through encouragement and snack foods. I would also like to thank my committee members, Dr. Robert Bringolf and Dr. Andrew Davis, for their assistance and advice concerning both my thesis and future career goals.

I also owe a great deal of gratitude to Rochelle Beasley and Jason O'Bryhim, who handled toad husbandry for another project while I was on campus. R. Wes Flynn was instrumental in the initial stage of this project and has since provided assistance with statistical analyses, manuscript edits, and advice. And a tremendous thanks to Erin Abernethy, Austin Coleman, Cara Love, Dr. Scott Weir, Megan Winzeler, and Dr. Shuangying Yu for their assistance with side projects, animal husbandry, the performance assay, statistical analyses, manuscript edits, and overall support. The work for this thesis would not have been as enjoyable without their presence. Praise Toadsus. Additionally, I would like to thank John Seaman and Rebecca Philipps for assistance with metals analysis. This research was partially supported by the U.S. Department of Energy under Award Number DE-FC09-07SR22506 to the University of Georgia Research Foundation,

and was also made possible by the status of the Savannah River Site as a National Environmental Research Park (NERP), as well as the protection of research wetlands in the SRS Set-Aside Program. Project funding was provided by the DOE National Nuclear Security Administration. Animals were collected under SCDNR permit #G-09-03 following IACUC procedures (AUP A2009 10-175-Y2-A0) from the University of Georgia.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Roughly eighty percent of animals have complex life histories (Pough et al. 2005) and metamorphosis marks the point at which organisms may change their morphology, physiology, and ecology in order to take advantage of environmental resources (Wilbur 1980). This change can be especially drastic for taxa that utilize both aquatic and terrestrial habitat types during their lifetimes. Most amphibians fall into this category and are thus susceptible to the effects of environmental stressors in multiple life stages and across habitat types (Todd et al. 2011a). Furthermore, there is also evidence indicating that stressor effects are not necessarily limited to the life stage in which an individual experiences them, rather effects can "carry over" or manifest in subsequent stages (Pechenik 2006). Specifically, stressors that affect larval growth, the timing of metamorphosis, and metamorphic body size can influence adult growth, survival, and reproduction (Prout and McChesney 1985). For many amphibians that utilize aquatic habitats as larvae before metamorphosing into terrestrial juveniles and adults, these factors can have long-lasting effects. However, with a majority of the available data focused on environmental stressor effects on amphibian aquatic stages, relatively little is known concerning terrestrial effects and how both affect populations over time (Vonesh and De La Cruz 2002; Salice et al. 2011).

The field of ecotoxicology seeks to determine the effects of environmental contaminants on wildlife populations as they are experienced in actual ecological

systems. Yet current models used for environmental risk assessment rely heavily on extrapolation from acute dose-response relationships tested in controlled laboratory settings. This approach, combined with the use of model organisms as surrogates for entire taxa, does not account for many ecological processes that ultimately affect exposure outcomes under field conditions (Boone and James 2005). With 2,500 new pre-manufacture notices being submitted to the U.S. EPA annually (U.S. EPA 1997), there is need for rapid testing of many chemicals across multiple taxa. Optimization of resources (e.g. funding, time) while minimizing variability in results for each chemical tested can make long-term or field-based testing logistically difficult. However, these data must be predictive on an environmentally relevant scale to determine the population-level effects of contaminants on wildlife, a central issue in the field of ecotoxicology and environmental risk assessment (Calow and Forbes 2003). While incorporating more intricate and ecologically informative testing and modeling techniques are more common, there remains a paucity of data for many non-model organisms and long-term effects on individuals (Calow and Forbes 2003).

Amphibians and reptiles are underrepresented in ecotoxicity data (Sparling et al. 2000) despite evidence that environmental contamination is contributing to global amphibian declines (Carey et al. 1995; Lannoo 2005). However, a majority of amphibian ecotoxicity data stems from aquatic testing, leaving terrestrial effects relatively underrepresented (Sparling et al. 2000; Boone and James 2005). The complex life histories of amphibians – including occupation of different ecological niches in aquatic and terrestrial habitats – should necessitate integrated aquatic/terrestrial ecotoxicity (Boone and James 2005). This is particularly misplaced when measurable endpoints from

aquatic testing are not predictive of adult life, such as time to and size at metamorphosis which can have weak predictive ability of population dynamics due to variable terrestrial survival (Folt et al. 1999; Earl and Semlitsch 2013; Kimberly and Salice 2013). Indeed post-metamorphic survivorship can be more variable and have greater impacts on population growth rates than embryonic or larval vital rates (Biek et al. 2002). Though beyond survivorship, the sublethal effects of contaminant or other stressor exposure can persist well into terrestrial life (Peckarsky et al. 1993; Pahkala et al. 2001; James and Semlitsch 2011). All told, these lethal and sublethal effects of early life-stage exposures suggest that incorporation of subsequent stages to make testing more accurate.

Therefore, we sought to address some knowledge gaps in the amphibian ecotoxicity literature by conducting a manipulative experiment incorporating greater environmental relevance. Utilizing outdoor mesocosms is an effective way to increase realism while retaining control of the factors being tested (Boone and James 2005). Any given contaminant will, in reality, be experienced along with multiple other stressors and persistent contaminants can affect multiple generations. Therefore, we designed a fully factorial mesocosm experiment in which we examined the singular and cumulative effects of exposure to multiple stressors by amphibian larvae and then observed individuals for five months post-metamorphosis. Because of the potential for interactions of aquatic stressors on larval growth and development, we chose to examine two chemical stressors – a metal contaminant and predator cue – which independently have been shown to cause similar effects in amphibian larvae. Additionally, as parental exposure to contaminants can be maladaptive for offspring (Marshall and Uller 2007), we were also interested in examining the impacts of stressor exposure in the parental

generation on offspring. Thus, we also incorporated the parental generation by utilizing offspring from adults sourced from wetlands with known differential contaminant histories to examine how parental metal exposure may influence offspring response to novel stressors. But before delving into the experiment, we will discuss some of the facets of amphibian life history and how environmental stressors may affect individuals and populations.

AMPHIBIAN LIFE HISTORIES

Most amphibian larvae are restricted to the aquatic habitat in which they hatched – subject to its specific environment and finite resources until metamorphosis. Thus, factors such as competition, resource availability, predation, and environmental stressors play important roles in larval survival, growth, and development. The timing of metamorphosis incorporates the potential costs and benefits of the aquatic and terrestrial environment, in order to minimize the risk of death and optimize growth in both (Werner 1986). Metamorphic size and timing are dependent upon growth and development rates (Wilbur and Collins 1973; Travis 1984) which are linked to the quality of the aquatic environment and available resources (Alford and Harris 1988). Therefore, in cases where environmental stressors affect larval physiology and behavior, the overall impacts may be seen in larval period and size upon metamorphosis. Factors that reduce foraging behavior, for example, may lead to slow larval growth (Relyea 2004) or development (Skelly 1992).

When amphibian larvae reach the minimum size at which metamorphosis can occur, they can either transition, resulting in small metamorphic size, or continue to grow

in order to maximize size at metamorphosis (Wilbur and Collins 1973). Although there may be benefits to metamorphosing at a smaller size – for example, to escape a drying pond, aquatic predators, disease, avoid predation or toxicants – emerging at a larger size increases fat reserves (Scott et al. 2007) and the ability to forage and compete for resources (Cabrera-Guzmán et al. 2013). Together, these often result in higher terrestrial survivorship (Berven 1990; Rothermel and Semlitsch 2006; Tarvin et al. 2015) and may reduce time to reach maturity (Berven and Gill 1983; Scott 1994). Furthermore, this can increase size at first reproduction (Semlitsch et al. 1988) and fecundity (Peckarsky et al. 1993; Vargas et al. 2012).

However, given adequate terrestrial environmental conditions, compensatory growth may allow small metamorphs to catch up and reach maturity at a comparable time and size as larger metamorphs (Morey and Reznick 2001; Gramapurohit 2009; Dahl et al. 2012). But this may not occur uniformly among sexes (Morey and Reznick 2001), which can affect the demography of a population as well as the reproductive success of individuals. Increased mating success has been associated with larger relative size in males (Howard 1980) and similarly, females can produce larger clutches and/or larger eggs with increased size (Semlitsch 1985). Yet overcoming this size discrepancy by increasing terrestrial growth may not produce the same results between similarly-sized individuals. Compensatory growth has been shown to reduce fecundity (Auer et al. 2010) and can also reduce reproductive investment for multiple breeding seasons (Lee et al. 2012). Further, negative effects of compensatory growth on longevity and accrued cellular damage that occur in invertebrates, vertebrates, and plants can impair individual fitness (Mangel and Munch 2005). Ultimately, the costs and benefits of growth strategies

depend on the environment (Orizaola et al. 2014), thus it is necessary to take other factors, such as contaminants or the threat of predation, into consideration.

AMPHIBIAN ECOTOXICOLOGY AND LATENT EFFECTS

Ultimately, to understand how stressors affect amphibian populations we must have data that are as environmentally realistic as possible. Larvae face the potential for lethal and sublethal effects from pond drying, predation, disease, UV exposure, competition, habitat loss and the effects of aquatic toxicants (Blaustein et al. 2011). Then upon metamorphosis novel exposure to stressors in the terrestrial environment can impact terrestrial survival (Brühl et al. 2013) and reproductive success (McCoy et al. 2008). As reviewed above, environmental conditions – which are increasingly altered by anthropogenic activity – impact amphibian life history characteristics and strategies. Many common stressors, such as predation, environmental contamination, disease, and wetland drying, can affect larval survival, growth and development (reviewed in Blaustein and Kiesecker 2002). The demography of a given population of amphibians can be altered by environmental stressors experienced in the larval environment with persistent effects throughout adulthood (Scott 1994).

Many stressors elicit carry-over or latent effects, the latter of which are the delayed effects of embryonic or larval experiences that only become apparent in subsequent life stages (Pechenik 2006). Pechenik (2006) highlighted that latent effects occur across broad range of taxa – from sea sponges to insects to mammals – and that various early life-stage experiences have been documented as causing these effects, including delayed metamorphosis, food or water deprivation, and environmental

contaminants. If latent effects occur during the larval stage due to embryonic contaminant exposure, this can be more readily documented. For example, Budischak et al. (2008) observed when embryonic exposure to malathion increased tadpole susceptibility to trematode infection seven weeks post-exposure. Nonetheless, due to the typically short testing periods, usually limited to early life-stages, involved in a majority of ecotoxicity testing, latent effects of contaminants may be missed. For example, maternally-derived polychlorinated biphenyl can affect juvenile snapping turtle (*Chelydra serpentina*) survival beginning eight months after hatching (Eisenreich et al. 2009). Likewise, chronic larval cadmium exposure can decrease terrestrial survival in southern leopard frog (*Lithobates sphenocephalus*) juveniles (James and Semlitsch 2011). Evidence of such occurrences are especially important for amphibians, for which Todd et al. (2012) noted ecotoxicity data is lacking for terrestrial effects and many studies have indicated that juvenile survivorship is critical for population growth or persistence (Biek et al. 2002; Vonesh and De La Cruz 2002; Harper et al. 2008).

COPPER AS A CONTAMINANT STRESSOR

Metal contamination occurs globally, and amphibians utilize wetlands that can be impacted by anthropogenic pollutants (Carey et al. 1995; Bishop et al. 1999). One common metal contaminant is copper (Cu), which is an essential micronutrient found naturally in the environment. However, it has many applied uses in pesticides (Zhou et al. 2011), algaecides (Song et al. 2011), antifouling agents (Simpson et al. 2013) and antimicrobial surfaces (Grass et al. 2011). Currently, Cu contamination by engineered Cu nanomaterials used in consumer goods has also erupted as a global issue (reviewed in

Bondarenko et al. 2013) due to potential toxic effects in non-target organisms such as invertebrates (Pradhan et al. 2012; Han et al. 2014), fishes (Griffitt et al. 2007; Isani et al. 2103), and amphibians (Nations et al. 2015). Due to intentional application to wetlands (e.g. noxious plant control) and agricultural fields, as well as unintentional discharge from mining and industry, Cu can be transported to surface waters (Banas et al. 2010; Wang et al. 2014). Even constructed wetlands designed to mitigate Cu input into the environment are used by wildlife (Lance et al. 2012, 2013), especially when loss of more suitable habitat encourages use for breeding (Pechmann et al. 2001). Additionally, because some metal contaminants, such as Cu, adhere to organic compounds, decreasing bioavailability in aquatic habitats, chronic exposure may not be emphasized. However, bioavailable Cu could occur often and chronically due to repeated or continual discharge or application (e.g. Zhou et al. 2011; Simpson et al. 2013). Therefore, exposure has the potential to occur chronically and across generations. The U.S. Environmental Protection Agency's (EPA) published water quality criteria reports that natural Cu concentrations in freshwater systems can range from 0.20 – 30 µg/L but in highly impacted areas, surface water concentrations can reach 200,000 µg/L Cu. Currently, the EPA has an established Criterion Maximum Concentration of 2.337 µg/L Cu for freshwater species, which is based on Final Acute Value (FAV) data of LC50 values from 350 tests. This FAV value should protect 95% of species; however, of the 27 genera represented in these data, only one amphibian species was represented (U.S. EPA 2007). Moreover, the one represented species' sensitivity is less than that observed in other North American amphibian species (Brown et al. 2012; Flynn et al. 2015).

Copper is ranked the second highest contaminant risk to amphibians due to its ubiquitous distribution and toxic effects at low concentrations (Fedorenkova et al. 2012). Exposure in aquatic organisms has been shown to increase oxygen consumption, reduce swimming speed, decrease lymphocytes and increase neutrophils, alter Cu-dependent and independent enzyme activities, and proliferation of epithelial cells to gills or intestines (Handy 2003). In amphibians, Cu can slow larval growth and increase larval period (Chen et al. 2007; García-Muñoz et al. 2009), reduce size at metamorphosis (Peles 2013), and decrease survivorship (Lance et al. 2013; Flynn et al. 2015). The larval stage may be most sensitive to metal stressors (Franco de Sá and Val 2014) due to lethality at low concentrations (Lance et al. 2012, 2013) and also sublethal effects which can increase vulnerability to other environmental threats, such as predation (McIntyre et al. 2012). As such, very few studies have examined post-metamorphic and terrestrial effects of contaminant exposure (see Bergeron et al. 2011; Todd et al. 2012). This knowledge gap is important because of the potential for latent effects associated with embryonic and larval exposure to metals, which can increase spinal malformation rate at the onset of metamorphosis (Todd et al. 2011a), and negatively affect juvenile survival (Bergeron et al. 2011) and growth (Todd et al. 2012). Such effects that influence survivorship and reproductive success (i.e., fitness) affect population dynamics (Willson et al. 2012) and, therefore, effects in this life stage should be examined more extensively, especially considering that very few studies have done so to date.

PREDATOR THREAT AS A NATURAL STRESSOR

Predation is a common, natural stressor that co-occurs with anthropogenic stressors, such as contaminants. Predation directly reduces prey numbers, which then indirectly impact density-dependent processes by alleviating conspecific competition. Further complexity arises when predator and conspecific alarm cues cause alterations in prey behavior. In the aquatic environment, exposure to these cues can elicit antipredator behavior, such as reduction in swimming speed (Relyea 2004), increased refuge use (Bridges 2002), and decreased activity (Ferrari et al. 2008). Furthermore, the combination of predator and conspecific alarm cues typically results in an even greater response (Maag et al. 2012). If larvae are able to avoid predation but continue to respond to cues, several other effects associated with avoidance behavior occur, including: altered morphology (Maher et al. 2013; McCollum and Leimberger 1997) and increased oxidative stress (Bopp et al. 2008; Janssens et al. 2014) which could make individuals more vulnerable to other stressors, such as disease. Thus, the indirect effects of predator exposure may outweigh the direct impacts of predation on population dynamics (McPeek and Peckarsky 1998) through decreased larval growth rate (Relyea and Werner 1999), prolonged larval period (Skelly 1992), and reduced size at metamorphosis (Kerby et al. 2011).

However, aquatic exposure to predators can produce beneficial carry-over effects that help metamorphs and juveniles avoid terrestrial predation. The transitory period of metamorphosis – when the presence of forelimbs or a tail can impede swimming or hopping ability – represents a time of increased vulnerability to attack (Wassersug and Sperry 1977; Arnold and Wassersug 1978). Factors, such as the time to forelimb

emergence and tail resorption, drastically affect an individual's risk of predation. Thus, it can be beneficial when prior exposure to predators decreases the time to complete metamorphosis and increases locomotor ability post-metamorphosis (Van Buskirk and Saxer 2001). However, anurans can compensate for impaired locomotor ability by reducing activity in the presence of a predator during both aquatic and semi-terrestrial transition stages. Reduced activity results in decreased predation rates overall (Touchon et al. 2013), but trade-offs to this decreased activity, such as decreased foraging and growth, can impact survival and reproductive success. Such indirect effects on survival make the threat of predation an important environmental stressor, but more has also yet to be revealed as to its interactions with anthropogenic contaminants of interest. Given the ubiquitous nature of both predation and metal contaminants, there is great potential for simultaneous exposure.

MULTIPLE STRESSORS

Ultimately we are interested in the how environmental stressors together impact populations – as this is how they are experienced in reality – but every wetland is different, thus it is necessary to determine how certain stressors act in combination in order to predict total effects in other scenarios. The cumulative effect is not necessarily additive, but may be synergistic or antagonistic. As reviewed by Crain et al. (2008), multi-stressor studies are underrepresented despite their environmental relevance and the prevalence and unpredictability of interactions. They further note that a high frequency (36%) of studies show synergistic population-level effects but that increasing the

replication of like-stressor studies can eventually render all interaction category types (i.e. additive, synergistic, antagonistic).

Copper and predator cue stressors can interact in ways that affect larval growth and development, as well as terrestrial behavior and survival. The actual ability to detect or respond to either stressor can be mediated by the other. In fish, even brief exposure to low concentrations (2-20 µg/L) of Cu can impair olfactory neuron receptors, resulting in a range of outcomes from increased detection limit thresholds to complete loss of olfaction (Sandahl et al 2007). The olfactory epithelium is present in all vertebrates and, similar to fish, amphibian tadpoles use this for chemoreception (Taniguchi et al. 1995), which enables them to sense predators and conspecific alarm cues (Belanger and Corkum 2009). Thus with olfactory impairment, heavy metal exposure can dampen fright response when predator cues are also present (Lefcort et al. 1998, 2013; Beyers and Farmer 2001; Hernández et al. 2006; Sandahl et al. 2007; McIntyre et al. 2012). But, alternatively, predator cues also decrease heavy metal avoidance when this behavior is possible (Lefcort et al. 2013). Further, exposure to both stressors do not have to occur simultaneously, as experimental predation trials have shown that prior exposure to Cu significantly decreases the ability of prey to detect predator and conspecific alarm cues, leading to loss of antipredator behavior and significantly greater capture rates (McIntyre et al. 2012).

The potential for specific interactions between Cu and predator cues merit further investigation, but the response to a combination of contaminant and predator stressors can be variable in ways that are not initially intuitive. For instance, Hanlon and Relyea (2013) found that exposing bullfrogs (*L. catesbeiana*) to endosulfan reduced mortality

when also exposed to predators, however the same did not apply to green frogs (*L. clamitans*), as predator presence increased mortality with endosulfan exposure. Thus, increasing the available data for multiple stressor effects in more than one species is essential to fully understand how contaminants impact wildlife within a given environment.

MATERNAL EFFECTS

Though the effects of early life stage stressor exposure across an individual's lifetime is crucial to know, these individual-level effects can also be influenced by stressor exposure history in prior generations. Many ecological studies have acknowledged the importance of parental effects, especially maternal effects and their relationship with offspring phenotypic plasticity. As defined in a review by Bernardo (1996a), maternal effects occur when the maternal phenotype directly impacts that of her offspring, and it has been suggested that maternal effects can drive the rate or direction of evolution. Indeed, maternal effects may be adaptive or non-adaptive for offspring (Wilbur 1977; Marshall and Uller 2007).

To date, examination of maternal effects has placed a strong emphasis on propagule size (i.e. egg size for amphibians) and its relationship to offspring survivorship, growth, and development (Laugen et al. 1992; Bernardo 1996b; King and Kaplan 1997). In amphibians, egg size is positively related to hatching success and early larval growth (Semlitsch and Gibbons 1990) but exposure to particular stressors can also alter offspring phenotypes or behaviors in other taxa as well. For instance, gravid *Gryllus pennsylvanicus* crickets exposed to *Hogna helluo* spiders will produce offspring with

greater relative antipredator response than offspring from unexposed mothers (Storm and Lima 2010). Additionally, parental predator exposure can affect tolerance to novel stressors in offspring; as Plautz et al. (2013) have found that freshwater snails (*Physa pomilia*) exposed to predator cues will yield offspring with greater tolerance to cadmium contamination.

Maternal effects can also produce negative effects in offspring. Contaminants can be transferred via the yolk from mother to offspring (Bergeron et al. 2010), which can reduce embryonic and larval offspring viability and health (Hopkins et al. 2006). Such transfer can negatively affect offspring size (Todd et al. 2011a; Bergeron et al. 2011) and increase larval period (Todd et al. 2011a). Because maternal effects can affect development time and an individual's ability to escape a drying wetland (Newman 1988), this can be especially detrimental to amphibians that utilize ephemeral wetlands (Mann et al. 2001). In addition, larval survival is also affected by maternal transfer. Bergeron et al. (2011) found differential mortality of small individuals completing metamorphosis – those from Hg-exposed females experienced high mortality compared to reference offspring. And Metts et al. (2012) found that maternal metal exposure, in addition to novel embryonic/larval metal exposure, can further reduce survivorship to metamorphosis than either exposure individually. Yet, while the transgenerational effects of contaminants are now becoming more widely studied, evaluation of interactions with other stressors present in biological systems is still rare (Pomati and Nizzetto 2013).

THESIS OBJECTIVES

To best understand how stressors affect amphibian populations we must have data that are as environmentally realistic as possible. Therefore, to increase the predictive value of a manipulative study we attempted to incorporate multiple life stages, maternal effects, and exposure to multiple stressors. We focused on the lethal and sublethal effects of aquatic exposure to an anthropogenic (Cu) and a natural (predator cue) stressor on larvae and juveniles of a common amphibian species in the southeastern United States, the southern toad (*Anaxyrus terrestris*). We used offspring of adults collected from heavy metal-contaminated and uncontaminated wetlands and exposed free-swimming larvae to Cu and predator cues until metamorphosis, at which point toads were moved to terrestrial mesocosms for five months. Our objectives were to examine the effects of parental contaminant history and larval exposure to Cu and predator cue stressors on 1) larval survivorship and time to/size at metamorphosis, and 2) juvenile performance, growth, and survivorship.

CHAPTER 2

LARVAL AND POST-METAMORPHIC CONSEQUENCES OF AQUATIC STRESSOR EXPOSURE IN SOUTHERN TOADS (ANAXYRUS TERRESTRIS)[1]

[1] Rumrill, C.T., Scott, D. E., and S. L. Lance. To be submitted to *Ecological Applications*.

ABSTRACT

The complex life histories of amphibians make them susceptible to both aquatic and terrestrial stressors, but we are only just piecing together how exposure in one life stage may affect another. Additionally, exposure to one stressor is almost always coupled with exposure to another, whether it be anthropogenic or natural, and the effects of one on an individual can be altered by interactions. Furthermore, parental exposure to one or many stressors can carry through to offspring, affecting their ability to cope with the same or novel environmental stressors. To examine these issues we employed a 2x2x2 factorial design in outdoor 1000 L mesocosms (n = 24), where larval southern toads (*Anaxyrus terrestris*) bred from parents collected from reference and heavy metal-contaminated sites were exposed to an anthropogenic stressor (copper – 0, 30 µg/L) and natural stressor (predator – present/absent). Upon metamorphosis survivors were transferred to terrestrial mesocosms for five months in order to examine the chronic effects of early life stage exposure. We measured larval and terrestrial survivorship, time to/size at metamorphosis, post-metamorphic growth, and conducted a physiological assay targeting sprinting and endurance performance at 0 and 1 mo post-emergence. We found a significant effect of parental source on size at metamorphosis, larval period, and juvenile survival. Larval Cu exposure also had a significant negative affect on size at metamorphosis, as well as a latent negative effect on juvenile survival. Additionally, a significant source*Cu*predator interaction was present at emergence for endurance performance, wherein larval predator cue exposure increased overall distance and time covered per trial. However, this beneficial effect on performance was negated when Cu was also present in the larval environment. The presence of parental effects, latent

survival effects of the aquatic habitat, and a three-way interaction on juvenile performance highlight the importance of conducting multistressor studies across generations.

Key Words: Amphibian; Ecotoxicology; Multiple stressors; Maternal effects; Latent effects; Copper; Predator cue

INTRODUCTION

Amphibians, as well as other taxa with complex life histories, are susceptible to abiotic and biotic stressors in multiple life stages. Importantly, the effects of stressors experienced at one stage may carry over and/or only become apparent in subsequent life stages (Pechenik 2006). Specifically, stressors that affect larval growth, the timing of metamorphosis, and metamorphic body size can influence adult growth, survival, and reproduction (Prout and McChesney 1985). For many anurans that utilize aquatic habitats as larvae before metamorphosing into terrestrial juveniles and adults, these aquatic stressors can have long-lasting effects.

Larval developmental plasticity is linked to the quality of the aquatic environment and available resources (Alford and Harris 1988). Factors that reduce foraging behavior, for example, may lead to slow larval growth (Relyea 2004) or development (Skelly 1992) which, in turn, affects time to and size at metamorphosis. When amphibian larvae reach the minimum size at which metamorphosis can occur, they can either transition, resulting in small metamorphic size, or continue to grow in order to maximize size at metamorphosis (Wilbur and Collins 1973). Although there may be benefits to metamorphosing at a smaller size – for example, to escape a drying pond, avoid predation or contaminants – emerging at a larger size increases fat reserves (Scott et al. 2007) and the ability to forage and compete for resources (Cabrera-Guzmán et al. 2013), and often results in higher terrestrial survivorship (Berven 1990; Tarvin et al. 2015). Additionally, large size at metamorphosis may reduce time to reach maturity (Berven and Gill 1983) and increase size at first reproduction (Semlitsch et al. 1988) and fecundity (Vargas et al. 2012). However, given adequate terrestrial environmental conditions, compensatory

growth may allow small metamorphs to catch up and reach maturity at a comparable time and size as larger metamorphs (Morey and Reznick 2001; Gramapurohit 2009; Earl and Whiteman 2015). Given the critical importance of the trade-offs that determine larval success and their carry-over effects in the terrestrial stage, we need to better understand the effects of stressors both in aquatic habitat and post-metamorphosis.

Metal contamination occurs globally and amphibians utilize wetlands that can be affected by anthropogenic pollutants (Bishop et al. 1999). The ubiquitous distribution and toxic effects of copper (Cu) at low environmental concentrations have it ranked the second highest contaminant risk to amphibians (Fedorenkova et al. 2012). In amphibians, Cu can slow larval growth and increase larval period (Chen et al. 2007; García-Muñoz et al. 2009), reduce size at metamorphosis (Peles 2013), and decrease survivorship (Lance et al. 2013; Flynn et al. 2015). The larval stage may be most sensitive to metal stressors (Franco de Sá and Val 2014) evident by direct lethality (Lance et al. 2012) and also sublethal effects which can increase vulnerability to other environmental threats, such as predation (McIntyre et al. 2012). However, there have been very few studies that have examined post-metamorphic and terrestrial effects of contaminant exposure (see Bergeron et al. 2011; Todd et al. 2011). Yet of the data available, there is evidence of latent effects in which early exposure to metals increases spinal malformation rate at the onset of metamorphosis (Todd et al. 2011a), and negatively affects juvenile survival (Bergeron et al. 2011) and growth (Todd et al. 2012).

Predation is a common, natural stressor that co-occurs with anthropogenic stressors, such as contaminants. While the direct effect of predation acts to reduce prey numbers, it indirectly impacts density-dependent processes by alleviating conspecific

competition. In addition, complexity arises when predators cause sublethal prey responses such as decreased activity (Ferrari et al. 2008) and increased refuge use (Bridges 2002). Yet latent effects of early predator exposure can induce beneficial terrestrial phenotypes which may improve juvenile predator avoidance (Van Buskirk and Saxer 2001). However, the indirect effects of predator exposure may outweigh the direct impacts of predation on population dynamics (McPeek and Peckarsky 1998) through decreased larval growth rate (Relyea and Werner 1999), prolonged larval period (Skelly 1992), and reduced size at metamorphosis (Kerby et al. 2011).

Environmental conditions – which are increasingly altered by anthropogenic activity – affect amphibian life history characteristics and strategies. Many common stressors, such as predation, environmental contamination, disease, and wetland drying, can affect larval survival, growth and development. Taken together, the interactions of stressors can have complex consequences that are not necessarily the cumulative effect of each component, but may include synergistic or antagonistic effects. Yet multi-stressor studies are underrepresented despite their environmental relevance and the prevalence and unpredictability of interactions (Crain et al. 2008). Given the ubiquitous nature of both predation and metal contamination, simultaneous exposure can occur. Interestingly, the actual ability to detect or respond to either stressor can be mediated by the other. Even brief exposure to low concentrations (2-20 µg/L) of dissolved Cu can impair olfactory neuron receptors, resulting in a range of outcomes from increased detection limit thresholds to complete loss of olfaction (Sandahl et al 2007). Thus, heavy metal exposure can dampen fright response when predator cues are also present (Lefcort et al. 1998, 2013) but, alternatively, predator cues also decrease heavy metal avoidance when

this behavior is possible (Lefcort et al. 2013). Thus, we were interested in the potential for either an additive or synergistic interaction, wherein both stressors reduce foraging activity of larvae and thus growth and development; or, alternatively, an antagonistic interaction wherein the presence of one stressor reduces the effect of the other.

Exposure to stressors in early life stages cannot only affect an individual's lifetime, but may also affect future generations. Many ecological studies have acknowledged the importance of parental effects of stressors, especially maternal effects and their relationship with offspring phenotypic plasticity (Bernardo 1996a). Indeed, maternal effects may be adaptive or non-adaptive for offspring (Marshall and Uller 2007), and typically maternal exposure to metals reduces embryonic and larval offspring viability and health (Hopkins et al. 2006). Furthermore, maternal metal exposure in addition to novel embryonic/larval metal exposure can further reduce survivorship to metamorphosis (Metts et al. 2012). Yet transgenerational examination of long-lived environmental contaminants interacting with other stressors present in biological systems is rare (Pomati and Nizzetto 2013). Because metal contaminants often occur in the environment chronically and the threat of predation is likely consistent over time, it is plausible that amphibians, as well as other taxa in aquatic ecosystems, experience these stressors over generations. As parental exposure to contaminants is likely non-adaptive for offspring, we were also interested in examining the implications of stressor exposure in the parental generation on offspring that were exposed to novel contaminant and predator cue stressors.

To understand how stressors affect amphibian populations we must have data that are as environmentally realistic as possible. Therefore, to increase the predictive value of

a manipulative study we attempted to incorporate multiple life stages, maternal effects, and exposure to multiple stressors. We focused on the lethal and sublethal effects of aquatic exposure to an anthropogenic stressor (Cu) and a natural stressor (predator cue) on larvae and juveniles of a common amphibian species in the southeastern United States, the southern toad (*Anaxyrus terrestris*). We used offspring of adults collected from heavy metal-contaminated and reference wetlands and exposed free-swimming larvae to Cu and predator cues until metamorphosis, at which point toads were moved to terrestrial mesocosms for five months. Our objectives were to examine the effects of parental contaminant history and larval exposure to singular and concurrent Cu and predator cue stressors on 1) larval survivorship and time to/size at metamorphosis, and 2) juvenile performance, growth, and survivorship.

METHODS

Study species

We collected adult southern toads at drift fences between March 31st and April 10th, 2014 at two locations on the U.S. Department of Energy's Savannah River Site (SRS), Aiken County, SC – one site with 30 years of coal fly ash contamination (Metts et al. 2012), D-Area, and one reference site, Ellenton Bay (E-Bay). Adults were moved to the University of Georgia's Savannah River Ecology Laboratory (SREL), Aiken, SC and bred via artificial insemination. To induce egg laying we injected gravid females with 250IU of human chorionic gonadotropin and moved them to plastic containers (33 x 20 x 13 cm) with simplified amphibian ringers solution on April 15 (D-Area) and April 17-18, 2014 (E-Bay). We massaged females to expel remaining eggs and distributed all eggs

into eight 60mL plastic weigh cups. To obtain sperm we first euthanized males in 3% MS-222 and then extracted testes and placed them into separate 1.5mL microcentrifuge tubes containing 500µL soft water – 48 mg/L $NaHCO_3$, 30 mg/L $CaSO_4$, 30 mg/L $MgSO_4$, and 2 mg/L KCl dissolved in nanopure Milli-Q water (Millipore). We gently homogenized each teste with a plastic pestle and diluted with 1.0mL soft water then immediately pipetted onto eggs. After 15 min, we flooded eggs with soft water and left them overnight at room temperature (24°C). After development was observed, embryos were transferred to plastic containers (33 x 20 x 13 cm) and allowed to develop and hatch in the SREL Animal Care Facility in water collected from E-Bay at 12:12 light dark cycles and approximately 22°C. On April 30 we pooled free-swimming larvae of parents from the same source location. Offspring from each source population were representative of 8 female and 32 male full/half sib crosses (total of 64 families/population) established for a separate study (Flynn et al. unpublished data). Larvae were acclimated to outdoor tank water and temperature prior to distribution by first placing into plastic containers with 50/50, E-Bay/control tank water for 2.5 hours, then to containers floating in tanks with 100% respective treatment tank water for 2.5 hours. After acclimation, we randomly distributed larvae to mesocosms (n = 120/tank), keeping each parental source separate (n = 12 tanks/source).

Aquatic mesocosms

We used a fully factorial design with randomized complete spatial blocking of two parental source populations (D-Area/E-Bay) x two levels of Cu (0, 30 µg/L) x two levels of predator (present/absent). Because larvae within each tank experienced the same conditions, tanks were considered the experimental unit (n = 3 tanks/treatment). We used

1000-L polyethylene cattle tanks located at SREL (n = 24 tanks), and randomly assigned three tanks to each treatment (n = 8). Each tank was filled with well water, 1-kg dry leaf litter, 3 L of algal inoculant collected from multiple sites on the SRS, one of which included algae from a Cu-contaminated wetland. Additionally, all tanks contained an iButton® protected within a Whirl-Pak®, which was randomly placed in the leaf litter/refugia and set to record temperature at 120-min intervals. Each tank also had an external standpipe to prevent overflowing during rain events, and a 60 percent shade cloth lid that doubled as shade and predator exclusion. All tanks sat with water, leaf litter, and algal inoculant for 24 days prior to the addition of larvae. We checked for and removed unintended predators daily and collected water quality data (pH, conductivity, DO) weekly using an YSI Professional Plus Quatro multiprobe. Over seven weeks the average water quality data across tanks were: 6.57 ± 0.21 (pH), 102.96 µS/cm ± 16.09 (conductivity), and 46.07% ± 12.82 (DO).

Copper treatments

We dosed tanks with copper sulfate ($CuSO_4$) solution 24 days before adding larvae and re-dosed weekly to reach a stable Cu concentration. We used a Cu concentration of 30 µg/L because it is within the range of environmental concentrations (Flynn et al. 2014) and previous studies demonstrated that it negatively affects southern toad larvae (Lance et al., 2013; Chen et al. 2007). Each week we analyzed water samples by either inductively coupled plasma mass spectrometry (ICP-MS) or optical emission spectroscopy (OES) to monitor concentrations and then added the necessary amount of $CuSO_4$ to recalibrate treatment tanks to 30 µg/L Cu. During the experimental period, tank

concentration averages ranged between 21.9 – 25.3 µg/L Cu with an overall average of 24.13 µg/L Cu (± 3.94).

Predator treatments

We collected larvae of the most abundant species of dragonfly (*Anax* sp.) present in the field to ensure relevant predator exposure. We housed dragonfly larvae in cylindrical mesh cages (approximately 15 cm in diameter by 60 cm in height) that allowed predatory and conspecific alarm cues to emanate while preventing predation of experimental toad larvae. Cages contained leaf litter and were fitted with floatation on one end and a small rock on the other to allow each cage to span the entire water column and move freely about the tank. Predator-present treatment tanks contained two cages, each with one dragonfly larvae that was fed approximately 300 mg of southern toad larvae (Maher et al. 2013; obtained as unused larvae from another experiment) 3x weekly and dragonflies were replaced upon death. We fed predators toad larvae for 21 days, finishing one day before the main pulse of toad metamorphosis ended. Predator-absent tanks contained two sham cages without dragonfly larvae but were constructed identically to those in predator-present tanks. We lifted and re-submerged all predator and sham cages during predator checks/feedings.

Emergence and terrestrial mesocosms

We checked tanks every morning for evidence of emerging metamorphs. We hand-captured metamorphs and combined up to 20 from each tank in 33 x 20 x 13 cm plastic containers (using multiple containers/tank as needed) with a paper towel moistened with tank water. Metamorphs were kept in these containers in the SREL

greenhouse facility until tail resorption – typically 2 days – but were monitored daily to remoisten paper towels.

Upon tail resorption, we moved metamorphs to durable 68-L plastic bin terrestrial mesocosms in the greenhouse. Terrestrial mesocosms contained 8 cm of sandy soil over small rocks to ensure drainage, 250 g of rehydrated coconut fiber for continued moisture, one water dish, two flower pot shelters, and a snapping mesh lid. Metamorphs from each aquatic mesocosm were divided between two terrestrial bins ("A" and "B" for n = 48 terrestrial bins), with "A" bins containing metamorphs that emerged during the main pulse (May 23-27, except for T2 on June 1-6) and "B" bins with toads post-emergence peak (median density: "A" = 34, "B" = 19; range: "A" = 7-58, "B" = 0-41). Approximately 2 mo after the main emergence pulse, we haphazardly redistributed a subset of toads from "A" bins to "B" bins to keep densities similar, at which point no bin exceeded 24 toads (i.e., before: density range = 4-47, median density = 19.5; after: density range = 4-24, median density = 16); two aquatic mesocosms (T16 and T18) required a third terrestrial bin ("C") due to the large number (>30 in a bin) of surviving metamorphs.

We monitored metamorphs and later, juveniles, daily to record and remove dead individuals, refill water dishes and moisten coconut fiber. Dead individuals were removed from bins, placed into Whirl-Paks® and frozen at -20°C to be used for metals analysis. We pooled individuals collected over defined time periods (i.e., 0-1 mo or 2-3 mo post-metamorphosis) to analyze body burdens of Cu for a separate study. Our measure of survivorship was based on the minimum known number alive using both daily checks and monthly bin counts (starting at 1 mo post-metamorphosis). Toads were

fed a combination of crickets and field-caught termites 3x weekly, with crickets receiving a dusting of RepCal® and Herpitivite® dietary supplements. We rationed the amount of food each bin received based on density to ensure an equal amount of available food per toad. We collected monthly measurements of mass and snout-vent length (SVL) for post-metamorphic growth on subsets of up to10 randomly collected toads from each "A" bin (with the exception of those from tank 16 (T16) – a husbandry error resulted in the release of six incorrect toads into T16-A at the end of month one, so we collected terrestrial survival and growth estimates for T16 from T16-B; 80% of toads in T16-B were from the main emergence pulse).

Performance assay

We conducted two performance trials approximately 1 wk (5-7 d) after tail resorption and 1 mo later (denoted 0 and 1 mo Trial, respectively) to test for any latent physiological effects of treatments that could affect terrestrial predation rates. Subsets of 10 randomly selected toads from each "A" bin (i.e., the main emergence pulse) were measured (mass and SVL) and up to 7 of these were randomly selected for the performance trial (n = 21 toads/treatment combined from three aquatic mesocosms). Measured toads were transferred to individual 10 x 10 cm plastic containers with a moist paper towel and fed 3 days before, then immediately after trial completion. Trials occurred from June 2-5, 2014 (0 mo Trial) and July 7-10, 2014 (1 mo Trial) with the exception of toads from larval tank 2 (T2), which emerged 1 wk after the main pulse, that we ran on June 12 and July 14, respectively.

The performance track consisted of a 3-m portion of vinyl fence rail, cut such that the center/top was open and the sides high enough to prevent escape. The track had a

starting line at one end and markings at 1 cm increments. To initiate a trial, we placed a toad at the starting line and began a stop watch. We "followed" toads down the track with a foam brush "predator" and when toads stopped, they were gently tapped on the urostyle. Each trial was terminated once a toad was tapped 10x and did not move. Tracks were rinsed with water and squeegeed between each trial. We recorded the distance covered in the first 30 s of the trail (sprint distance), the overall distance covered before the end of the trial (endurance distance), the time to reach the end of the trial (endurance time), and the number of taps a toad received when encouraged to hop (taps); distances were recorded to the nearest cm. Toads were returned to their appropriate terrestrial bins immediately after trials.

Statistical analyses

For our analyses of all response variables we used the aquatic tank (or corresponding terrestrial bin) as our experimental unit to avoid psuedoreplication; i.e., we averaged data collected on individuals to obtain tank means or, for survival, we used proportion surviving per tank. For terrestrial escape behavior and growth we also used only individuals from the emergence pulse by sampling from "A" bins (excepting T16-B). We analyzed size at metamorphosis, larval period and larval survivorship using ANOVA (Type III SS) of fitted linear mixed effects models using the anova.lme() function in the nlme package of R (v. 3.0.3). We tested residuals for normality using a Shapiro Wilk's test but did not find deviation from normality. We used Tukey's HSD for post hoc comparisons analysis for ANOVAs. Originally, we included larval spatial block as a factor in our full models but removed it as it was not significant for any endpoint. Similarly, since we hypothesized the potential for interactions we initially included all

three main effects, plus all 2-way and the 3-way interaction term into our models, but if interactions were non-significant we removed them and examined only main effects in the reduced model.

For terrestrial performance we used analysis of covariance (ANCOVA) to account for body size before examining treatment main effects. As with the aquatic response variables, we used tank means to test the main effects of parental source, Cu, and predator treatments. We tested residuals for normality using a Shapiro Wilk's test and log transformed endurance distance/time and tap data across 0 and 1 mo assays to meet assumptions of normality. We tested parental source, Cu, and predator cue effects on juvenile mass and SVL across time (0-5 mo post-emergence) using a multivariate analysis of variance (MANOVA) with the manova() function in R (v. 3.0.3). Finally, we examined the effects of parental source and larval treatment on juvenile survivorship from the time toads were placed into terrestrial bins to the end of the study (5 mo). We used all juveniles (i.e. from bins "A" and "B") for survivorship analysis because the majority of toads emerged during between May 21-June 7 and were, thus, approximately the same age. For our analysis of juvenile time-to-death we used time-to-event analysis (i.e., survival analysis; PROC LIFETEST, SAS v.9.3), which is a nonparametric log-rank test that uses a chi-square test statistic to determine whether survival curves differ among treatment groups; because we expected mortality rates to be greatest soon after metamorphosis we used the Gehan-Wilcoxon test, which is more sensitive to survival differences at earlier times (Dixon and Newman 1991). We assessed time-to-death differences for each main effect (i.e., between Cu levels, parental source populations, and predator levels); we treated juveniles that survived to 5 mo as right-censored data. For

main effects that significantly affected time-to-death, then we also tested 2-way interactions.

To test effects on overall juvenile survivorship we used a generalized linear mixed model (PROC GLIMMIX, SAS v. 9.3) beginning with a full model with three main effects and all 2- and 3-way interactions. We removed non-significant ($P > 0.05$) interactions from the final model, and ran a reduced model that tested solely for the main effects of Cu, predator level, and parental source on survivorship. Because our treatment groups had only two levels each, there was no need to conduct post hoc Bonferroni pairwise tests of treatment means. In these models we used a random statement to define terrestrial bin as the subject to account for the nesting of toads within bins.

RESULTS

Larval effects

We found no significant effect of parental source or larval treatment on survivorship to metamorphosis (Fig.1; Table 1). The effects of Cu and predator cue stressors individually had the same relative effect on average survivorship regardless of source indicating that, in this case, it was the presence of a stressor and not the type that led to such an outcome. Interestingly, the combination of these stressors yielded contrasting trajectories depending on parental source compared to either single stressor. Here, E-Bay offspring responded to multiple novel stressors with the greatest number of survivors on average while D Area offspring had the lowest average survivorship compared to all control and treatment groups.

The average time for larvae to reach metamorphosis, however, was significantly affected by parental source ($F_{1, 20} = 11.527$, $P = 0.003$) in our reduced model (Table 1). All E-Bay treatment groups emerged earlier than corresponding D Area treatments, except for the Cu treatment where there was no significant difference between sources (Fig.2). A multiple comparisons test with a full model indicated significant differences between sources for larval control treatments ($P < 0.009$) and predator cue-only treatments ($P = 0.016$), where E-Bay offspring emerged before D Area offspring in both cases.

There was a significant effect of parental source on average metamorph size (mass and SVL) 1 wk post-emergence (Mass: $F_{1, 20} = 7.136$, $P = 0.015$; SVL: $F_{1, 20} = 13.245$, $P < 0.002$), where offspring of parents from D-Area were smaller than those from E-Bay (Fig. 3 and 4-A; Table 1). Additionally, metamorph SVL was also negatively affected by larval Cu treatment ($F_{1, 20} = 4.768$, $P = 0.041$), where 30 µg/L Cu treatments were smaller than those exposed to 0 µg/L Cu (Fig. 4-B).

Performance assay

Our ANCOVAs indicated that body size (SVL) significantly affected sprint and endurance distance and endurance time at 0 and 1 mo post-metamorphosis (Table 2). After accounting for body size, at metamorphosis there was a significant 3-way interaction of parental source*Cu*predator on endurance time ($F_{1,15} = 6.564$, $P = 0.022$), which was also marginally significant for endurance distance ($F_{1,15} = 4.158$, $P = 0.06$). Here, it was the E-Bay–0 Cu–predator group that traveled the farthest, for the greatest total time, and in so doing accrued the greatest number of taps on average (Fig. 5 A-D).

The performance trials occurring 1 mo post-emergence indicated a general loss of larval treatment effects, as there were no significant results due to main effects (Fig. 5 E-H).

Terrestrial survivorship and growth

Post-metamorphic survivorship was measured at 6 time intervals – 11, 59, 101, 121, 148, and 170 d post-emergence – using the total number of emerged metamorphs as the starting value. Both parental source and larval Cu exposure affected post-metamorphic time-to-death of juveniles (source: Wilcoxon $\chi^2 = 13.638$, $P = 0.0002$; Cu: Wilcoxon $\chi^2 = 7.31$, $P = 0.0068$); there was no effect of larval predator presence on post-metamorphic time-to-death (Wilcoxon $\chi^2 = 0.08$, $P = 0.7729$). The four Cu by source treatment combinations also differed in time-to-death, with time-to-death significantly delayed in the E-Bay – 0 µg Cu/L treatment compared to all other groups (Wilcoxon $\chi^2 > 9.2$, $P < 0.01$) (Fig. 5). We found a significant effect of both parental source ($F_{1,42} = 4.6$, $P = 0.0384$) (χ^2 (1, N = 1189) = 12.4, $P \leq 0.0001$) and larval Cu treatment ($F_{1,42} = 10.5$, $P = 0.0024$) (χ^2 (1, N = 1189) = 5.1, $P \leq 0.0241$) on overall survival to 170 d post-emergence (Fig. 5). The odds of surviving 5 mo post-metamorphosis were 53% higher for juveniles without larval Cu exposure, and 25% lower for individuals of parents from a contaminated source population (D Area). As such, parental exposure to heavy metals or larval exposure to Cu reduced survivorship significantly 5 mo post-metamorphosis. Examining the simplified model (Fig. 6), we see that the E-Bay–30µg/L Cu and D Area–0 µg/L Cu have a similar negative trend in survival, but that the fewest proportion surviving were from the D Area–30µg/L Cu treatments. The highest percent of juveniles surviving five months post-metamorphosis were offspring of reference source parents who were not exposed to Cu as tadpoles (39% ± 0.061), with predator cue exposure

slightly decreasing survival (36% ± 0.039) (see Table 3). The lowest survival was in the groups exposed to larval Cu but not predator cues, regardless of parental source (18 and 21% for E-Bay and D Area, respectively). Similar proportions (26-27%) of juveniles survived to 5 mo post-emergence in the groups from both parental sources exposed to both larval stressors, as well as the D Area offspring control.

Our MANOVAs identified that parental source had a significant effect on post-metamorphic SVL (Wilk's $\lambda = 0.329$, $F_{5,12} = 4.895$, $P = 0.011$) and marginally significant effect on mass (Wilk's $\lambda = 0.381$, $F_{5,12} = 3.903$, $P = 0.025$) between 0-5 mo post-emergence (Fig. 4-A). Given these significant multivariate effects, we further investigated differences for each time point using a univariate approach. Significant differences in mass between parental source treatments occurred upon emergence ($F_{1,16} = 6.6433$, $P = 0.02$) with offspring of adults sourced from E-Bay emerging larger on average, but at 1 mo ($F_{1,16} = 4.7499$, $P = 0.045$) and at 3 mo post-emergence ($F_{1,16} = 11.766$, $P = 0.003$) this trend switched with offspring of adults collected from D-area being significantly larger on average. Similarly, parental source affected juvenile SVL at emergence ($F_{1,16} = 12.063$, $P = 0.003$) with E-Bay offspring larger than D Area offspring (Fig. 3) but at 3 mo post-emergence ($F_{1,16} = 10.385$, $P = 0.005$) D Area offspring are significantly larger than E-Bay (Fig.4). Copper treatment also had a marginally significant effect on metamorph SVL at emergence ($F_{1,16} = 4.343$, $P = 0.054$), with toads from the 0 µg/L Cu treatments emerging larger on average than those from the 30 µg/L Cu treatment. For both mass and SVL the largest difference in average size occurred at 3 mo yet after that point size did not differ significantly. Additionally, when examining growth rate from one month to the next there was a significant effect of larval predator

(SVL: $F_{1,16} = 5.199$, $P = 0.037$) and parental source (SVL: $F_{1,16} = 15.683$, $P = 0.001$; Mass: $F_{1,16} = 10.147$, $P = 0.006$) between 0-1 mo post-emergence; and, additionally parental source between 2-3 mo post-emergence (Mass: $F_{1,16} = 15.883$, $P = 0.001$).

DISCUSSION

Environmental contaminant and predator stressors have typically been examined singularly rather than in combination, which makes predicting their true ecological consequences a challenge. We saw an antagonistic interaction between larval Cu and predator stressors for metamorph performance. Additionally, the combination of being exposed to Cu during the larval stage and having parents from a metal-contaminated environment had synergistic negative effects on juvenile survival. Considering this, more investigation is merited to determine how these common, co-occurring stressors interact. Both Cu and predator cues elicit similar effects in aquatic organisms including reduced swimming speed (Handy 2003; Relyea 2004) and activity (Redick and LaPoint 2004; Ferrari et al. 2008) and increased oxidative stress (Handy 2003; Bopp et al. 2008; Janssens et al. 2014). However, the indirect costs of Cu exposure can increase the risk of predation due to increased rates of malformations (Zhu et al. 2014), erratic swimming (García-Muñoz et al. 2011), lateral line damage (Hernández et al. 2006), and impaired olfaction (McIntyre et al. 2012; Dew et al. 2014). A recent study by Hayden et al. (2015) saw that the number of fatal attacks by dragonfly (*Aeshna sitchensis*) larvae on *Lithobates sylvaticus* tadpoles increased 77% in only 1.85 µg/L Cu. Because we did not test larval predation rates, we cannot say whether the Cu treatment we used could increase predation rate, per say, but our concentration was within or above the range

wherein vulnerability to predation (via malformation or behavior) increases for various invertebrates (Clements 1999; Reza et al. 2012), fishes (Hernández et al. 2006; Sandahl et al. 2007; McIntyre et al. 2012), and amphibians (Redick and LaPoint 2004; Lance et al. 2013). In fact, due to its effects on antipredator behavior in low concentrations, copper has even been tested as a potential treatment for malaria eradication via vector control. Reza et al. (2013) suggest the use of 0.26 mg/L Cu for larval *Anopheles stephensi* mosquito control because it significantly increases predation rates by medaka (*Oryzias latipes*).

Our data suggest that the combination of larval Cu and predator cue exposure can affect terrestrial predation rates, as our performance assay indicates that the presence of Cu negates beneficial, latent effects of larval predator exposure. We show here that prior exposure to a singular aquatic stressor can impact escape performance terrestrially, specifically that the predator cue-only larval treatment performed to a greater degree in sprint and endurance distance and time. The effects of larval environmental stressors had the greatest impact on metamorphs one week post-emergence, but if individuals survived to one month post-metamorphosis, these effects were lost. The transitional period of metamorphosis – when the presence of forelimbs or a tail can impede swimming or hopping ability – represents a time of increased vulnerability to predation (Wassersug and Sperry 1977; Arnold and Wassersug 1978). Thus, it is beneficial when prior exposure to predators decreases the time to complete metamorphosis and increases locomotor ability post-metamorphosis (Van Buskirk and Saxer 2001). Our data are consistent with this in that exposure to predator cues as larvae corresponded to increased metamorph sprint and endurance distances and overall endurance time. Alternatively, larvae with prior exposure

to Cu showed decreased performance, suggesting that exposure to this particular stressor could indirectly lead to mortality by reducing an individual's ability to avoid predation.

Importantly, when larvae were exposed to the combination of predator cues and Cu, the presence of Cu negated the positive effects of prior predator exposure for metamorph performance. This could be due to copper's deleterious effects on olfaction, and such impairment can dampen fright response when predator cues are also present (Lefcort et al. 1998, 2013; Beyers and Farmer 2001; Hernández et al. 2006; Sandahl et al. 2007; McIntyre et al. 2012). However, Cu can also impair other mechanisms by which larval prey detect predators. Toxicological effects due to metal contamination, including Cu, can disrupt retinoid metabolism, leading to impaired vision (reviewed in Defo et al. 2014); thus, prey may not recognize visual predator cues. Additionally, aquatic prey can detect predators via mechanosensory means using the lateral line system which can also be damaged due to Cu exposure (Hernández et al. 2006). Thus, exposure to Cu in the aquatic environment can lead to reduced detection of chemical, visual, and mechanical cues of predators. Hence, the combination of Cu with predator cues in our study could have been affected by any one or all of these various detection impairments. Considering that this short period after emergence is critical for predator avoidance (Arnold and Wassersug 1978) and dispersal (Breden 1987, Berven and Grudzien 1990) our data indicate that this may be a critical time at which indirect mortality due to larval stressors occurs.

Juvenile survivorship may even be more critical to population persistence than larval survivorship, as indicated by the sensitivity of amphibian population models to this parameter (Biek et al. 2002; Harper et al. 2008). Thus, indirect effects on survival (e.g.

escape performance, as discussed above) or latent effects of parental and larval stressor exposure that directly affect juvenile survivorship are important to population persistence. In general, our juvenile survival in the control group (i.e., E-Bay–0 Cu–predator absent) was comparable to terrestrial survival observed in a similar species (*Anaxyrus americanus*), which was found to be 25% after one year in one study (Harper and Semlitsch 2007) and only 9% after ten months in another study (Earl and Semlitsch 2013). Similar to our data, the latter study also found a drastic decrease in juvenile survival after the first month, with 17% surviving (Earl and Semlitsch 2013). Our study indicates latent effects of parental source and larval metal exposure on juvenile survivorship. Here, our time-to-death analysis suggests that either parental or larval metal contaminant exposure will result in similar deleterious effects, which are increased with the combination of the two (Fig. 6). However, examining the final proportion surviving from each treatment group may suggest more intricate trends, especially concerning the predator cue stressor which was removed from the model due to non-significance. Here, reference source offspring always experienced deleterious effects of larval stressors, singularly and in combination, on juvenile survivorship compared to the control, but the presence of predator cues did appear to ameliorate some of the deleterious effects of Cu (Table 3). Yet for D Area offspring, larval predator cue exposure alone increased the proportion surviving while exposure to Cu decreased survival after five months. The latent nature and ameliorative effects of predator cue on Cu effects, further highlights the importance of examining combinations of stressors beyond the larval period. Additionally the differential effects, between source populations suggests that caution be made in determining parental exposure history and its potential impact on aquatic stressor

effects in offspring. Further investigation into the biological significance of these results should also be made because it is yet unclear whether these differences between treatment groups would result in altered population dynamics.

We found significant differences in larval period, size at metamorphosis, juvenile performance, growth and survivorship due to parental source. Overall, offspring from D Area experienced a longer larval period, smaller size at metamorphosis, reduced performance, juvenile growth "catch up", and reduced juvenile survivorship compared to the reference source offspring. We attempted to control for environmental factors such as hydroperiod, predators, and vegetative land cover between wetlands to isolate contamination differences; though, there could be other factors not accounted for that affected offspring. Ultimately we cannot attribute these observed effects to differential contamination history because we had no replication in source wetlands. However, previous studies concerning D Area wetlands have indicated the presence of maternal transfer of metals which correlated with reduced embryonic and larval viability (Hopkins et al. 2006; Metts et al. 2013). Although we did not measure maternal transfer, our data are consistent with other studies that have found negative effects of maternal transfer of heavy metals on offspring size (Todd et al. 2011a, 2012; Bergeron et al. 2011) and increased time to complete metamorphosis (Todd et al. 2011a). Interestingly, our data also suggest that offspring of parents exposed to metals have a similar larval period as tadpoles currently exposed to a metal stressor. This is consistent with previous work by Metts et al. (2012) who found that maternal exposure to coal combustion waste (CCW) as well as larval exposure to CCW both individually increased time to reach metamorphosis in southern toads. Due to amphibian utilization of ephemeral wetlands (Mann et al. 2001)

it is important that larvae are able to metamorphose before pond drying, thus any stressor that impedes this process can potentially lead to catastrophic reproductive failure.

Additionally, emerging larger can increase a juvenile's probability of survival and decrease the time to first reproduction (Scott et al. 2007) but if food is abundant small juveniles can close the gap in early size differences to reach a comparable size by maturity (Morey and Reznick 2001). We found that offspring of D Area parents emerged smaller than E-Bay offspring but, due to compensatory growth, were at a comparable size five months after emerging. However, this may not occur uniformly among sexes (Morey and Reznick 2001) and it is known that for females, body size is correlated with fecundity (Peckarsky et al. 1993). Juveniles in our study did experience compensatory growth, however, we did not extend our study far enough to examine whether this growth occurred differentially between sexes, or if it affected time to maturity or reproductive output. Compensatory growth has been shown to reduce fecundity (Auer et al. 2010) and can also reduce reproductive investment for multiple breeding seasons (Lee et al. 2012). Further, negative effects of compensatory growth on longevity and accrued cellular damage that occur in invertebrates, vertebrates, and plants can impair individual fitness (Mangel and Munch 2005). Ultimately, the costs and benefits of growth strategies are context-depend (Orizaola et al. 2014), thus it is necessary to take other factors, such as environmental stressors in this case, into consideration.

Additionally, studies may also take into account responses to novel stressors later in an individual's lifetime or across generations. Prior studies have suggested that latent effects of developmental exposure to stressors, especially metals, can negatively affect adult tolerance to novel stressors and even affect reproductive success. Kimberly and

Salice (2014) found that developmental exposure to cadmium (Cd) reduced adult heat stress tolerance in *Physa pomilia* and that parental developmental exposure to Cd resulted in greater reduction of offspring hatching success than compared to parental adult exposure to Cd. Given that populations are exposed to different stressors over time, more work is required to fully understand the costs and benefits of interactions of all environmental factors that affect amphibian population dynamics.

As there are several factors implicated in amphibian population declines (Lannoo 2005), more studies have begun to address the lethal and sublethal consequences of environmental stressors but there is still a paucity of data for terrestrial effects and the effects of multiple stressors. Given that our findings included interactive effects of parental source and aquatic stressor exposures on terrestrial performance and survival, it is clear that effects of stressors should be tested in combination and over multiple life stages. As described by Crain et al. (2008), multistressor studies are underrepresented despite their environmental relevance and the prevalence and unpredictability of interactions. They indicate a high frequency (36%) of studies showing synergistic population-level effects while also indicating that increasing the replication of like-stressor studies can eventually yield additive, synergistic, and antagonistic interactions. Thus, we suggest further investigation into the impacts of environmental stressors together, across life stages and generations.

Table 1. ANOVA results for larval survival, larval period, and size at metamorphosis. All models shown here are reduced, with residual errors incorporated into the error term to examine main effects (source, Cu, predator cue). Significance ($P < 0.05$) is designated in bold.

	Larval Survival			Larval Period			SVL at Metamorphosis			Mass at Metamorphosis		
Factor	df	F	*P*	df	F	*P*	df	F	*P*	df	F	*P*
Source	1,20	1.653	0.213	1,20	11.527	**0.003**	1,20	13.245	**0.002**	1,20	7.136	**0.015**
Cu	1,20	0.010	0.921	1,20	1.894	0.184	1,20	4.768	**0.041**	1,20	1.591	0.222
Predator	1,20	0.040	0.843	1,20	1.128	0.301	1,20	0.763	0.393	1,20	0.270	0.609

Table 2. ANCOVA for performance assays for trials at 0 and 1 mo post-metamorphosis. All models include main effects (source, Cu, predator cue) as well as the covariate (SVL) and all interactions. Performance measurements include the distance covered in the first 30 s of the trail (Sprint Distance), the overall distance covered before the end of the trial (Endurance Distance), the time to reach the end of the trial (Endurance Time), and the number of taps a toad received when encouraged to hop (Taps); distances were recorded to the nearest cm. Performance measurements that are significance ($P < 0.05$) are designated in bold.

0 Month Trial

Factor	Sprint Distance			Endurance Distance			Endurance Time			Taps		
	df	F	*P*	df	F	*P*	df	F	*P*	df	F	*P*
SVL	1,15	22.352	**0.0003**	1,15	12.523	**0.003**	1,15	4.796	**0.045**	1,15	3.701	0.074
Source	1,15	0.658	0.430	1,15	0.091	0.767	1,15	0.246	0.627	1,15	0.174	0.682
Cu	1,15	0.006	0.940	1,15	1.166	0.297	1,15	1.620	0.223	1,15	0.635	0.438
Predator	1,15	0.364	0.555	1,15	0.725	0.408	1,15	0.371	0.551	1,15	0.066	0.801
Source*Cu	1,15	0.850	0.371	1,15	3.173	0.095	1,15	3.944	0.066	1,15	1.507	0.239
Source*Predator	1,15	0.039	0.847	1,15	0.079	0.783	1,15	0.134	0.719	1,15	0.067	0.799
Cu*Predator	1,15	0.060	0.810	1,15	1.605	0.225	1,15	1.711	0.211	1,15	0.554	0.468
Source*Cu*Predator	1,15	1.578	0.228	1,15	4.158	**0.06**	1,15	6.564	**0.022**	1,15	3.095	0.099

1 Month Trial

Factor	Sprint Distance			Endurance Distance			Endurance Time			Taps		
	df	F	*P*	df	F	*P*	df	F	*P*	df	F	*P*
SVL	1,15	20.338	**0.0004**	1,15	20.796	**0.0004**	1,15	15.821	**0.001**	1,15	3.157	0.096
Source	1,15	1.933	0.185	1,15	0.013	0.912	1,15	0.015	0.904	1,15	1.157	0.299
Cu	1,15	0.050	0.826	1,15	0.005	0.945	1,15	0.685	0.421	1,15	0.535	0.476
Predator	1,15	1.620	0.223	1,15	0.284	0.602	1,15	0.113	0.741	1,15	1.039	0.324
Source*Cu	1,15	0.793	0.387	1,15	0.028	0.870	1,15	0.013	0.911	1,15	2.159	0.162
Source*Predator	1,15	0.874	0.365	1,15	0.476	0.500	1,15	0.002	0.969	1,15	0.070	0.795
Cu*Predator	1,15	1.694	0.213	1,15	0.565	0.464	1,15	0.347	0.564	1,15	0.671	0.425
Source*Cu*Predator	1,15	2.131	0.165	1,15	0.394	0.540	1,15	0.025	0.877	1,15	2.427	0.140

Table 3. Final proportion of surviving juveniles at 5 mo post-metamorphosis (±SE). Proportions are averaged within treatments, where each treatment contains three experimental units (i.e., aquatic tanks). The two source populations are indicated (E = E-Bay; D = D Area) as are the two level of Cu treatment (0, 30 µg/L Cu) and the two levels of predator cue treatment (absent "-", present "+").

			Survival	
Source	Cu (µg/L)	Predator	Proportion	SE
E	0	-	0.39	0.061
D	0	-	0.26	0.096
E	30	-	0.18	0.099
D	30	-	0.21	0.029
E	0	+	0.36	0.039
D	0	+	0.30	0.021
E	30	+	0.27	0.067
D	30	+	0.26	0.099

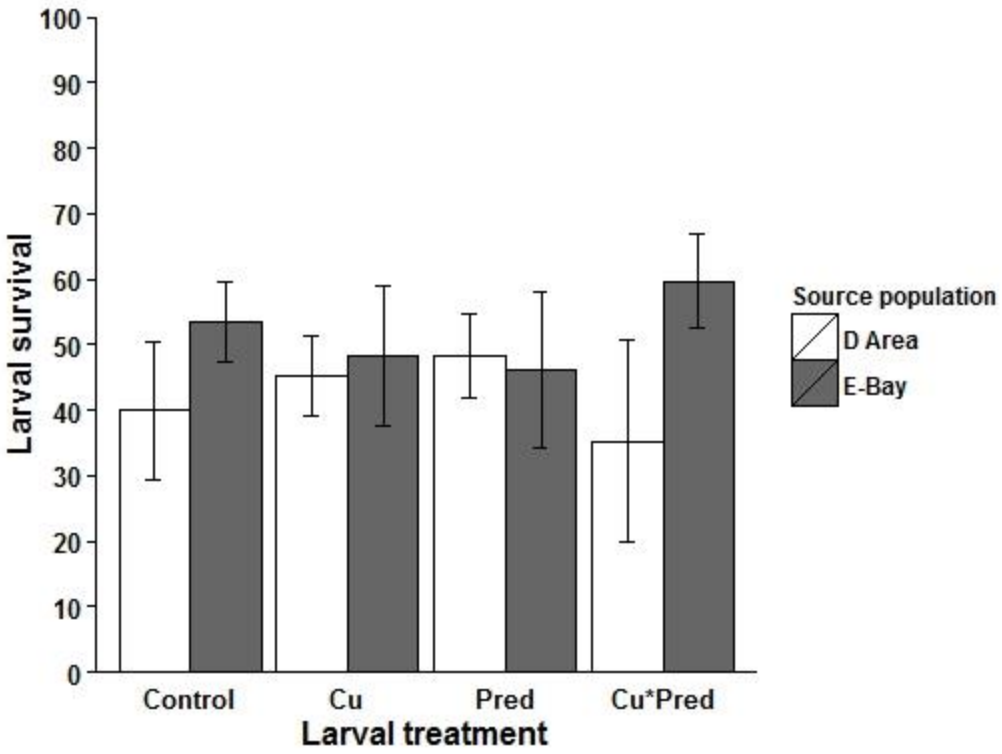

Figure 1. Larval survivorship (±SE) estimated as the total number of metamorphs by larval treatment (*n* = 3 reps) captured with shading by parental source (light = D Area; dark = E-Bay). The four larval treatments applied to each source population are: Control (0 µg Cu/L, no predators), Cu (30 µg/L, no predators), Pred (0 µg Cu/L, predators present), and Cu*Pred (30 µg Cu/L, predators present).

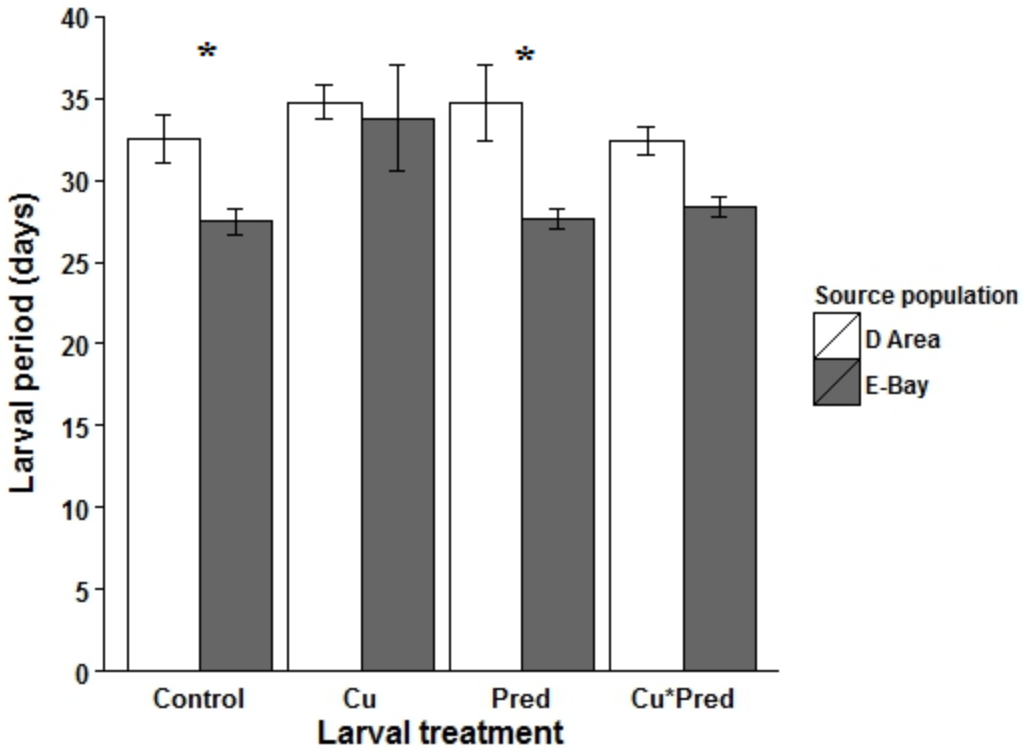

Figure 2. Days to first emergence (±SE) by larval treatment (*n* = 3 reps) and shading according to parental source population (light = D Area; dark = E-Bay). The four larval treatments applied to each source population are: Control (0 µg Cu/L, no predators), Cu (30 µg/L, no predators), Pred (0 µg Cu/L, predators present), and Cu*Pred (30 µg Cu/L, predators present). Letters correspond to significantly different group averages. Asterisks (*) indicate significant pairwise differences between source populations for indicated larval treatments.

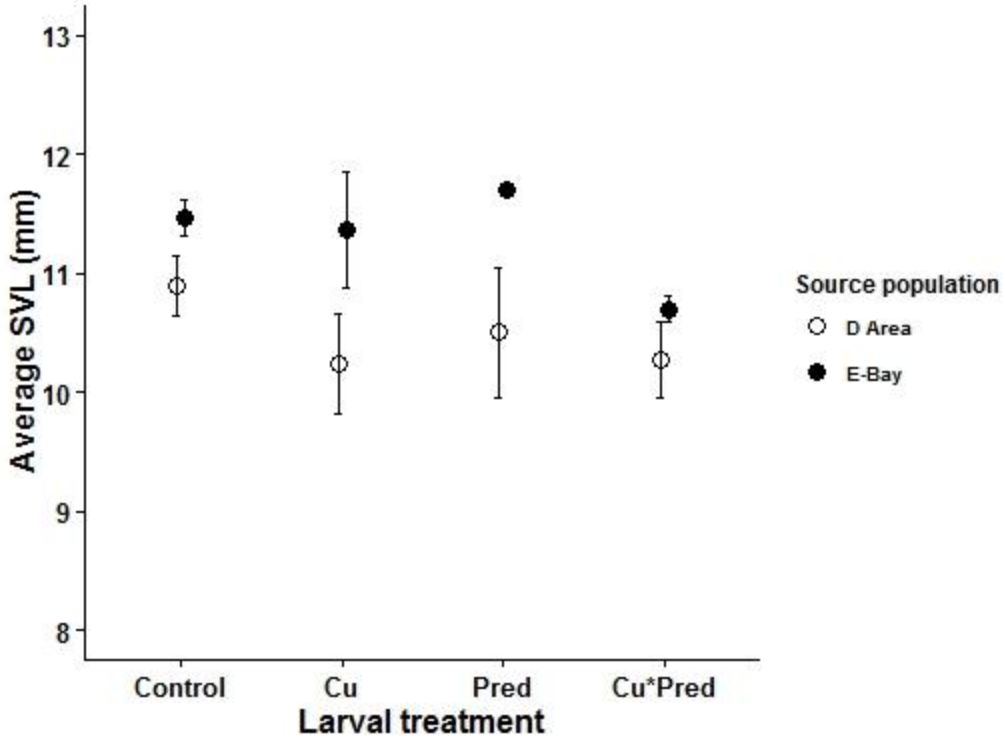

Figure 3. Average size (SVL) at metamorphosis (±SE) by larval treatment with shading according to parental source population (light = D Area; dark = E-Bay). The four larval treatments applied to each source population are: Control (0 µg Cu/L, no predators), Cu (30 µg/L, no predators), Pred (0 µg Cu/L, dragonflies present), and Cu*Pred (30 µg Cu/L, dragonflies present).

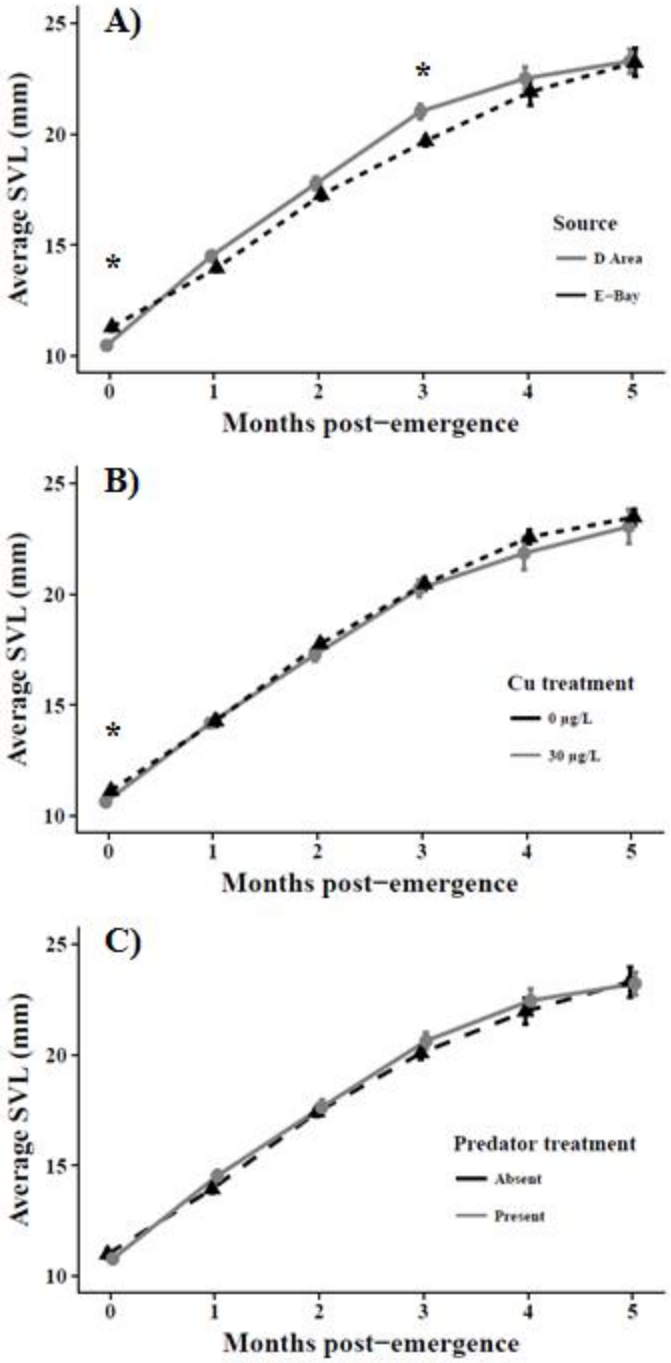
A)
Average SVL (mm)
Months post−emergence
Source
D Area
E−Bay
*
*
B)
Average SVL (mm)
Months post−emergence
Cu treatment
0 µg/L
30 µg/L
*
C)
Average SVL (mm)
Months post−emergence
Predator treatment
Absent
Present

Figure 4. Average juvenile SVL (±SE) from 0-5 mo post-metamorphosis by parental source (A; dark = E-Bay, light = D Area), larval Cu (B; dark = 0 µg/L, light = 30 µg/L), and larval predator cue (C; dark = absent, light = present). Asterisks (*) signify a time period wherein there was a significant difference between tested main effects (source, Cu, or predator cue).

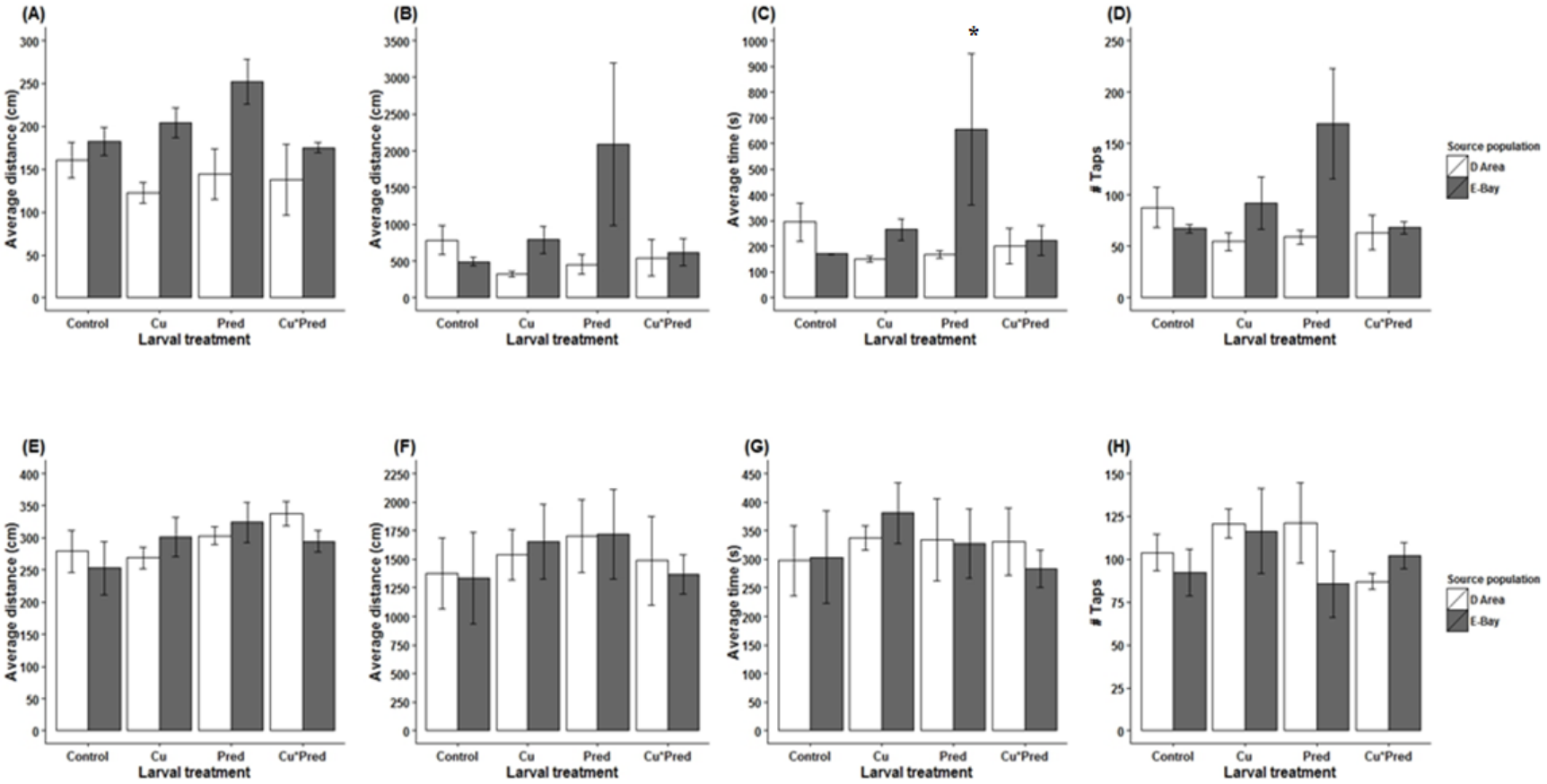
(A)
Average distance (cm)
Control
Cu
Pred
Cu*Pred
Larval treatment
(B)
Average distance (cm)
Larval treatment
(C)
*
Average time (s)
Larval treatment
(D)
Taps
Larval treatment
Source population
D Area
E-Bay
(E)
Average distance (cm)
Larval treatment
(F)
Average distance (cm)
Larval treatment
(G)
Average time (s)
Larval treatment
(H)
Taps
Larval treatment
Source population
D Area
E-Bay

Figure 5. Average juvenile performance measurements (±SE) for Trial 1 (A-D), 0 mo post-metamorphosis, and Trial 2 (E-H), 1 mo post-metamorphosis, by larval treatment (x-axes) and parental source population (light = D Area/contaminated; dark = E-Bay/uncontaminated). Measurements include sprint distance (A, E), endurance distance (B, F), endurance time (C, G), and taps (D, H). The four larval treatments applied to each source population are: Control (0 µg Cu/L, no predators), Cu (30 µg/L, no predators), Pred (0 µg Cu/L, predators present), and Cu*Pred (30 µg Cu/L, predators present). Asterisks (*) indicate significant treatment effects due to main effects.

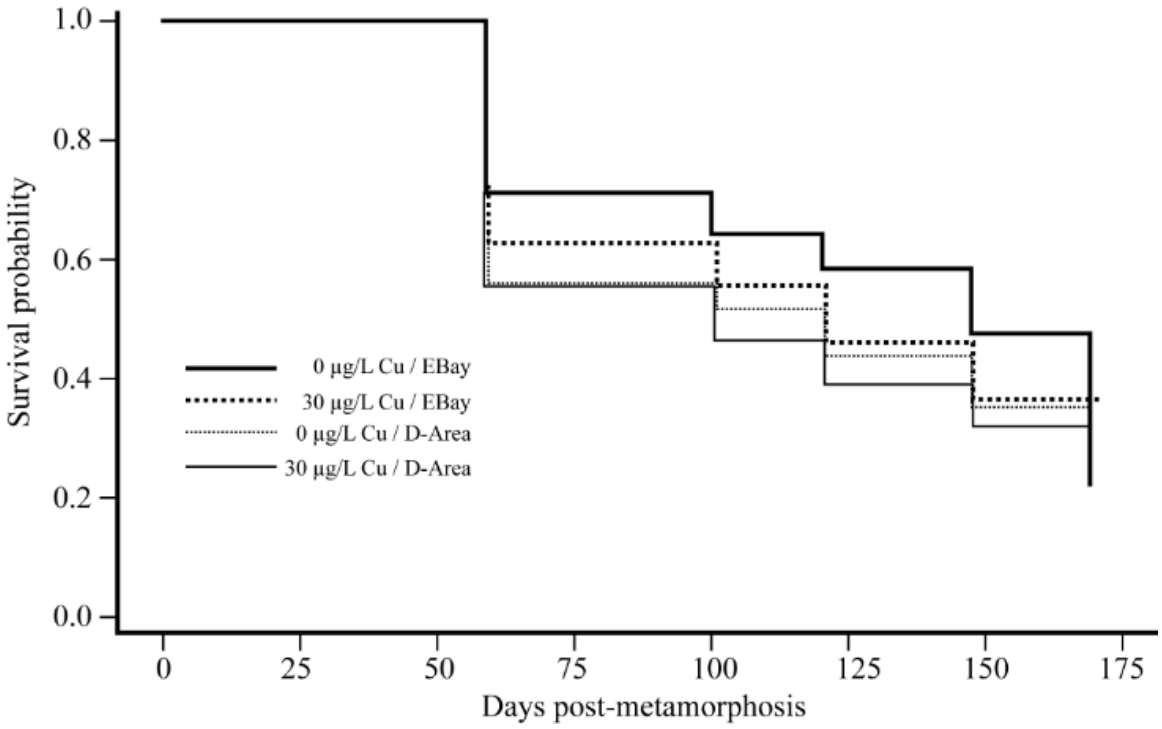

Figure 6. Proportion of surviving juveniles up to 5 mo post-metamorphosis using time-to-event analysis. There were significant effects due to parental source (E-Bay, D Area) and larval Cu exposure but not larval predator cue exposure (treatments not shown) on juvenile survival probability.

CHAPTER 3

CONCLUSION

The complex life histories of amphibians make them susceptible to both aquatic and terrestrial stressors (Todd et al. 2011), but we are only just piecing together how exposure in one life stage may affect another (Calow and Forbes 2003). Additionally, exposure to one stressor is almost always coupled with exposure to another, whether it be anthropogenic or natural, and the effects of one on an individual can be altered by interactions (reviewed in Crain et al. 2008). Furthermore, parental exposure to one or many stressors can carry through to offspring, affecting their ability to cope with novel stressors (Plautz et al. 2013). Because amphibians are affected by human activity especially environmental contamination (Fedorenkova et al. 2012), these issues are important to incorporate into ecotoxicity testing to further understand contaminant impacts on amphibian populations over time. Therefore, with this work we sought to address the effects of parental and multiple aquatic stressor exposure on amphibian larval and terrestrial life history traits.

To examine these issues we employed a fully factorial experiment in outdoor 1000-L mesocosms (n = 24), where larval southern toads (*Anaxyrus terrestris*) bred from parents collected from reference and heavy metal contaminated wetlands were exposed to an anthropogenic stressor (Cu) and natural stressor (predator cue). Upon metamorphosis we transferred survivors were transferred to terrestrial mesocosms to examine the chronic and/or latent effects of parental source and early life stage stressor exposure. Our

objectives were to examine the effects of parental contaminant history and larval exposure to Cu and predator cue stressors on 1) larval survivorship and time to/size at metamorphosis, and 2) juvenile performance, growth, and survivorship.

We found no statistically significant effects of larval stressor exposure or parental source on larval survivorship. Our data do, however, show intriguing trends which if examined with more replicates (i.e., >3/treatment) may result in statistical significance. On the other hand, larval period was significantly affected by parental source, wherein E-Bay offspring emerged earlier than D Area offspring on average, except for the E-Bay–30 µg/L Cu treatment which emerged at a similar time to all D Area treatments. Size at metamorphosis was also significantly affected by parental source, with E-Bay emerging larger than D Area metamorphs. However, our data indicated compensatory growth in juveniles, also significantly affected by parental source. D Area juveniles were larger than E-Bay juveniles three months post-metamorphosis, but both sources were comparable in size at five months.

One week post-emergence there was a significant three-way interaction with the E-Bay–0 Cu–predator group having greater endurance distance, time and accrued taps than other groups. Our data also suggest that larval Cu exposure can affect terrestrial predation rates, as our performance assay indicated that the presence of Cu can negate beneficial, latent effects of larval predator exposure. The effects of larval environmental stressors had the greatest impact on metamorphs one week post-emergence, but most effects were lost one month post-emergence. The transitory period of metamorphosis – when the presence of forelimbs or a tail can impede swimming or hopping ability – represents a time of increased vulnerability to predation our data indicate that this may be

a critical time at which indirect mortality due to larval stressors occurs. Therefore, we suggest ecotoxicity testing incorporate this time period as it is sensitive and potentially more predictive of population dynamics.

Finally, parental source and Cu exposure both significantly affected juvenile survivorship up to five months post-metamorphosis. Here, D Area offspring experienced greater mortality (25%) than E-Bay, and juveniles exposed to 30 µg/L Cu experienced greater mortality (53%) than controls. Juvenile survivorship may even be more critical to population persistence than larval survivorship and our study indicated latent effects of parental source and larval Cu exposure on juvenile survivorship. It is crucial, especially for ecotoxicity testing, that measureable endpoints are tested during sensitive life stages and are predictive of effects in subsequent life stages in order to ultimately determine population-level effects of contaminant exposure. Importantly, had we examined only the effects of parental source, Cu and predator cue stressors up to metamorphosis we would have predicted an entirely different outcome for the terrestrial stage than we actually observed.

REFERENCES

Alford, R. A., & Harris, R. N. (1988). Effects of larval growth history on anuran metamorphosis. *The American Naturalist*, *131*(1), 91–106.

Arnold, S. J., Wassersug, R. J., & Wassersug, R. J. (1978). Differential Predation on Metamorphic Anurans by Garter Snakes (Thamnophis): Social Behavior as a Possible Defense. *Ecology*, *59*(595), 1014–1022. Retrieved from http://www.jstor.org

Auer, S. K., Arendt, J. D., Chandramouli, R., & Reznick, D. N. (2010). Juvenile compensatory growth has negative consequences for reproduction in Trinidadian guppies (Poecilia reticulata). *Ecology Letters*, *13*(8), 998–1007. doi:10.1111/j.1461-0248.2010.01491.x

Belanger, R. M., & Corkum, L. D. (2009). Review of Aquatic Sex Pheromones and Chemical Communication in Anurans Published By : The Society for the Study of Amphibians and Reptiles Review of Aquatic Sex Pheromones and Chemical Communication, *43*(2), 184–191. http://doi.org/10.1670/08-054R1.1

Bergeron, C. M., Bodinof, C. M., Unrine, J. M., & Hopkins, W. A. (2010). Bioaccumulation and maternal transfer of mercury and selenium in amphibians. *Environmental Toxicology and Chemistry / SETAC*, *29*(4), 989–97. http://doi.org/10.1002/etc.125

Bergeron, C. M., Hopkins, W. A., Todd, B. D., Hepner, M. J., & Unrine, J. M. (2011). Interactive effects of maternal and dietary mercury exposure have latent and lethal consequences for amphibian larvae. *Environmental Science and Technology*, *45*(8), 3781-3787. doi:10.1021/es104210a

Bernardo, J. (1996a). Maternal Effects in Animal Ecology. *American Zoologist*, *36*, 83–105.

Bernardo, J. (1996b). The Particular Maternal Effect of Propagule Size, Especially Egg Size: Patterns, Models, Quality of Evidence and Interpretations. *Integrative and Comparative Biology*, *36*(2), 216–236. http://doi.org/10.1093/icb/36.2.216

Berven, K. A. (1990). Factors Affecting Population Fluctuations in Larval and Adult Stages of the Wood Frog (Rana Sylvatica). *Ecology*, *71*(714), 1599–1608.

Berven, K. A., & Gill, D. E. (1983). Interpreting Geographic Variation in Life-History Traits Interpreting Geographic Variation in Life-History Traits. *American Zoologist*, *23*(1), 85–9785. Retrieved from http://www.jstor.org

Berven, K. A., & Grudzien, T. A. (1990). Dispersal in the Wood Frog (Rana sylvatica): Implications for Genetic Population Structure. *Evolution*, *44*(8), 2047–2056. Retrieved from http://www.jstor.org

Beyers, D. W., & Farmer, M. S. (2001). Effects of copper on olfaction of Colorado pikeminnow. *Environmental Toxicology and Chemistryoxicology*, *20*(4), 907–912.

Biek, R., Funk, W. C., Maxell, B. a, & Mills, L. S. (2002). Is Missing from Insights Amphibian Decline Ecological Sensitivity Analysis. *Conservation Biology*, *16*(3), 728–734.

Bishop, C. A., Mahony, N. A., Struger, J., Ng, P., & Pettit, K. E. (1999). Anuran development, density and diversity in relation to agricultural activity in the Holland River watershed, Ontario, Canada (1990-1992). *Environmental Monitoring and Assessment*, *57*, 21–43. doi:10.1023/A:1005988611661

Blaustein, A. R., Han, B. A., Relyea, R. A., Johnson, P. T. J., Buck, J. C., Gervasi, S. S., & Kats, L. B. (2011). The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. *Annals of the New York Academy of Sciences*, *1223*, 108–19. http://doi.org/10.1111/j.1749-6632.2010.05909.x

Blaustein, A. R., & Kiesecker, J. M. (2002). Complexity in conservation: lessons from the global decline of amphibian populations. *Ecology Letters*, *5*(4), 597–608. http://doi.org/10.1046/j.1461-0248.2002.00352.x

Bondarenko, O., Juganson, K., Ivask, A., Kasemets, K., Mortimer, M., & Kahru, A. (2013). Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: A critical review. *Archives of Toxicology*, *87*(7), 1181–1200. http://doi.org/10.1007/s00204-013-1079-4

Budischak, S. A., Belden, L. K., & Hopkins, W. A. (2008). Effects of malathion on embryonic development and latent susceptibility to trematode parasites in ranid tadpoles. *Environmental Toxicology and Chemistry / SETAC*, *27*(12), 2496–2500. http://doi.org/10.1897/08-018.1

Boone, C. M., & James, S. M. (2005). Aquatic and terrestrial mesocosms in amphibian ecotoxicology. *Applied Herpetology*, 2:231-257

Bopp, S. K., Abicht, H. K., & Knauer, K. (2008). Copper-induced oxidative stress in rainbow trout gill cells. *Aquatic Toxicology*, *86*, 197–204. http://doi.org/10.1016/j.aquatox.2007.10.014

Breden, F. (1987). The Effect of Post-Metamorphic Dispersal on the Population Genetic Structure of Fowler's Toad, Bufo woodhousei fowleri. *Source: Copeia*, *1987*(2), 386–395. Retrieved from http://www.jstor.org

Bridges, C. M. (2002). Tadpoles Balance Foraging and Predator Avoidance: Effects of Predation, Pond Drying, and Hunger. *Journal of Herpetology*. doi:10.1670/0022-1511(2002)036[0627:TBFAPA]2.0.CO;2

Brown, J. R., Miiller, T., & Kerby, J. L. (2013). The interactive effect of an emerging infectious disease and an emerging contaminant on Woodhouse's toad (Anaxyrus woodhousii) tadpoles. *Environmental Toxicology and Chemistry*, *32*(9), 2003–2008. doi:10.1002/etc.2266

Brown, M. G., Dobbs, E. K., Snodgrass, J. W., & Ownby, D. R. (2012). Ameliorative effects of sodium chloride on acute toxicity among Cope's gray tree frog (Hyla chrysoscelis) and green frog (Rana clamitans) embryos. *Environmental Toxicology and Chemistry/SETAC*, *31*(4), 836-842. doi:10.1002/etc.1751.

Cabrera-Guzmán, E., Crossland, M. R., Brown, G. P., & Shine, R. (2013). Larger Body Size at Metamorphosis Enhances Survival, Growth and Performance of Young Cane Toads (Rhinella marina). *PLoS ONE*, *8*(7). doi:10.1371/journal.pone.0070121

Carey, C., & Bryant, C. J. (1995). Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. *The National Institute of Environmental Health Sciences (NIEHS)*, *103*(May), 13–17.

Chen, T.-H., Gross, J. A., & Karasov, W. H. (2007). Adverse effects of chronic copper exposure in larval northern leopard frogs (Rana pipiens). *Environmental Toxicology and Chemistry / SETAC*. doi:10.1897/06-487R.1

Clements, W. H. (1999). Metal tolerance and predator-prey interactions in benthic macroinvertebrate stream communities. *Ecological Applications*, *9*(3), 1073–1084. doi:doi:10.1890/1051-0761(1999)009[1073:MTAPPI]2.0.CO;2

Crain, C. M., Kroeker, K., & Halpern, B. S. (2008). Interactive and cumulative effects of multiple human stressors in marine systems. *Ecology Letters*, *11*, 1304–1315. doi:10.1111/j.1461-0248.2008.01253.x

Dahl, E., Orizaola, G., Nicieza, A. G., Laurila, A., & Meiri, S. (2012). Time constraints and flexibility of growth strategies: geographic variation in catch-up growth responses in amphibian larvae. *The Journal of Animal Ecology*, *81*(6), 1233–43. http://doi.org/10.1111/j.1365-2656.2012.02009.x

Defo, M. A., Spear, P. A., & Couture, P. (2014). Consequences of metal exposure on retinoid metabolism in vertebrates : A review. *Toxicology Letters*, *225*(1), 1–11. http://doi.org/10.1016/j.toxlet.2013.11.024

Dew, W. A., Azizishirazi, A., & Pyle, G. G. (2014). Contaminant-specific targeting of olfactory sensory neuron classes: Connecting neuron class impairment with behavioural deficits. *Chemosphere*, *112*, 519–525. doi:10.1016/j.chemosphere.2014.02.047

Earl, J. E., & Semlitsch, R. D. (2013). Carryover effects in amphibians: Are characteristics of the larval habitat needed to predict juvenile survival? *Ecological Applications*, *23*(6), 1429–1442. http://doi.org/10.1890/12-1235.1

Earl, J. E., & Whiteman, H. H. (2015). Are Commonly Used Fitness Predictors Accurate? A Meta-analysis of Amphibian Size and Age at Metamorphosis. *Copeia*, *103*(2), 297–309. doi:10.1643/CH-14-128

Eisenreich, K. M., Kelly, S. M., & Rowe, C. L. (2009). Latent Mortality of Juvenile Snapping Turtles from the Upper Hudson River, New York, Exposed Maternally and Via the Diet to Polychlorinated Biphenyls (PCBs). *Environmental Science & Technology*, *43*(15), 6052–6057. http://doi.org/10.1021/es9008344

Fedorenkova, A., Vonk, J. A., Lenders, H. J. R., Creemers, R. C. M., Breure, A. M., & Hendriks, a J. (2012). Ranking ecological risks of multiple chemical stressors on amphibians. *Environmental Toxicology and Chemistry / SETAC*, *31*(6), 1416–21. doi:10.1002/etc.1831

Ferrari, M. C. O., Messier, F., & Chivers, D. P. (2008). Larval amphibians learn to match antipredator response intensity to temporal patterns of risk. *Behavioral Ecology*. doi:10.1093/beheco/arn056

Flynn, R., Scott, D., Kuhne, W., Soteropoulos, D., & Lance, S. (2015). Lethal and sublethal measures of chronic copper toxicity in the eastern narrowmouth toad, Gastrophryne carolinensis, reveal within and among population variation. *Environmental Science & Technology*, *34*(3), 575–582. doi:10.1002/etc.2835

Folt, C. L., Chen, C. Y., Moore, M. V., & Burnaford, J. (1999). Synergism and antagonism among multiple stressors. *Limnology and Oceanography*, *44*(3_part_2), 864–877. http://doi.org/10.4319/lo.1999.44.3_part_2.0864

Franco-de-Sá, J. F. O., & Val, A. L. (2014). granulosa (Amphibia : Anura) of the Amazon : Potential of Biotic Ligand Model to predict toxicity in urban streams. *ACTA Amazonica*, *44*(4), 491–498.

García-Muñoz, E., Guerrero, F., & Parra, G. (2009). Effects of copper sulfate on growth, development, and escape behavior in epidalea calamita Embryos and Larvae. *Archives of Environmental Contamination and Toxicology*. doi:10.1007/s00244-008-9201-y

García-Muñoz, E., Guerrero, F., & Parra, G. (2011). Larval escape behavior in anuran amphibians as a wetland rapid pollution biomarker. *Marine and Freshwater Behaviour and Physiology*, *44*(2), 109–123. doi:10.1080/10236244.2011.557855

Gramapurohit, N. P. (2009). Catch-up growth during juvenile life can compensate for the small metamorphic size in Euphlyctis cyanophlyctis. *Current Science*, *97*(8), 1243–1246.

Grass, G., Rensing, C., & Solioz, M. (2011). Metallic copper as an antimicrobial surface. *Applied and Environmental Microbiology*, *77*(5), 1541–1547. http://doi.org/10.1128/AEM.02766-10

Griffitt, R. J., Weil, R., Hyndman, K. a., Denslow, N. D., Powers, K., Taylor, D., & Barber, D. S. (2007). Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (Danio rerio). *Environmental Science and Technology*, *41*(23), 8178–8186. http://doi.org/10.1021/es071235e

Han, X., Geller, B., Moniz, K., Das, P., Chippindale, A. K., & Walker, V. K. (2014). Monitoring the developmental impact of copper and silver nanoparticle exposure in Drosophila and their microbiomes. *Science of the Total Environment*, *487*(1), 822–829. http://doi.org/10.1016/j.scitotenv.2013.12.129

Handy, R. (2003). Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process?. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology*, 135A(1), 25-38.

Hanlon, S. M., & Relyea, R. (2013). Sublethal Effects of Pesticides on Predator–Prey Interactions in Amphibians. *The American Society of Ichthyologists and Herpetologists*, *2013*(4), 691–698. http://doi.org/10.1643/CE-13-019

Harper, E. B., Rittenhouse, T. A. G., & Semlitsch, R. D. (2008). Demographic consequences of terrestrial habitat loss for pool-breeding amphibians: Predicting extinction risks associated with inadequate size of buffer zones. *Conservation Biology*, *22*(5), 1205–1215. doi:10.1111/j.1523-1739.2008.01015.x

Harper, E. B., & Semlitsch, R. D. (2007). Density dependence in the terrestrial life history stage of two anurans. *Oecologia*, *153*(4), 879–89. http://doi.org/10.1007/s00442-007-0796-x

Hayden, M. T., Reeves, M. K., Holyoak, M., Perdue, M., King, A. L., & Tobin, S. C. (2015). Thrice as easy to catch! Copper and temperature modulate predator-prey interactions in larval dragonflies and anurans. *Ecosphere*, *6*(4), 1–17.

Hernández, P. P., Moreno, V., Olivari, F. A., & Allende, M. L. (2006). Sub-lethal concentrations of waterborne copper are toxic to lateral line neuromasts in zebrafish (Danio rerio). *Hearing Research*, *213*(1-2), 1–10. doi:10.1016/j.heares.2005.10.015

Hopkins, W. A., DuRant, S. E., Staub, B. P., Rowe, C. L., & Jackson, B. P. (2006). Reproduction, Embryonic Development, and Maternal Transfer of Contaminants in the Amphibian Gastrophryne carolinensis. *Environmental Health Perspectives*, *114*(5), 661–666. doi:10.1289/ehp.8457

Howard, R. D. (1980). Mating behaviour and mating success in woodfrogs Rana sylvatica. *Animal Behaviour*, *28*(3), 705–716. http://doi.org/10.1016/S0003-3472(80)80130-8

Isani, G., Falcioni, M. L., Barucca, G., Sekar, D., Andreani, G., Carpenè, E., & Falcioni, G. (2013). Comparative toxicity of CuO nanoparticles and CuSO4 in rainbow trout. *Ecotoxicology and Environmental Safety*, *97*, 40–46. http://doi.org/10.1016/j.ecoenv.2013.07.001

James, S. M., & Semlitsch, R. D. (2011). Terrestrial Performance of Juvenile Frogs in Two Habitat Types after Chronic Larval Exposure to a Contaminant. *Journal of Herpetology*, *45*(2), 186–194. http://doi.org/10.1670/10-032.1

Janssens, L., & Stoks, R. (2014). Chronic predation risk reduces escape speed by increasing oxidative damage: A deadly cost of an adaptive antipredator response. *PLoS ONE*, *9*(6), 1–6. doi:10.1371/journal.pone.0101273

Kerby, J. L., Hart, A. J., & Storfer, A. (2011). Original contribution Combined Effects of Virus , Pesticide, and Predator Cue on the Larval Tiger Salamander (Ambystoma tigrinum). *EcoHealth*, *8*, 46–54. http://doi.org/10.1007/s10393-011-0682-1

Kimberly, D. A., & Salice, C. J. (2013). Interactive effects of contaminants and climate-related stressors: High temperature increases sensitivity to cadmium. *Environmental Toxicology and Chemistry*, *32*(6), 1337–1343. http://doi.org/10.1002/etc.2198

Kimberly, D. A., & Salice, C. J. (2014). If you could turn back time: Understanding transgenerational latent effects of developmental exposure to contaminants. *Environmental Pollution*, *184*, 419–425. doi:10.1016/j.envpol.2013.09.018

King, E. G., & Kaplan, H. (1997). Society for the Study of Amphibians and Reptiles Estimating Ovum Size in Amphibians : Egg Size Increases Differently among Species during Early Development. *Journal of Herpetology*, *31*(2), 299–302.

Lance, S. L., Erickson, M. R., Flynn, R. W., Mills, G. L., Tuberville, T. D., & Scott, D. E. (2012). Effects of chronic copper exposure on development and survival in the southern leopard frog (Lithobates [Rana] sphenocephalus). *Environmental Toxicology and Chemistry*, *31*(7), 1587-1594. doi:10.1002/etc.1849

Lance, S. L., Flynn, R. W., Erickson, M. R., & Scott, D. E. (2013). Within- and among-population level differences in response to chronic copper exposure in southern toads, Anaxyrus terrestris. *Environmental Pollution, 177*, 135-142. doi:10.1016/j.envpol.2013.02.009

Lannoo M, ed. 2005. Amphibian Declines: The Conservation Status of United States Species. University of California Press, Berkeley, CA, USA, p 1094.

Laugen, A. T., Laurila, A., & Merilä, J. A. (2002). Maternal and genetic contributions to geographical variation in Rana temporaria larval life-history traits. *Biological Journal of the Linnean Society*, *76*(1), 61–70. http://doi.org/10.1046/j.1095-8312.2002.00048.x

Lee, W. S., Monaghan, P., & Metcalfe, N. B. (2012). The pattern of early growth trajectories affects adult breeding performance. *Ecology, 93*(4), 902-912. doi:10.1890/11-0890.1

Lefcort, H., Meguire, R. A., Wilson, L. H., & Ettinger, W. F. (1998). Heavy metals alter the survival, growth, metamorphosis, and antipredatory behavior of Columbia spotted frog (Rana luteiventris) tadpoles. *Archives of Environmental Contamination and Toxicology*. *456*, 447–456. doi:10.1007/s002449900401

Lefcort, H., Wehner, E. A., & Cocco, P. L. (2013). Pre-exposure to heavy metal pollution and the odor of predation decrease the ability of snails to avoid stressors. *Archives of Environmental Contamination and Toxicology*. *64*, 273–280.doi:10.1007/s00244-012-9821-0

Maag, N., Gehrer, L., & Woodhams, D. C. (2012). Sink or swim: A test of tadpole behavioral responses to predator cues and potential alarm pheromones from skin secretions. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *198*(11), 841–846. http://doi.org/10.1007/s00359-012-0750-1

Maher, J. M., Werner, E. E., & Denver, R. J. (2013). Stress hormones mediate predator-induced phenotypic plasticity in amphibian tadpoles. *Proceedings. Biological Sciences / The Royal Society*, *280*(1758), 20123075.

Mangel, M., & Munch, S. B. (2005). A life-history perspective on short- and long-term consequences of compensatory growth. *The American Naturalist*, *166*(6), 155–176. doi:10.1086/444439

Mann, R. M., Bidwell, J. R. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and two exotic frogs. *Environmental Pollution*, 114:195–205.

Marshall, D. J., & Uller, T. (2007). When is a maternal effect adaptive? *Oikos*, *116*(12), 1957–1963. doi:10.1111/j.2007.0030-1299.16203.x

Metts, B. S., Buhlmann, K. A., Scott, D. E., Tuberville, T. D., & Hopkins, W. A. (2012). Interactive effects of maternal and environmental exposure to coal combustion wastes decrease survival of larval southern toads (Bufo terrestris). *Environmental Pollution (Barking, Essex : 1987)*, *164*, 211–8. doi:10.1016/j.envpol.2012.01.042

Metts, B. S., Buhlmann, K. A., Tuberville, T. D., Scott, D. E., & Hopkins, W. A. (2013). Maternal transfer of contaminants and reduced reproductive success of southern toads (Bufo [Anaxyrus] terrestris) exposed to coal combustion waste. *Environmental Science & Technology*, *47*(6), 2846–53. doi:10.1021/es303989u

McCollum, S. A., & Van Buskirk, J. (1996). Costs and Benefits of a Predator-Induced Polyphenism in the Gray Treefrog Hyla chrysoscelis. *Source: Evolution*, *50192114*(2), 583–593.

McIntyre, J. K., Baldwin, D. H., Beauchamp, D. A., & Scholz, N. L. (2012). Low-level copper exposures increase visibility and vulnerability of juvenile coho salmon to cutthroat trout predators. *Ecological Applications*, *22*(5), 1460-1471. doi:10.1890/11-2001.1

McPeek, M., & Peckarsky, B. L. (1998). Life histories and the strength of species interactions: combining mortality, growth, and fecundity effects. *Ecology*, *79*(3), 867–879. doi:10.1890/0012-9658(1998)079[0867:LHATSO]2.0.CO;2

Morey, S., & Reznick, D. (2001). Effects of larval density on postmetamorphic spadefoot toads (*Spea Hammondii*). *Ecology*, *82*(2), 510–522.

Nations, S., Long, M., Wages, M., Maul, J. D., Theodorakis, C. W., & Cobb, G. P. (2015). Subchronic and chronic developmental effects of copper oxide (CuO) nanoparticles on Xenopus laevis. *Chemosphere*, *135*, 166–174. http://doi.org/10.1016/j.chemosphere.2015.03.078

Newman, R. A. (1988). Genetic variation for larval anuran (Scaphiopus couchii) development time in an uncertain environment. *Evolution*, *42*(4), 763–773. http://doi.org/10.2307/2408867

Newman, M. C., & McIntosh, A. W. (1991). *Metal Ecotoxicology: Concepts & Applications*. Chelsea, Mich.: Lewis Publishers, c1991, p214-222.

Orizaola, G., Dahl, E., & Laurila, A. (2014). Compensatory growth strategies are affected by the strength of environmental time constraints in anuran larvae. *Oecologia*, *174*(1), 131–137. doi:10.1007/s00442-013-2754-0

Pahkala, M., Laurila, A., & Merilä, J. (2001). Carry-over effects of ultraviolet-B radiation on larval fitness in Rana temporaria. *Proceedings. Biological Sciences / The Royal Society*, *268*(1477), 1699–1706. http://doi.org/10.1098/rspb.2001.1725

Pechenik, J. A. (2006). Larval experience and latent effects - Metamorphosis is not a new beginning. *Integrative and Comparative Biology*, *46*(3), 323–333. doi:10.1093/icb/icj028

Peckarsky, B. L., Cowan, C. A., Penton, M. A., & Anderson, C. (1993). Sublethal consequences of stream-dwelling predatory stoneflies on mayfly growth and fecundity. *Ecology*, *74*(6), 1836–1846.

Peles, J. D. (2013). Effects of chronic aluminum and copper exposure on growth and development of wood frog (Rana sylvatica) larvae. *Aquatic Toxicology (Amsterdam, Netherlands)*, *140-141*, 242–8. doi:10.1016/j.aquatox.2013.06.009

Plautz, S. C., & Salice, C. J. (2013). Plasticity in offspring contaminant tolerance traits: developmental cadmium exposure trumps parental effects. *Ecotoxicology (London, England)*, *22*(5), 847–53. http://doi.org/10.1007/s10646-013-1076-7

Pomati, F., & Nizzetto, L. (2013). Assessing triclosan-induced ecological and trans-generational effects in natural phytoplankton communities: a trait-based field method. *Ecotoxicology (London, England)*, *22*(5), 779–94. doi:10.1007/s10646-013-1068-7

Pough, F. H. (2004). Herpetology. Upper Saddle River, NJ: Pearson Prentice Hall, c2004, p 322.

Prout, T., & McChesney, F. (1985). Competition Among Immatures Affects Their Adult Fertility: Population Dynamics. *The American Naturalist, 126*(4), 521-528. doi:10.1086/284436

Redick, M. S., & La Point, T. W. (2004). Effects of sublethal copper exposure on behavior and growth of Rana pipiens tadpoles. *Bulletin of Environmental Contamination and Toxicology*, *72*(4), 706–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15199983

Relyea, R. A. (2004). Fine-tuned phenotypes: Tadpole plasticity under 16 combinations of predators and competitors. *Ecology, 85*(1), 172-179. doi:10.1890/03-0169

Relyea, R. A., & Werner, E. E. (1999). Quantifying the relation between predator-induced behavior and growth performance in larval anurans. *Ecology*, *80*(6), 2117–2124.

Reza, M., Yamamoto, D. S., & Matsuoka, H. (2012). Low-concentration copper solution jeopardizes larval movement and ability to survive predation: New insight into malaria eradication via vector control. *Medical Entomology and Zoology*, *63*(3), 217–222. doi:10.7601/mez.63.217

Reza, M., Yamamoto, D. S., & Matsuoka, H. (2013). Laboratory testing of larvivorous fish Japanese medaka (Oryzias latipes) predation ability to copper-treated Anopheles stephensi larvae: An alternative method for vector control under low concentration of copper. *Medical Entomology and Zoology*, *64*(2), 67–71. doi:10.7601/mez.64.67

Rohr, J. R., & Palmer, B. D. (2005). Aquatic herbicide exposure increases salamander desiccation risk eight months later in a terrestrial environment. *Environmental Toxicology and Chemistry / SETAC*, *24*(5), 1253–1258. http://doi.org/10.1897/04-448R.1

Rothermel, B. B., & Semlitsch, R. D. (2006).) Salamanders. *Canadian Journal of Zoology*, *84*(6), 797–807. http://doi.org/10.1139/z06-056

Salice, C. J., Rowe, C. L., Pechmann, J. H. K., & Hopkins, W. A. (2011). Multiple stressors and complex life cycles: insights from a population-level assessment of breeding site contamination and terrestrial habitat loss in an amphibian. *Environmental Toxicology and Chemistry / SETAC*, *30*(12), 2874–82. http://doi.org/10.1002/etc.680

Sandahl, J. F., Baldwin, D. H., Jenkins, J. J., & Scholz, N. L. (2007). A Sensory System at the Interface between Urban Stormwater Runoff and Salmon Survival. *Environmental Science & Technology*, *41*(8), 2998–3004. doi:10.1021/es062287r

Scott, D. E. (1994). The Effect of Larval Density on Adult Demographic Traits in Ambystoma. *Ecology*, *75*(755), 1383–1396.

Scott, D. E., Casey, E. D., Donovan, M. F., & Lynch, T. K. (2007). Amphibian lipid levels at metamorphosis correlate to post-metamorphic terrestrial survival. *Oecologia*, *153*(3), 521–32. doi:10.1007/s00442-007-0755-6

Semlitsch, R. D. (1985). International Association for Ecology Reproductive Strategy of a Facultatively Paedomorphic Salamander Ambystoma talpoideum. *Oecologia*, *65*(3), 305–313.

Semlitsch, R. D., & Gibbons, J. W. (1990). Effects of Egg Size on Success of Larval Salamanders in Complex Aquatic Environments. *Ecology*, *71*(5), 1789–1795.

Semlitsch, R. D., Scott, D. E., & Pechmann, J. H. K. (1988). Time and Size at Metamorphosis Related to Adult Fitness in Ambystoma Talpoideum. *Ecology*, *69*(1), 184–192. Retrieved from http://www.jstor.org

Simpson, S. L., Spadaro, D. A., & O'Brien, D. (2013). Incorporating bioavailability into management limits for copper in sediments contaminated by antifouling paint used in aquaculture. *Chemosphere*, *93*(10), 2499–2506. http://doi.org/10.1016/j.chemosphere.2013.08.100

Skelly, D. K. (1992). Field Evidence for a Cost of Behavioral Antipredator Response in a Larval Amphibian. *Ecology*, *73*(2), 704–708.

Song, L., Marsh, T. L., Voice, T. C., & Long, D. T. (2011). Loss of seasonal variability in a lake resulting from copper sulfate algaecide treatment. *Physics and Chemistry of the Earth*, *36*(9-11), 430–435. http://doi.org/10.1016/j.pce.2010.04.016

Sparling, D.W. (2000). Ecotoxicology of organic contaminants to amphibians. In: Ecotoxicology of Amphibians and Reptiles. SETAC Press, Pensacola, FL USA, pp. 461e494.

Storm, J. J., & Lima, S. L. (2010). Notes and Comments Mothers Forewarn Offspring about Predators : A Transgenerational Maternal Effect on Behavior. *The American Naturalist*, *175*(3), 382–390. http://doi.org/10.1086/650443

Taniguchi, K., Toshima, Y., Saito, T. R., & Taniguchi, K. (1996). Development of the Olfactory Epithelium and Vomeronasal Organ in the Japanese Reddish Frog, Rana japonica. *The Journal Of Veterinary Medical Science / The Japanese Society Of Veterinary Science*, *58*(1), 7–15. http://doi.org/10.1248/cpb.37.3229

Tarvin, R. D., Silva Berm Udez, C., Briggs, V. S., & Warkentin, K. M. (2015). Carry-over Effects of Size at Metamorphosis in Red-eyed Treefrogs: Higher Survival but Slower Growth of Larger Metamorphs. *Biotropica*, *47*(2), 218–226. doi:10.1111/btp.12198

Todd, B. D., Bergeron, C. M., Hepner, M. J., & Hopkins, W. A. (2011a). Aquatic and terrestrial stressors in amphibians: a test of the double jeopardy hypothesis based on maternally and trophically derived contaminants. *Environmental Toxicology and Chemistry / SETAC*, *30*(10), 2277–84. doi:10.1002/etc.617

Todd, B. D., Bergeron, C. M., Hepner, M. J., Burke, J. N., & Hopkins, W. A. (2011b). Does maternal exposure to an environmental stressor affect offspring response to predators? *Oecologia*, *166*(1), 283–90. doi:10.1007/s00442-011-1961-9

Touchon, J. C., Jiménez, R. R., Abinette, S. H., Vonesh, J. R., & Warkentin, K. M. (2013). Behavioral plasticity mitigates risk across environments and predators during anuran metamorphosis. *Oecologia*, *173*(3), 801–11. http://doi.org/10.1007/s00442-013-2714-8

Travis, J. (1984). Anuran size at metamorphosis: experimental test of a model based on intraspecific competition. *Ecology 65*, 1155–1160.

Todd, B. D., Willson, J. D., Bergeron, C. M., & Hopkins, W. A. (2012). Do effects of mercury in larval amphibians persist after metamorphosis? *Ecotoxicology (London, England)*, *21*(1), 87–95. doi:10.1007/s10646-011-0768-0

USEPA, 1997. Chemistry assitance manual for premanufacture notification submitters. Office of Pollution Prevention and Toxics (7406), EPA-744-F-97-003, p6.

USEPA, 2007. Aquatic life ambient freshwater quality criteria – copper. Office of Water Report EPA-822-R-07-001, p1-19.

Van Buskirk, J., & Saxer, G. (2001). Delayed costs of an induced defense in tadpoles? Morphology, hopping, and development rate at metamorphosis. *Evolution*, *55*(4), 821–829.

Vargas, G., Michaud, J. P., & Nechols, J. R. (2012). Cryptic maternal effects in Hippodamia convergens vary with maternal age and body size. *Entomologia Experimentalis et Applicata*, *146*, 302–311. doi:10.1111/eea.12027

Vonesh, J. R., & De la Cruz, O. (2002). Complex life cycles and density dependence: Assessing the contribution of egg mortality to amphibian declines. *Oecologia*, *133*(3), 325–333. http://doi.org/10.1007/s00442-002-1039-9

Wassersug, R., & Sperry, D. (1977). The relationships of locomotion to differential predation on Pseudacris triseriata (Anura: Hylidae). *Ecology*, *58*(4), 830–839. doi:10.2307/1936218

Werner, E. E. (1986). Amphibian Metamorphosis: Growth Rate, Predation Risk, and the Optimal Size at Transformation. *The American Naturalist, 128*(3), 319-341. http://doi.org/10.1086/284565

Wilbur, H. M. (1980). Complex life cycles. *Annual Review Ecological Systems*, *11*, 67–93.

Wilbur, H. M., & Collins, J. P. (1973). Ecological Aspects of Amphibian Metamorphosis. *Science*, *182*(4119), 1305–1314.

Zhou, D. M., Wang, Q. Y., & Cang, L. (2011). Free Cu2+ Ions, Cu fractionation and microbial parameters in soils from apple orchards following long-term application of copper fungicides. *Pedosphere*, *21*(2), 139–145. http://doi.org/10.1016/S1002-0160(11)60111-2

Zhou, Y., Zha, J., & Wang, Z. (2012). Occurrence and fate of steroid estrogens in the largest wastewater treatment plant in Beijing, China. *Environmental Monitoring and Assessment*, *184*(11), 6799–813. http://doi.org/10.1007/s10661-011-2459-y

Zhu, B., Liu, L., Li, D. L., Ling, F., & Wang, G. X. (2014). Developmental toxicity in rare minnow (Gobiocypris rarus) embryos exposed to Cu, Zn and Cd. *Ecotoxicology and Environmental Safety*, *104*(1), 269–277. doi:10.1016/j.ecoenv.2014.03.018