UNDERSTANDING HOW AMHIBIANS COPE WITH PERSISTENT CHEMICAL STRESSORS

by

ROBERT WESLEY FLYNN

(Under the Direction of STACEY L. LANCE)

ABSTRACT

Human activities have radically altered environments globally. One of the prominent, but less visible impacts of human activities is the chemical contamination of aquatic habitats. Aquatic organisms are often highly susceptible to changes in the chemical composition of the water they rely on for survival. This dissertation investigates mechanisms by which a highly susceptible species, the southern toad (*Anaxyrus terrestris*), copes with the chronic contamination of wetlands with toxic trace elements. Using an interdisciplinary approach, I assessed the evidence for the evolution of adaptive tolerance in a population living in a contaminated habitat for >60 years, physiological mechanisms underlying tolerance, and the role of gut microbial communities in mediating toxicity. I found evidence of adaptive tolerance to trace element stressors corresponded with physiological divergence in response to these chemical stressors and life history trade-offs in their absence. Gut microbial communities in metamorphic toads were altered by rearing in the contaminated environment, which was associated with greater abundances of potentially pathogenic bacterial genera. Further, the richness and structure of gut communities were related to within environment variation in life history

traits and accumulation of toxic trace elements. Lastly, I examined larval metal tolerance across toad populations varying in their history of contaminant exposure to assess whether adaptive tolerance to trace elements observed in the field study was common across the landscape. Overall, offspring from trace element contaminated sites were more tolerant to aquatic metal exposure, but the extent of this tolerance depended on the specific nature of the contamination across sites. These results suggest the potential for evolutionary rescue to mitigate some negative effects associated with environmental contaminants, but not without costs.

INDEX WORDS: phenotypic plasticity; phenotypic divergence; environmental contaminants; amphibian; microbiome; life history; genetic variation; quantitative genetics; heavy metal; trace element

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BS, Penn State Erie, 2009

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

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2017

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December 2017

DEDICATION

This dissertation is dedicated to all the toads that gave their lives for science.

ACKNOWLEDGEMENTS

I first need to thank the friends and family that have supported me over the course of my adventures in graduate school and research. You have all made this not only possible, but an incredible experience. Over the course of my degree I have been lucky to have amazing people supporting me and the opportunity to meet so many other lovely people to whom I will always carry with me.

I must give special thanks to my advisor Stacey Lance, who had enough faith in me to let me fail when I needed to and help me climb back up afterwards. The time I spent working with her at SREL as a research technician inspired me push myself and develop confidence in myself as a scientist. To Mr. David Scott for seeing something in my questionable technician application package and pushing the hiring committee to give me an interview. David has been a source of inspiration for me from the beginning.

I would also like to thank my committee: Allison Welch, Richard Shefferson, and Gene Rhodes. You all facilitated my intellectual growth and provided invaluable feedback and discussion for all the research I have done over the last five years.

I would also like to thank Austin Coleman, David Scott, Cara Love, Phil Vogrinc, Caitlin Rumrill, and Rochelle Beasley for their invaluable assistance in the field and lab. I had a tendency to design studies, I would only later realize, that were far too much for a single person to do and these people made it possible for me to pursue these projects. A big thank you to John Seaman for his invaluable expertise and assistance in the elemental analysis of tissues and water samples.

This research was funded in part by the U.S. Department of Energy National Nuclear Security Administration (Grant no: 1021RR267432) and the University of Georgia's Odum School of Ecology, Small Grants program. Animals were collected under SCDNR permit #G-09-03 following IACUC procedures (AUP A2009 10-175-Y2-A0) from the University of Georgia.

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CHAPTER 1

INTRODUCTION

Humans have been a dominant force in shaping the structure and function of ecosystems globally (Vitousek, Mooney, Lubchenco, & Melillo, 1997). Collectively, the impact of human activities on the environment fall under the heading of human induced environmental change (HIEC). As the human population continues to grow, the impacts of HIEC are only expected to increase in their extent and intensity (Millenium Ecosystem Assessment, 2010). Human induced environmental change presents challenges for the persistence of wildlife (Vitousek et al., 1997), especially species with specialized habitat needs (Tingley, Hitchmough, & Chapple, 2013), limited dispersal ability (Blaustein, Wake, & Sousa, 1994; Tingley et al., 2013), and high site fidelity (Blaustein et al., 1994). Amphibians are a group of particular conservation concern as they exhibit all of these characteristics and are experiencing declines across species and geographic regions (Collins & Storfer, 2003). The main drivers of amphibian declines are habitat loss and emerging infectious disease (Collins & Storfer, 2003; Kiesecker, Blaustein, & Belden, 2001; Stuart et al., 2004). However, many populations have continued to decline or go extinct even when they retain seemingly suitable habitat (Stuart et al., 2004). Given most severe and pervasive environmental changes are directly tied to human activity, understanding how HIEC influences the health of amphibian populations will be critical to developing conservation strategies that will best retain biodiversity.

Amphibians are a diverse group of organisms that are critical to the function of aquatic ecosystems (Ranvestel, Lips, Pringle, Whiles, & Bixby, 2004; Whiles et al., 2006) and the

transfer of nutrients and energy between aquatic and terrestrial systems (Gibbons et al., 2006). Declines of amphibian populations often stem from complex interactions between natural and anthropogenic environmental factors (Blaustein, Romansic, Kiesecker, & Hatch, 2003; Caruso & Lips, 2013; Collins & Storfer, 2003). Habitat degradation associated with HIEC can create novel, stressful conditions outside the range of conditions to which many organisms are adapted (Sih, Ferrari, & Harris, 2011). Rates of environmental change associated with HIEC are rapid relative to those experienced by organisms over their evolutionary history and thus could outpace the ability for populations to adapt to the new conditions (Palumbi, 2001). One of the most pervasive, but less visible features of HIEC that have been implicated in declines is the degradation of habitats from chemical contamination (Blaustein et al., 2003; Carey & Bryant, 1995). Chemical contaminants are of particular concern for aquatic organisms that rely on clean water for osmoregulation and respiration. Facing stressful, fitness reducing conditions such as those created by chemical contaminants, populations can either respond by migrating for more favorable habitats, exhibiting adaptive plasticity, or adapting to the local conditions. Adaptation to such stressors has been documented in fish and invertebrates inhabiting contaminated environments (Klerks & Bartholomew, 1991; Kolok & L'Etoile-Lopes, 2005; Lopes, Baird, & Ribeiro, 2005; Maroni, Wise, Young, & Otto, 1987; Shirley & Sibly, 1999; Xie & Klerks, 2004), but most of these studies did not account for toxicity derived from multiple routes of exposure to contaminants or for multiple chemical stressors. Exposure to multiple chemical stressors simultaneously is likely to constrain adaptation (Rolshausen et al., 2015).

One globally ubiquitous source of environmental contaminants is the solid waste generated by the combustion of coal for energy. Coal is the single largest source of electricity globally and produces vast amounts of waste in the form of fly ash that must be stored in the environment.

One of the most common strategies to mitigate the environmental impacts of coal ash is to store it in ponds, where the ash can settle out before the water is discharged into the surrounding waterways. However, these permanent sources of freshwater are also attractive to wildlife, which are then put in direct contact with water and sediments containing high levels of numerous toxic trace elements (heavy metals and metalloids; Hopkins, Mendonça, Rowe, & Congdon, 1998; Metts, Buhlmann, Scott, Tuberville, & Hopkins, 2012; Roe, Hopkins, & Jackson, 2005). In chapter 2, I examine whether a population of southern toads (*Anaxyrus terrestris*) inhabiting a system of ponds used to manage coal combustion wastes for > 60 years showed signs of adaptation to the contaminated conditions. Incorporating a quantitative genetic approach with an *in situ* reciprocal transplant, allowed me to 1) compare phenotypic responses between a populations with and without long histories of contaminant exposure; 2) assess potential life history trade-offs associated with any observed differences in contaminant tolerance; and 3) assess how multigenerational exposure to environmental contaminants may have impacted the additive genetic variation necessary for adaptive responses to future environmental change.

The coal combustion waste contaminated habitat is characterized by having high levels of multiple, potentially toxic trace elements (heavy metals and metalloids). These heavy metal trace elements can elicit acute toxicity in aquatic organisms by impairing osmoregulation, which causes the depletion of essential sodium (Na) and potassium (K) ions that ultimately result in death (Grosell, Nielsen, & Bianchini, 2002). As such, tolerance to some heavy metals is associated with the ability to maintain internal levels of these essential elements in the presence of metal stressors (Grosell et al., 2002; Lauren & McDonald, 1987). In Chapter 3, I quantify the elemental compositions of metamorphic toads from trace element-tolerant and -susceptible populations reared in contaminated and reference conditions to assess their physiological

responses to simultaneous aquatic and dietary exposure to multiple contaminants. Comparing how the elemental make up of toads reared in reference and contaminated environments differed between populations provide insight into physiological mechanisms underlying tolerance to complex chemical stressors. I followed this by examining relationships between levels of trace elements and essential electrolytes (e.g. Na and K) with individual phenotypic variation to determine how accumulation of toxic elements and reductions in essential electrolytes contribute to exposure outcomes.

In chapters 2 and 3 I focus on adaptation of amphibians to environmental contaminants. However, the ability of wildlife to tolerate stress are tied not only to their own metabolism, but also to the complex microbial communities with which they are associated (Bordenstein & Theis, 2015). Microbial communities inhabiting the digestive tracts of organisms are particularly important as they contribute to the metabolic pathways available to the hosts (Round & Mazmanian, 2014). Interestingly, a number of microbes known to associate with wildlife hosts are capable of reducing the toxicity of trace elements by metabolizing them or sequestering them in less bioavailable forms (Anderson & Cook, 2004; Combs et al., 1996; Madhaiyan, Poonguzhali, & Sa, 2007). Given the important role of gut microbiota in influencing host phenotypes and their potential to influence the toxicity of chemical stressors, in Chapter 4 I examine how interactions between these microbial communities and the environment could influence exposure outcomes for the amphibian hosts. I quantified how development in a contaminated environment affected the composition of these microbial communities, and how variation in their diversity and structure relates to fitness related traits and accumulation of toxic trace elements in amphibian hosts.

The fifth chapter of my dissertation examines the prevalence of adaptive tolerance to trace element contaminants across multiple populations of southern toads varying in their exposure history to trace element stressors. Trace elements are common contaminants in aquatic habitats, though the concentrations and mixtures of elements can vary considerably. Given a number of trace elements elicit toxicity via similar mechanisms (e.g. oxidative stress and impairment of osmoregulation) and that cross-tolerance to stressors has been observed in other systems, I hypothesized that the three populations from contaminated habitats would be more tolerant to the heavy metal copper (Cu) than the three populations with no history of exposure. I quantified tolerance to Cu using acute, 48 h time-to-death assays replicated within and among populations. I also included the size of adults and eggs for each clutch, as well as concentrations of trace elements in egg masses to account for potential non-genetic maternal effects that could influence tolerance. This approach provided the opportunity to assess the potential for widespread adaptive tolerance to these ubiquitous aquatic stressors, which could mitigate some of the negative impacts associated with environmental contaminants on amphibian populations.

Environmental contaminants are widespread in aquatic habitats and pose a significant threat to aquatic life, including amphibians (Sparling, Linder, Bishop, & Krest, Sherry, 2010). Numerous human activities contribute to the distribution of chemical contaminants into the environment, including energy production, agriculture, manufacturing, and urbanization (Nelson, 2005). These chemical stressors can negatively affect survival (Egea-Serrano, Relyea, Tejedo, & Torralva, 2012; Flynn, Scott, Kuhne, Soteropoulos, & Lance, 2015; Lance et al., 2012; Lance, Flynn, Erickson, & Scott, 2013), growth and development (Carey & Bryant, 1995; Flynn et al., 2015; Lance et al., 2012), and increase susceptibility to disease (Hua et al., 2017) and other stressors. While the immediate impacts of contaminants have been extensively studied, we are

still a long way from understanding the long-term implications of such exposures on amphibians. Understanding how environmental exposures to chemical stressors translate into population-level outcomes for wildlife is the ultimate goal of ecotoxicology and risk assessment. However, these fields have traditionally relied on a number of assumptions that limit their applicability to natural systems. Together, these chapters offer a more holistic approach to understanding the implications of persistent environmental contaminants for amphibian populations.

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CHAPTER 2

PHENOTYPIC DIVERGENCE, PLASTICITY, AND GENETIC VARIATION ASSOCIATED WITH A HUMAN-IMPACTED ENVIRONMENT[1]

[1] R. Wesley Flynn, Allison M. Welch, and Stacey L. Lance. To be submitted to *Evolutionary Applications*.

ABSTRACT

Spatial and temporal change are inherent components of natural environments; however human activities have greatly accelerated the rate and intensity of such changes and created novel conditions unlikely to have been encountered in species evolutionary histories. These relatively recent and often rapid changes present a unique challenge that wildlife must cope with. We employed an *in situ* reciprocal transplant design within a quantitative genetic framework to examine how decades of selection in an environment characterized by high levels of toxic trace elements has affected the life history and quantitative genetic variation in an amphibian population. We found evidence of phenotypic divergence suggesting local adaptation to this contaminated environment and potential trade-offs in the absence of contaminants. Specifically, offspring derived from the population with a history of contaminant exposure had elevated larval growth and survival to metamorphosis in the contaminated environment relative to offspring from the naïve population. While survival in the uncontaminated environment was not compromised in offspring from the putatively adapted population, they did show delayed development and reduced early larval growth and growth rate averaged over larval development. We saw no evidence of reduced additive genetic variation in the divergent population, suggesting long-term selection in a novel environment has not reduced the evolutionary potential of that population. Investigations of local adaptation in response to simultaneous exposure to multiple anthropogenic selection pressures are rare and have generated often conflicting results. Our results demonstrate that adaptation to such complex selective forces is possible in wild populations, which does not appear to be facilitated by adaptive phenotypic plasticity.

INTRODUCTION

Human induced environmental change has modified ecosystems on a global scale and presents challenges for the persistence of wildlife populations (Vitousek et al. 1997). Specifically, habitat degradation can create novel, stressful environments outside the range of conditions to which many organisms are adapted (Sih et al. 2011). For populations to avoid local extinction when confronted with these stressful environments that reduce fitness, organisms can respond by migrating to more favorable habitats, exhibiting adaptive phenotypic plasticity, or adapting to the local conditions. However, the current rates of environmental change are often rapid relative to those experienced by organisms over their evolutionary history (Palumbi 2001b) and thus may outpace the ability of organisms and populations to adjust to new conditions. Comparisons of plastic responses of naïve populations to novel environments and populations having contended with such habitats for multiple generations is a critical step in understanding how wildlife cope with and persist in the face of human induced environmental change.

One of the most pervasive contributors to human induced environmental change is the contamination of habitats with chemical compounds due to human activities (Nelson 2005). These chemical stressors can negatively impact wildlife by reducing growth and development, altering behavior, increasing susceptibility to infectious disease, and generally elevating the risk of mortality (Carey and Bryant 1995; Egea-Serrano et al. 2012). Certain classes of contaminants, such as persistent organic pollutants and trace elements, do not readily degrade in the environment and thus can result in multigenerational exposure. Persistent chemical stressors have the potential to act as strong directional selection pressures that can result in the evolution of tolerance (Klerks and Weis 1987; Groeters et al. 1994; van Straalen and Donker 1994; Merilä et al. 2004).

Adaptive evolution in response to chemical contaminants has been documented across bacterial (de Lima e Silva et al. 2012), plant (Jasieniuk et al. 1996) and animal (Groeters et al. 1994; Shirley and Sibly 1999; Meyer and Di Giulio 2003; Räsänen et al. 2003) lineages. Adaptation resulting in reduced survival costs in the presence of anthropogenic chemicals can be extremely rapid and occur within only a few generations (Klerks and Levinton 1989; Coldsnow et al. 2017). However, there is considerable uncertainty regarding the adaptive potential of species highly susceptible to environmental perturbations or under conditions where multiple anthropogenic stressors are present. Further, adaptive evolution in response to anthropogenic stressors can coincide with divergence in life history traits compared to 'unselected' populations (Klerks and Levinton 1989; Roelofs et al. 2006)

The potential for trade-offs associated with adaptation to anthropogenic stressors has important implications for population viability. Evolved tolerance to chemical stressors can coincide with reductions in fitness in the absence of the chemical stressor, including increased susceptibility to infection (Hua et al. 2017) and reduced reproductive output (Shirley and Sibly 1999; Xie and Klerks 2004), growth (Shirley and Sibly 1999), and development (Xie and Klerks 2004). Reductions in fecundity and delayed development can reduce population growth rates putting populations at greater risk of extinction (McArthur and Wilson 1967; Hutchings et al. 2012) and delays in growth and development can put organisms at greater risk of predation (Wilbur 1980; Werner 1986). Thus, while populations able to adapt to chemical contaminants may decrease their risk of mortality associated with those degraded habitats, it may come with costs that make them more susceptible to other environmental factors. Many organisms, including amphibians, have evolved the capacity for extreme phenotypic plasticity in response to the variability inherent in their environments (Wilbur 1980). This plasticity, or acclimation,

provides organisms the ability to respond rapidly to changing conditions, including chemical contamination.

Phenotypic plasticity allows individuals to alter allocation of resources and developmental course when conditions change or become unfavorable to remaining in the ecological niche associated with a given life stage. In an adaptive context, plasticity can shift phenotypes closer to the optima in the new environment (West-Eberhard 2003). For example, exposure to sublethal levels of some pesticides and heavy metals early in development can increase tolerance to that stressor later on (Lauren and McDonald 1987; Herkovits and Pérez-Coll 2007; Tate-Boldt and Kolok 2008; Hua et al. 2013). However, plasticity, especially in response to novel stressors, is not always adaptive. Environmental exposure to contaminants often results in reduced growth, development, and survival (Egea-Serrano et al. 2012; Lance et al. 2013; Flynn et al. 2015; Hintz and Relyea 2017). Interestingly, species with complex life cycles, like amphibians, stressful conditions in the aquatic environment that increase mortality risk (e.g. pond drying and predation) are expected to result in more rapid larval development, albeit at some cost to size at metamorphosis (Wilbur and Collins 1973; Werner 1986). Thus, instead of adaptive developmental plasticity in response to many contaminants that would reduce the exposure time to aquatic contaminants and risk of contaminant related mortality; non-adaptive plastic responses that increase exposure time to the stressor are common. This suggests that the stress elicited by some compounds at certain concentrations can inhibit the ability for individuals to respond with adaptive plasticity. However, non-adaptive plasticity can actually facilitate adaptive evolution by effectively increasing the strength of the selection pressure or inducing a broader array of phenotypes for selection to act on (Ghalambor et al. 2007, 2015). Though both adaptive and non-adaptive plasticity can facilitate genetic adaptation to novel

environments, populations must have genetic variation in the traits under selection for evolution to occur.

Additive genetic variation is especially important as it can facilitate or constrain evolutionary responses to selection. A number of studies have reported extensive within- and among-population variation in susceptibility to environmental contaminants, which suggests there is genetic variation in contaminant tolerance in wild populations (Bridges and Semlitsch 2000; Barata et al. 2002; Piola and Johnston 2006; Lance et al. 2012, 2013; Metts et al. 2012; Flynn et al. 2015). However, far fewer studies have been able to determine whether this natural variation is due to additive genetic variation underlying tolerance and general phenotypic response to chemical stressors (Klerks and Levinton 1989; Posthuma et al. 1993; Semlitsch et al. 2000; Klerks and Moreau 2001; Räsänen et al. 2003; Roelofs et al. 2006; Bridges and Semlitsch 2011). Further, the majority of these studies have relied on estimates of genetic variation obtained from laboratory studies under artificial conditions that are often not reflective of complex environments experienced by populations in nature. Incorporating reciprocal transplant and quantitative genetic approaches using natural populations reared *in situ* in the environments in which selection has been occurring allows for more realistic estimates of phenotypic and genetic variation in chemical tolerance and life history traits, as well as potential trade-offs associated with tolerance. While additive genetic variation is critical for adaptation to novel environments, multigenerational selection for tolerance has the potential to reduce genetic variation, which could hinder populations' ability to respond to future environmental change.

Amphibians are an ideal system to assess the relative importance of plasticity and adaptation in response to anthropogenic stressors. Amphibians are especially susceptible to a number of factors associated with human induced environmental change, in part due to their

unique physiological constraints, limited dispersal capability, and high site fidelity (Blaustein et al. 1994). Because many amphibians rely on aquatic habitats for larval development and reproduction, habitat degradation resulting from chemical contamination is of particular concern for aquatic organisms. Trace element (TEs) contaminants, including heavy metals and metalloids, are especially common in aquatic environments and because they do not degrade they lead to multi-generational exposure. Exposure to elevated TEs in aquatic environments is common and associated with a broad range of negative effects (Linder and Grillitsch 2000; Fedorenkova et al. 2012). In response to TE exposure, amphibians often display reduced survivorship (Rowe et al. 2001; Metts et al. 2012; Lance et al. 2013), growth (Snodgrass et al. 2004; Chen et al. 2006; Metts et al. 2012; Peles 2013), and development (Snodgrass et al. 2004; Metts et al. 2012; Flynn et al. 2015), as well as altered stress response (Hopkins et al. 1999) and elevated metabolic costs (Rowe et al. 1998). Despite these immediate negative impacts on fitness, some populations continue to persist in the presence of these persistent stressors (Weis and Weis 1989). Both adaptive plasticity and evolution can mitigate some of the negative impacts on fitness associated exposure to chemical stressors, including anthropogenic acidification (Hangartner et al. 2012), salinization (Brady 2012; Wu et al. 2014), and agricultural chemicals (Cothran et al. 2013; Hua et al. 2015). However, it remains unclear how common these mechanisms are in natural systems across species and environments characterized by multiple chemical stressors.

Given the detrimental effects of even short term TE exposure and widespread within population variation in tolerance (Lance et al. 2012, 2013; Flynn et al. 2015), we hypothesized that a population subjected to multigenerational exposure of toxic TEs would exhibit elevated survival in that environment relative to a nearby population without a history of exposure. We

also hypothesized that this adaptive divergence would be associated with trade-offs in the absence of toxic TEs and reduced genetic variation in life history traits due to directional selection for tolerance. We selected coal combustion wastes (CCWs) as our stressor because they are a globally pervasive source of TEs in the environment. Coal combustion waste is often stored in surface impoundments (USEPA, 2010) which provide permanent sources of freshwater that can be attractive to wildlife for food, water, and reproduction. In these impoundments wildlife come in direct contact with CCWs which can negatively impact reproduction, survival, and development (Raimondo et al. 1998; Rowe et al. 2001; Metts et al. 2012). We designed our study to 1) determine if an amphibian population inhabiting a CCW disposal site has diverged phenotypically from a nearby population inhabiting an environment without a history of contamination, 2) characterize patterns of phenotypic plasticity between populations to assess evidence of local adaptation and life history trade-offs, and 3) quantify quantitative genetic variation in life history traits and their plasticity to make inferences about past selection and assess their future evolutionary potential.

METHODS

Study species

Southern toads (*Anaxyrus terrestris,* Bonnaterre) are widely distributed across the southeastern United States and can be one of the most abundant anuran species in wetlands within their range (e.g. representing up to 29% of terrestrial amphibian community (Bennett, Gibbons, and Glanville 1980). *Anaxyrus terrestris* are considered indiscriminate breeders, using a variety of ephemeral, permanent, natural, and constructed aquatic habitats (Wright and Wright 1949; Gibbons and Semlitsch 1991; Hopkins et al. 1997) and they remain common in areas even

in the face of severe habitat destruction and urbanization (Bartlett and Bartlett 1999a). Larvae are grazers, scraping algae, biofilms, and decaying organic matter from submerged surfaces, putting them into direct contact with sediments (Jensen 2008).

Study populations and sites

The study sites consisted of a CCW disposal site (ASH) and a large (>10 acre) temporary wetland (REF) located ~3.0 km from ASH, both located on the United States' Department of Energy Savannah River Site (SRS) in Aiken county South Carolina. The ASH site was created in the 1950's to manage waste from a nearby coal-fired power plant designed to generate steam for DOE activities on the SRS (US Environmental Protection Agency 2007). This site consists of influent, primary settling, and secondary settling basins, where a slurry of coal fly ash and water are pumped in. The system is designed so that the ash settles out from the water column over time as it slowly flows through the three successive basins, before the water is discharged into a nearby stream system which empties into the Savannah River. *Anaxyrus terrestris* use these basins for reproduction though very few recently metamorphosed individuals have been observed emerging (*pers. comm.* WA Hopkins; Rowe, Hopkins, and Coffman 2001). The water chemistry at the ASH site differs from natural wetlands (e.g. REF), having exceedingly high conductivity (>700 μS/cm vs. <100 μS/cm), low levels of dissolved organic carbon, and more basic pH (Appendix Table A1). The water, sediment, and biofilms at ASH contain highly elevated levels of a number of TEs, including arsenic, selenium, chromium, and cadmium (see Appendix Table A2 and Hopkins, Mendonça, Rowe, & Congdon, 1998; Metts et al., 2012; Roe, Hopkins, & Jackson, 2005). Adult *A. terrestris* living in and around the ASH site (i.e. ASH population) have elevated levels of many TEs associated with CCWs (Metts et al. 2012, 2013)

suggesting that dietary exposure in the terrestrial environment may be an important route of exposure.

Artificial fertilization design and methods

We used drift fences installed at each site to capture adults migrating to breed from 3/31/14 - 4/10/14. We recorded mass and snout-vent length (SVL) for all adults, which were used to calculate body condition indices for each animal (see Methods: *Statistical and quantitative genetic analyses*). Females were injected with 250 IU of human chorionic gonadotropin to induce ovulation and placed individually in 6 qt plastic shoeboxes containing simplified amphibian ringers solution (ASH: 0730 on 4/15/14; REF: 2400 on night of 4/17/14 - 4/18/14). Females began laying eggs 10-15 h post-injection. As eggs became available we euthanized males in 3% MS-222, rinsed them with water and removed their testes. Each testis was placed into a separate 1.5 mL microcentrifuge tube containing 500 μL of reconstituted soft water (US Environmental Protection Agency 2002) and macerated before diluting with an additional 1.0 mL of soft water. In addition to the previously euthanized males, following fertilization, female toads were also euthanized, frozen, and lyophilized to determine TE body burdens.

The overall breeding design consisted of 32 total sires and eight total dams in four blocks per site, each block consisting of eight sires and two dams (Appendix Figure A1). The breeding within each block was fully crossed, which resulted in 16 full sibling families per block, 64 full sibling families per population, and 128 families total. This breeding design maximized the power to estimate sire effects, but reduced the statistical power to estimate dam and dam x sire effects. To achieve this breeding design we cut strings of eggs into sections of approximately equal length and distributed them among eight 60 mL plastic weigh cups (i.e. one cup for each

sire x dam combination). Then, to fertilize eggs, we immediately (i.e. within one minute) added and then gently swirled 600 μL of the sperm suspensions pooled from both testis onto the eggs of each of the two females in each breeding block. After 15 min, the weigh cups containing the eggs and sperm were flooded with soft water and left overnight. We preserved a subset of ten eggs from each female (except for one female from the REF population due to low fertilization success) in 4% formalin to later photograph and determine mean egg size for each clutch using the program ImageJ (Schneider et al. 2012).

Embryo trial

After 24 h we removed any obviously dead or non-developing embryos and divided the remaining embryos roughly equally among replicates containing either water collected from REF or ASH ponds (ASH: 4/17/15 and REF: 4/20/15; i.e. two days post-fertilization). We used a full factorial design that included 2 source populations x 64 families (generated from 8 dams and 32 sires from each population) x 2 water treatments x 3 spatial blocks, resulting in 768 experimental units. Each replicate corresponded to a shelving unit in our climate controlled Animal Care Facility. Previous research in this room has shown that the temperature is consistent vertically, but can vary slightly based on distance from the door/HVAC unit. Each family and treatment was represented once in each block. There were too few successfully fertilized eggs from some clutch x treatment combinations to fill any or all of the replicates (see Appendix Table A3), but otherwise each experimental unit contained 5-45 viable embryos. Experimental units consisted of 0.5 L plastic containers containing 400 mL of water collected from either the REF or ASH sites. Water samples were taken from a subset of experimental units at the start and end of the embryonic study to quantify for major cations and TEs in the test solutions (Appendix Table A4). We checked containers daily and counted and removed any dead embryos. Though time to

the free-swimming stage, Gosner stage 25 (GS 25) varied slightly among clutches and populations (not reported), this stage was reached for all normally developing embryos by five days post-fertilization. Any malformed or unresponsive embryos were counted as "dead" on day five. After final counts, we pooled all replicates within a family x treatment and haphazardly sampled ten larvae (in some cases fewer when survivorship was low for a family x treatment group) to determine mean size (total length) at GS 25 for each family x treatment combination.

Larval Field Enclosures and Study Design

Subsets of six larvae (fewer in some cases due to mortality; see Appendix Figure A1) from each family x rearing solution combination from the embryo trial were carried through to the field portion of the study. We reared these larvae to metamorphosis *in situ* in field enclosures in each environment, maintaining the rearing solution they experienced in embryonic development (i.e. individuals reared through embryonic development in water collected from the REF environment were continued on in the REF environment in the field). We designed the enclosures to allow individual rearing of larvae and regular exchange of water. The general design consisted of a 26.5 L plastic storage bin (58 x 41 x 15 cm) into which six smaller 1 L plastic containers (14.5 x 14.5 x 11.5 cm) were nested. We removed the centers of lids and container bottoms of both the small and large containers and replaced them with non-metal screen to keep larvae in and exclude predators, while allowing for exchange of water, suspended sediments, and air with surrounding environment (see Appendix Figure A2 for details). The enclosures were deployed two weeks prior to the start of the field trial to allow the insides of the containers to be colonized with resident algae and biofilms that would provide a food source for developing larvae.

Field enclosures were grouped into six spatial blocks in each environment, where one larva from each family (i.e. one replicate) was represented in each block. We used a random number generator to assign families to containers within each block. Due to low fertilization and survivorship to the larval stage in several families from the REF population and an experimental error, offspring from two breeding blocks were not used in the field study. This resulted in an unbalanced design for the field portion of the study, with 64 families represented from the ASH population and 32 from REF (96 families total).

We transferred larvae to field enclosures two days after all surviving larvae reached GS 25 (ASH: 4/25/14, REF: 4/28/14). Initial photos of individual larvae in the field were taken seven days later (ASH: 5/2/14, REF: 5/5/14) and a final set of photographs 14 d after the initial photos (or 21 days after being in the field; ASH: 5/16/14, REF: 5/19/14). Initially, we made observations three times per week at which time we exchanged water by gently lifting each enclosure up until only ~1 cm of water remained and setting it back down. After the first larvae began developing rear legs we began checking enclosures daily. Individuals were determined to have metamorphosed upon the emergence of at least one forelimb (GS 42). All metamorphic individuals were brought back to the laboratory and maintained at 23^{o}C (±1.5^{o}C) in 0.5 L plastic containers with an unbleached paper towel dampened with water from the individual's field container. We terminated the field study on 08/07/17 (101st and 104th day in the field for REF and ASH populations respectively) when only eight larvae remained and had showed no signs of further development for several weeks.

Throughout the field study we monitored water temperature with iButtons® temperature loggers sealed with clear Plastidip ® rubber coating. Loggers were placed in the bottom of every other field enclosure and programmed to log a point every hour to obtain a temperature profile

for every spatial block within each environment. On 5/2/14 and 6/9/14, at a subset of blocks we measured pH and specific conductivity (μS/cm) with a YSI Pro-Plus instrument with a Quattro cable in two randomly selected bins and the surrounding water (see Appendix Table A5) and collected two 14 mL water samples at ~4 cm below the water surface (one inside and one outside the bins for subsequent analyses of total levels of major elements and TEs.

Metamorphic toad processing protocol

After metamorphic individuals were returned to the lab, we observed them daily to monitor tail resorption. Two days after individuals reached GS46 (GS46 was determined when < 1.0 mm of tail remained) animals were gently blotted dry before being weighed (± 0.01 mg) and measured for SVL to the nearest 0.5 mm. We euthanized individuals within two minutes of initial handling by immersion in a 4% solution of MS-222. Following euthanasia, we dissected and preserved liver, gut, and limb tissues for future analyses.

Sample prep and elemental analysis

To prepare water samples collected in the lab and field for analysis we added 140 μL of trace metal-grade nitric acid (HNO_3) to the 14 mL of each sample in trace element-free certified 15 mL conical tubes (VWR®) to yield a final concentration of 1% HNO_3. Acidified samples were run on an inductively coupled plasma mass spectrometer (ICP-MS, Nexion 300X ICP-MS; Perkin Elmer, Norwalk, CT, USA) for TE (aluminum [Al], arsenic [As], barium [Ba], beryllium [Be], cadmium [Cd], cobalt [Co], copper [Cu], nickel [Ni], tin [Sb], selenium [Se], strontium [Sr], thorium [Th], uranium [U], vanadium [V], and zinc [Zn]) and major cation concentrations (sodium [Na], calcium [Ca], magnesium [Mg], and potassium [K]).

We prepared toe and liver samples collected from parental toads by freeze drying followed by homogenizing with non-metallic pestle if sample was >15 mg dry weight. We

weighed dried samples to the nearest 0.01 mg before digesting in 1.5 mL clear microcentrifuge tubes by adding 300 μL HNO_3 and placing on a heat block at 80^{o}C for two hours. We also digested reference standards (TORT-3, National Research Council Canada) and blanks for quality control and determination of minimum detection limits. Digested samples were diluted with ultrapure water before analyzing on ICP-MS (3.33% HNO_3 final sample concentration). Due to small dry masses associated with the tissue samples, only a subset of elements was analyzed.

Statistical and Quantitative genetic analyses

All statistical analyses were carried out in R (Team 2013). We examined differences in water quality (pH, spc, Na, Ca, Mg, and K) and TE concentrations (Al, As, Ba, Be, Cd, Co, Cu, Ni, Sb, Se, Sr, Th, U, V, and Zn) between environments and among blocks within environments using MANOVAs followed by univariate ANOVAs. We performed principal component analysis (PCA; *prcomp* in *stats* package; Team, 2013) with scaling, centering and varimax rotation to provide a more concise representation of differences within and between rearing environments. We tested for differences in adult body burdens of TEs (As, Ba, Cd, Cu, Se, Sr, V, Zn) using MANOVA with population of origin and sex as fixed effects using type III sums of squares. The interaction between population and sex was not significant ($p = 0.8063$), so it was not included in the final models. Body condition indices (BCI) for each adult were derived by taking residuals from models regressing SVL on mass (Schulte-Hostedde et al. 2005). These residuals were then used as the response variables in a simple linear regression including population of origin and sex as fixed effects.

We determined differences between populations and environments in larval traits (size at GS 25, early larval size and growth, time to metamorphosis, size at metamorphosis, and growth

rate over entire larval period [metamorphic mass with time to metamorphosis as a covariate], and probability of metamorphosis), by fitting generalized linear mixed models that included fixed effects for population of origin (hereafter 'population'), rearing environment (hereafter 'environment'), and their interaction, as well as a random effects structure including within environment spatial block and environment-specific terms for sire, dam, and sire x dam (*glmer* in *lme4* package; Bates, Machler, Bolker, & Walker, 2015). We did not include water temperature in our models as we determined it was correlated with spatial block and therefore opted to use block alone as it accounted for spatial differences in temperature within environment as well as other microenvironmental differences. Continuous response variables were log-transformed prior to analysis and visually inspected to confirm data followed an approximately Gaussian distribution. Significance of fixed effects are reported as Wald-χ^2 tests with Type III error (*car* package: *Anova;* Fox & Weisberg, 2011). As *lme4* does not support multivariate distributions, embryonic survival was modeled as a bivariate response of successes and failures using a bivariate distribution (family = "multinomial2") with weak, non-informative parameter expanded priors in the R package *MCMCglmm* (Hadfield, 2010, 2015). As the number of viable embryos differed among clutches and experimental units due to variation in fertilization success and can influence early survival, it was included as a covariate in these analyses (Lance et al. 2013). Mean embryo sizes for each clutch of eggs was included as a covariate in initial models of embryonic survival, but removed from final models as it was not significant (pMCMC = 0.310) and did not improve model fit.

To assess population-specific responses of traits to the two environments and obtain estimates of quantitative genetic parameters associated with plasticity, we modeled embryonic survival and metamorphic probabilities and larval traits separately by fitting generalized linear

mixed models in a Bayesian framework using Markov Chain Monte Carlo sampling in the R package *MCMCglmm* (Hadfield 2010). We used weak, non-informative priors and optimized each model to ensure adequate mixing and negligible autocorrelation (Hadfield 2015). These plasticity models included rearing environment as a fixed effect, while sire, dam, sire x dam, and spatial block were treated as environment-specific random effects. We fit binary response variables (i.e. metamorphosis probability) to a probit model (family = "threshold"). Embryonic survivorship was modeled as a bivariate trait of dead and surviving embryos (family = "multinomial2") and included initial egg number as a covariate as it improved precision of variance estimates and improved model mixing.

We estimated environment-specific quantitative genetic variance components associated with each trait by fitting separate univariate *MCMCglmm* models for each population x environment combination. Models included only the intercept as a fixed effect, with a random effects structure including sire, dam, sire x dam, and block. We estimated causal variance components as follows:

$$V_P = \sigma^2_{sire} + \sigma^2_{dam} + \sigma^2_{sire:dam} + \sigma^2_{block} + \sigma^2_{residual}$$

$$\sigma^2_{sire} = \frac{1}{4}V_A$$

$$\sigma^2_{dam} = \frac{1}{4}V_A + V_M$$

$$\sigma^2_{sire:dam} = \frac{1}{4}V_D$$

$$\sigma^2_{residual} = \frac{1}{2}V_A + \frac{3}{4}V_D + V_E$$

Additive genetic (V_A), non-genetic maternal (V_M), and non-additive genetic (V_D) contributions to total phenotypic variance of traits were population- and rearing environment-

specific, with total phenotypic variation (V_P) expressed as the sum of variances for all random effects in a given model, including residual variance (see Appendix Table A6 for estimates of each component). We calculated the proportion of phenotypic variance explained by quantitative genetic components by dividing V_A, V_M, and V_D by V_P, yielding estimates of narrow-sense heritability (h^2), non-genetic maternal, and non-additive genetic contributions respectively. These parameters were estimated for the plasticity of traits using the random terms for the interactions of rearing environment with sire, dam, and sire x dam. All confidence intervals for Bayesian analyses are given as 95% highest posterior density intervals (HPDI). The heritability of plasticity, h^2_{pl}, was calculated following the approach of Becker (1964) and Scheiner & Lyman (1989):

$$h^2_{pl} = \frac{4\,\sigma^2_{S\,X\,E}}{\sigma^2_P}$$

To obtain estimates of the contribution of other quantitative genetic parameters associated with the plasticity of population-specific life history traits across the two rearing environments, we followed a similar approach, except parental factors nested within rearing environment and site was used as a fixed effect. We used the MCMC-derived slopes and p-values from model 'environment' terms (i.e. β -estimates) to determine statistical significance, direction, and slope of the plastic response of traits to rearing environment.

We also used Cox's proportional hazard models (Therneau 2015) to test how rearing environment affected probability of metamorphosis. This is a non-parametric analysis that incorporates a binary value for the event (i.e. 0 = died prior to metamorphosis, 1 = successful metamorphosis) and time to the event (i.e. metamorphosis). In these analyses, rearing environment, population of origin, and their interaction were included as fixed effects and sire,

dam, and block within environment as random effects. Individuals that had not metamorphosed by the end of the study (i.e. 104 days, $n = 8$), were considered unsuccessful and censored. This approach generates hazard ratios associated with metamorphic probability for a given population in a given environment that have a convenient direct interpretation. Significant hazard ratios less than 1 show that the probability of metamorphosis is reduced relative to a reference environment, while those greater than 1 show metamorphic probability was elevated. Specifically, a hazard ratio of 0.5 would suggest that the 'risk' of metamorphosing in that environment was half that relative to the other environment.

RESULTS

Adult size and body burden

Adults from the two populations did not differ significantly in terms of SVL ($F_{1,71} = 1.3795$, $p = 0.244$) or mass ($F_{1,71} = 1.4168$, $p = 0.238$). Interestingly, adults from the ASH population had greater body condition indices (BCI) than those from REF ($F_{1,71} = 8.5926$, $p = 0.004$) and post-oviposition dams had greater BCI than sires ($F_{1,71} = 4.972$, $p = 0.029$). Though the population by sex interaction was not significant and was removed from the model, the differences in BCI between populations appear largely due to population level differences in BCIs of males (Appendix Figure A3).

We found adults from the ASH population accumulated higher levels of trace elements (i.e. liver) than those from REF (MANOVA: $F_{1,68} = 12.3085$, $p < 0.0001$) and that overall, females tended to accumulate higher levels of elements than males (MANOVA: $F_{1,68} = 7.1131$, $p < 0.0001$). Univariate models showed that body burdens of ASH adults were greater than (As,

Cu, Se, and Sr) or not different (Cd) from REF adults. Additionally, in adults from ASH, accumulation of some elements (i.e. As, Se) was positively associated with body size.

Water quality and chemistry

The aquatic environments at the two sites differed markedly in their water chemistries (MANOVA: $F_{1,24} = 7266.2$, $p < 0.0001$), but there was little variation among spatial blocks within environment (MANOVA: $F_{4,24} = 1.2$, $p = 0.268$). Water pH, specific conductance, Ca, Na, K, and Mg were always greater in ASH than REF, and there was relatively little within environment variation (Appendix Table A1). Similarly, TE levels in ASH were either greater than or not different from REF, however the ASH environment was significantly more variable than REF (with exception of Zn; Appendix Table A2). Principal components analysis revealed the two environments differed most markedly along PC1, which was associated with elevated pH, conductivity, As, Ba, Ni, Se, and Sr (see Appendix Table A7). Temperature profiles differed among environments with ASH being consistently warmer and having less daily fluctuation in temperature than REF (Appendix Figure A5).

Phenotypic divergence

Embryonic laboratory trial

Embryo size differed significantly between populations ($F_{1,149} = 184.405$, $p < 0.0001$), such that embryos from ASH dams were significantly larger than those from REF, even after accounting for variation in dam size (dam size: $F_{1,149} = 3.777$, $p = 0.054$; , Appendix Figure A6). Survival through embryonic development was not influenced by rearing solution ($p = 0.857$), population of origin ($p = 0.426$) or their interaction ($p = 0.503$). Initial number of embryos in had a significant positive effect on survival ($p = 0.024$), but mean egg size did not.

Size at GS 25 was affected by rearing solution (Wald-χ^2 = 24.508, p < 0.0001), but not by population of origin (Wald-χ^2 = 0.962, p = 0.327). However, there was a significant population by rearing solution interaction (Wald-χ^2 = 23.948, p < 0.0001) such that larvae from the ASH population were of similar size in both solutions, but REF larvae were larger in the ASH solution, which suggests the effects of rearing solution on size at GS 25 are dependent on population of origin.

Larval field trial

Size at seven and 21 days of larval development were greater in the ASH-environment (7d: Wald-χ^2 = 40.543, p < 0.0001; 21d: Wald-χ^2 = 8.7113, p = 0.003, Fig. 2.1A,B) and there was a significant population x environment interaction at day seven, such that mean size was comparable for both populations in the REF environment, but in the ASH environment larvae from the REF population were slightly larger than those from the ASH population (Wald-χ^2 = 20.616, p < 0.00001; Fig. 2.1A). Early larval growth was marginally greater in the ASH population (Wald-χ^2 = 3.8349, p = 0.050). However the population by environment interaction (Wald-χ^2 = 31.8499, p < 0.0001) revealed that ASH larvae reared in the ASH environment experienced the most rapid growth, but ASH larvae reared in the REF environment grew the slowest (Figure 2.1C).

Time to metamorphosis was affected by rearing environment (Wald-χ^2 = 8.8921, p = 0.003) and population of origin (Wald-χ^2 = 5.7532, p = 0.017). Development was delayed in the ASH environment and the ASH population developed more slowly than the REF population (Figure 2.1D). There was a marginal population by environment interaction (Wald-χ^2 = 3.3268, p = 0.068) as a result of the ASH population developing more slowly than the REF population when reared in the REF environment. Size at metamorphosis (SVL) did not differ by population

of origin (Wald-χ^2 = 0.1215, p = 0.727) or rearing environment (Wald-χ^2 = 0.0867, p = 0.768) and there was no evidence of a population by environment interaction (Wald-χ^2 = 0.0044, p = 0.947, Fig 2.1E). Though not reported, we ran models using mass at metamorphosis as the response variable which generated similar results.

Growth rate over the entire developmental period (mg/d) was significantly greater in the REF population (Wald-χ^2 = 6.3651, p = 0.011) and the REF environment (Wald-χ^2 = 5.3948, p = 0.020, Fig 2.1F). Although there was no statistically significant population by environment interaction (Wald-χ^2 = 1.811, p = 0.178), pairwise comparisons within each environment showed offspring from the ASH-population were significantly smaller than their REF-population counterparts when reared in the REF environment.

Using Cox proportional hazard models incorporating not only whether metamorphosis was achieved, but the time that was required to reach metamorphosis, we found that individuals in the ASH environment were half as likely to metamorphose compared with those in the REF environment (hazard quotient = 0.4864, p < 0.0001) (Figure 2.2). However, a significant population x environment interaction showed that the ASH population experienced less of a cost, in terms of metamorphic probability in the ASH environment, than the REF population (hazard quotient = 1.4361, p = 0.024).

Phenotypic plasticity

Population-specific models of trait plasticity between environments revealed that plastic responses were similar between populations for several traits and generally congruent with observations of population x environment interactions observed in the full models. We did not find any evidence of consistent phenotypic responses in either population for embryonic survival, size at GS25, size at metamorphosis, or probability of metamorphosis. However, both

populations showed significant plasticity in larval size at day 7 and time to metamorphosis, such that larvae were larger and took longer to develop in the ASH environment (Figure 2.1 A, D).

Plasticity in larval size at day 21 and early larval growth were markedly different between populations. While the REF population reaction norms were not significantly different from zero for either trait, ASH population reaction norms were significantly positive. Offspring from the ASH population were significantly larger and grew more in early larval development in the ASH environment. So while the ASH environment did not seem to negatively affect offspring from the REF population, the ASH population actually performed better in ASH conditions for these traits. However, it should be noted that ASH larvae at day 21 were not significantly larger in the ASH environment or smaller in the REF environment than the REF population. This suggests that the lack of a significant slope for size at day 21 in the REF population may be more related to the reduced statistical power to detect effects due to the smaller sample size for that REF population (i.e. N = 273 vs. 557).

Quantitative genetic variation in life history traits and their plasticity

We did not find survival through embryonic development to be heritable; however we detected strong non-genetic maternal effects for both populations in both rearing conditions even after accounting for initial number of embryos (Table 2.1). Size at GS 25 was moderately heritable for all populations and rearing conditions (h^2 = 0.41 – 0.56) and maternal effects were moderate, but generally had confidence intervals approaching zero (Table 2.1). Non-additive genetic effects (dom) were overall similar to additive genetic contributions to phenotypic variance. Mean non-additive effects were ~25% lower for GS25 than additive genetic effects, but well within confidence intervals for both. Estimates of h^2_{pl} for both traits were similar for both populations, but estimates for size at GS25 were roughly 50% lower than within environment

estimates (Table 2.2). This apparent reduction is likely not statistically significant as confidence intervals are heavily overlapping.

Heritability and non-additive genetic contributions to early larval size and growth were in general moderate to low respectively. Non-genetic maternal effects appeared to contribute negligibly to phenotypic variation in these traits, as means estimates were low and confidence intervals overlapped zero. These population x environment-specific heritability estimates were similar overall, but means were nearly twice as high for REF population reared in the ASH environment compared to other population x environment estimates (Table 2.1). Non-additive genetic effect estimates were similar to additive genetic contributions for size at day 7, but differed markedly for early larval growth for the REF population. In both environments, non-additive genetic effects were nearly 100% greater than those estimated for additive genetic effects. These non-additive effects were also considerably larger than those for the ASH population, though confidence limits overlapped.

We detected low heritability for developmental time that was comparable to the estimated non-additive genetic effects (Table 2.1). The heritability and non-additive genetic estimates for developmental time in the ASH population in the ASH environment were substantially lower than other estimates (~ 0.10 vs. >0.25). Estimates of maternal effects were consistently very low and had confidence limits overlapping zero, suggesting limited evidence for non-genetic effects contributing to phenotypic variation. Additive genetic variation contributing to plasticity in developmental time was lower than environment specific-estimates (Table 2.2). Further, h^2_{pl} for developmental time in the ASH population was roughly 50% of that estimated for the REF population.

Size at metamorphosis, was moderately heritable and non-additive genetic effects contributed similarly as additive genetic effect to phenotypic variation (Table 2.1). Estimates of maternal effects contributing to phenotypic variation all overlapped zero, but mean estimates were twice as large in the REF population as ASH. Though there was no significant population level plastic response to rearing environment for size at metamorphosis, we did find both populations to show low heritability for this trait (Table 2.2). Like developmental time, h^2_{pl} estimates were lower than those obtained for environment specific h^2.

Both additive and non-additive genetic variation contributed moderately to growth rate, while non-genetic maternal contributions were extremely low (Table 2.1). We saw no marked differences in means estimates between population and rearing environment combinations. Additive genetic contributions to plastic responses between rearing environments were low and roughly half those for environment specific estimates.

Estimates of additive genetic contributions to probability of metamorphosis were low (Table 2.1). Non-additive genetic variation was similar to that for additive genetic variation, except for estimates obtained for the ASH population reared in the REF environment (Table 2.1). Though confidence intervals were large and approached zero, the mean estimate of non-additive genetic effects was twice that for additive genetic effects. Maternal effects were extremely small and overlapped zero. Similarly, additive genetic contributions to plasticity in metamorphic probability were very low and imprecise (Table 2.2).

DISCUSSION

Our results demonstrate phenotypic divergence of an amphibian population originating from an environment impacted by CCWs from a nearby population with no known history of

contaminant exposure. Consistent with local adaptation to CCWs, offspring from the ASH population had a nearly 50% greater probability of successful metamorphosis in the ASH environment than the REF population. Further, there was no evidence that elevated tolerance to CCWs carried direct survival costs in the absence of those stressors, though the compromised early larval growth and rate of development we observed could translate to survival costs under conditions allowing for predation and competition. This is consistent with observations across a number of taxa where populations residing in environments impacted by contaminants show improved survival in presence of related chemical stressors relative to populations naïve to those stressors (Klerks and Levinton 1989; Xie and Klerks 2004; Roelofs et al. 2009; Hangartner et al. 2012; Hua et al. 2013). While survival though aquatic development is most closely tied to fitness, other phenotypic measures can contribute substantially to the probability of survival to reproductive maturity (Smith 1987).

Phenotypic divergence in a contaminated environment

In organisms with biphasic life histories, including many amphibians and invertebrates, early aquatic development is followed by juvenile and adult terrestrial stages in which reproduction occurs. In amphibians, survival to first reproduction is tied to time to- and size at the metamorphic transition (Smith 1987). We saw no differences in time to metamorphosis or size at metamorphosis between populations reared in the ASH environment. Larval development was significantly delayed in the presence of CCWs, but it did not correspond with larger size at metamorphosis. This translated into an overall decrease in growth rate in terms of average mass gain per day of development. While metamorphosing at larger sizes generally conveys greater probability of survival in the terrestrial environment, optimal size at this transition is also dependent on the environmental conditions in the aquatic environment (Wilbur and Collins 1973;

Werner 1986). For example, when aquatic conditions are optimal for growth and survival (i.e. abundance food resources with limited competition and predation) prolonged aquatic development can allow individuals to maximize growth and survival across life stages. However, when conditions in the aquatic environment sufficiently elevate mortality risk or reduce growth, fitness may be maximized by developing rapidly and metamorphosing at smaller sizes (Werner 1986). Given that aquatic exposure to CCWs significantly increases the risk of mortality for developing amphibian larvae, under this theory we would predict adaptive plasticity would result in shorter larval periods and consequently smaller sizes at metamorphosis. We saw no evidence of this and instead saw extended larval periods with no change in size at metamorphosis. This suggests that the stressors in the ASH environment were severe enough that they delayed development by constraining daily growth rate, meaning it took longer to reach the minimum size at which metamorphosis can occur.

One explanation for our observations of delayed development in the ASH environment for both populations and in the REF environment for the ASH population is that the high levels of TEs in the ASH environment delayed development via perturbation of the hypothalamus-pituitary-thyroid (HPT) axis. The HPT-axis plays a critical role in amphibian development and can be disrupted by exposure to a number of common environmental contaminants (Hopkins et al. 1999; Goleman et al. 2002; Kashiwagi et al. 2009; Wang et al. 2015). Exposure to these chemicals prolongs larval development, which is tied to concomitant reductions in thyroid hormone levels. While some of these studies see thyroid hormone mediated extension of larval periods coinciding with larger sizes at metamorphosis, we saw rather they metamorphosed at similar sizes as those developing in shorter times. As these responses to a stressful environment would not be expected under accepted theory of metamorphosis (Wilbur and Collins 1973), it is

possible adaptive plasticity in response to this contaminated environment is constrained by the action of CCWs on the HPT-axis. Interestingly, adult southern toads from the ASH population have been found to have altered function of the HPT-axis (Hopkins et al. 1999). Compared to a reference population, ASH adults had higher corticosterone levels that showed no response to treatments inducing corticosterone release, which was observed in a reference population. Though these observations were made in adults, if this altered HPT-function has a genetic component, it is possible that the ASH larvae reared in the REF environment in our study experienced developmental delays through perturbations of this system.

Plastic responses to environmental change

Rearing offspring from two populations inhabiting two distinct aquatic environments in both their natal and foreign environments provided insight into the consequences of long-term environmental contamination and adaptation on life history traits. The increased tolerance of the ASH population to the CCW-associated stressors in the ASH environment may come with costs in the absence of those stressors. Developmental rate and growth through aquatic life stages was greater in the REF environment for both populations, however the populations did not respond to the lack of CCW-stressors to the same extent. In both cases, the REF population developed and grew more rapidly in the REF environment. These traits are closely tied to fitness, as quickly developing and growing larvae reduce their risk of mortality by reducing risks associated with predation (Travis et al. 1985) and desiccation as aquatic habitats dry (Travis and Trexler 1986). Thus, the reduced performance of the ASH population relative to REF in the absence of contaminants could represent potential fitness costs associated with adaptation to a human impacted environment by indirectly influencing survival. Fitness-related costs in the absence of chemical stressors appear to be a common consequence of adaptation to chemical stressors

(Groeters et al. 1994; Shirley and Sibly 1999; Xie and Klerks 2004; Agra et al. 2011). However, our study adds critical data to the few studies that examined these consequences in populations adapted under field- rather than laboratory selection regimes (Räsänen et al. 2008; Agra et al. 2011). Overall, the population x environment interactions we observed in heritable traits suggest developmental rate and early larval growth are controlled in part by interactions between genetics and the environment.

While we did not directly examine the physiological responses of these populations to the two environments, established mechanisms of tolerance to TE stressors have been well characterized across a wide range of taxa and can provide insight into the nature of the trade-offs observed for the ASH population in the REF environment. For example, elevated expression of metallothionein (Mt) genes has been associated with local adaptation to TE contaminants. Metallothionein codes for intracellular, cysteine rich proteins that have a high affinity for TEs (Kagi and Vallee 1961) and is involved in homeostasis and detoxification. Generally, increased expression of Mt can be induced by environmental exposure to TEs (i.e. a plastic response), however in some cases evolved metal tolerance has been associated with constitutive expression of Mt in adapted populations even in the absence of inducing stressors (Roelofs et al. 2006, 2009). The genetic assimilation of tolerance to environmental contaminants (i.e. constitutive tolerance) and/or transcriptional regulation of pathways associated with tolerance has been demonstrated in a number of natural populations (Roelofs et al. 2006, 2009), including amphibians (Hua et al. 2013). The induction of metal detoxification pathways is energetically costly, thus expression of these pathways in absence of the stressors normally required for their induction would necessarily allocate resources away from growth and development and may partially explain the trade-offs observed in the absence of CCW stressors.

Quantitative genetic variation in life history traits and their plasticity

Adaptation to anthropogenic stressors requires sufficient additive genetic variation on which selection can act. However, sustained directional selection is also expected to reduce additive genetic variation if the selection pressure is strong enough. Given the constructed ASH environment had been in place for over sixty years (>20 generations assuming conservative estimate of 3 years to first reproduction) and the toxic TEs present there do not degrade, we hypothesized the sustained contaminant-induced mortality in that environment would reduce genetic variation in the traits we measured.

Most traits had low to moderate heritability, which did not differ substantially among population x environment-specific estimates. While mean heritability estimates for probability of successful metamorphosis were low and had lower intervals approaching zero, we saw no evidence that additive genetic variance had been eroded in the ASH population relative to the CCW- naïve REF population for this or other traits as would be predicted if directional selection for increased tolerance to the toxic TEs in the ASH environment has eroded genetic variation. There are several explanations for this observation. First, the selective force exerted by CCWs in the aquatic environment may be modest relative to the immigration, mutation, and recombination that would maintain genetic variation. Second, adaptive tolerance to anthropogenic chemical stressors is often associated with genes of large effect (Macnair 1991) rather than the many genes of small effect assumed under quantitative genetic theory (Lande 1982), which has been documented in cases of evolution in response to TE contaminants found in CCWs in both plants and animals (Macnair 1991; Shirley and Sibly 1999). In reality, both of these processes likely contribute to the apparent maintenance of genetic variation in this population. Some studies have suggested that heavily contaminated habitats, like ASH, could act as ecological sinks (Rowe et

al. 2001). In this scenario, breeding adults are drawn to the degraded habitat from the surrounding area, but their reproductive efforts result in insignificant recruitment into the population due to excessive larval mortality associated with the contaminants. While we cannot rule this out as a possibility, based on our results, offspring from the ASH population had relatively high survival in the ASH environment relative to reference conditions, which would suggest that this environment may not be acting as an ecological sink as reported in other *in situ* studies (Rowe et al. 2001). Further studies would be necessary to determine relative rates of immigration and recruitment from surrounding populations that could resolve the conflicting results of this study from earlier studies.

Our study lacked the statistical power to detect minor changes in additive genetic variation, especially when coupled with the extensive environmental variation inherent to *in situ* field studies. However, rearing offspring from natural populations in the field also provided a unique opportunity to obtain more realistic estimates of heritability and non-genetic maternal effects, as laboratory studies tend to reduce phenotypic variation, which results in inflated estimates of heritability and maternal effects. Additive genetic variation is crucial to maintaining the evolutionary potential of populations necessary for response to future environmental change. Thus identifying reduced survival costs to an anthropogenic selection pressure without reduction in standing genetic variation is of importance for conservation managers seeking to maintain viable populations and understanding the consequences of human induced environmental change in natural populations. Future studies coupling transcriptomic approaches and parallel laboratory studies in which the effects of the chemical stressors could be isolated from those of other environmental variables would provide considerable insight into how genetic variation is maintained under anthropogenic selection.

While there was extensive additive genetic variation for a number of fitness-related traits, we found very limited evidence of non-genetic maternal effects. In fact, the only maternal effect estimates that did not overlap zero were for embryonic survival and size at GS 25, with a trend that maternal effects decreased with developmental time and became undetectable by the initiation of the field portion of the study. While this could be due in part to field conditions introducing too much environmental variation to detect maternal effects with our breeding design, this pattern is also consistent with a number of other studies that have found maternal effects to dominate early in development, but dissipate as development progresses (Montalvo and Shaw 1994; Cruz and Ibarra 1997). Further, embryonic survival was the only trait for which maternal effects surpassed genetic effects. In addition to nutrients, hormones, mRNAs, and other molecules (reviewed in (Toth 2015)), female amphibians can transfer TEs to their eggs, which can correlate with reduced reproductive success (Metts et al. 2013) and increased frequency of malformations (Hopkins et al. 2006). Though our results support the notion that non-genetic maternal effects can significantly contribute to offspring survival, it is interesting to note that the magnitude of maternal effects for both populations was comparable. This suggests the bulk of these maternal effects may be unrelated to the maternal transfer of contaminants, as suggested by previous studies. To our knowledge, only three other studies have investigated the effects of parental exposure to CCWs on the survival and performance of embryonic and larval amphibians (Hopkins et al. 2006; Metts et al. 2012, 2013) and only one of these assessed offspring performance under both reference and CCW contaminated conditions (Metts et al. 2012). Though there are experimental differences between these studies and ours, we did not see any sign of reduced embryonic survival (Hopkins et al. 2006; Metts et al. 2013) or reduced survival to or size at metamorphosis (Metts et al. 2012) in offspring from ASH dams compared to REF

dams. However, we saw similar patterns where offspring derived from the ASH population had delayed development and growth rate (i.e. mg/d) relative to the REF population in the absence of CCW-stressors (Metts et al. 2012). Our study adds critical information to the understanding of the complexity of how multigenerational exposure to environmental contaminants influences life history traits. Though our results are congruent with previous studies findings of differential performance of offspring from the ASH population relative to a CCW-naive population, they suggest that 1) non-genetic maternal effects dominate in early development and not necessarily due to maternal transfer and 2) that divergence in larval and metamorphic traits between populations is highly variable and due more to additive genetic effects than maternal effects.

Quantitative genetic variation in the plasticity of traits was generally similar to that for environment-specific trait estimates. While changes in mean trait values are indicative of past selection, plasticity provides more insight into the potential for coping with current environmental variation. Additive genetic variation for plasticity in this analysis is the variation in the slopes of reaction norms by genotype (i.e. cross environmental means by sire). Mean heritability estimates of plasticity for life history traits was low overall and similar for most traits and populations. Embryonic survival and metamorphic probability had substantially lower estimates of h^2_{pl} than other traits (i.e. < 0.04 and 0.13 respectively), which is in line with the low environment-specific h^2 estimates for these traits and suggests that additive genetic variation for traits most closely tied to fitness (i.e. survival) is low compared to other life history traits. Though uncertainty around mean estimates was rather large, h^2_{pl} for time to metamorphosis appeared somewhat reduced in the ASH population relative to REF. Reduced variation in sire-specific slopes between environments and a flatter overall reaction norm in the ASH population could indicate either selection in the ASH environment has reduced V_A. In fact, the heritability of

developmental time in the ASH population in the ASH environment, in which past selection occurred was also reduced relative to the estimate in the REF environment or either estimate in the REF population. Though speculative, the ASH environment could represent a reduced selection pressure on developmental time for the southern toads. The ponds constructed to hold the CCWs are a permanent source of water in landscape characterized largely by ephemeral wetlands. As the frequency and severity of drought in the southeastern U.S. continues to increase, these constructed freshwater systems may be even more attractive to amphibians seeking a breeding site. However, as the life histories of amphibians like the southern toad have been shaped by seasonal and annual variability in precipitation over millennia, it seems unlikely that relaxing constraint on developmental time over the course of decades would significantly reduce their capacity for developmental plasticity. Over all, we did not see any evidence that past selection in the ASH environment has canalized trait plasticity in the ASH population. Though reaction norms for some traits differed between populations (i.e. early growth, size at day 21) there was no evidence h^2_{pl} was reduced for any traits in the ASH population. Both populations appear to have equivalent capacity for future selection on plasticity to result in evolution of trait reaction norms, despite evolving under unique selection regimes.

Conclusions

By using a quantitative genetic breeding design, coupled with a reciprocal transplant, we were able to control for environmental effects to reveal intrinsic differences between these populations. Our results, coupled with previous studies (e.g. Metts et al., 2012; Rowe et al., 2001), have documented that the unique aquatic environment associated with CCW disposal sites could act as a strong selection pressure causing elevated larval mortality in *A. terrestris* larvae relative to development in natural, uncontaminated wetlands in the area. Exposure to CCWs in

the aquatic environment reduces larval *A. terrestris* survival by 20 – 100% (Rowe et al. 2001; Metts et al. 2012) relative to development in environments in the absence of CCWs. We found mean heritability for survival to metamorphosis in the ASH environment to be ~0.16 in both populations and rearing environments; suggesting the drastic reductions in survival associated with the ASH environment observed in ours and previous studies could be effectively selecting for increased CCW-tolerance. The direction of phenotypic divergence in our study suggests that the ASH population is better adapted to the local CCW-contaminated environments than the naïve REF population. Probability of metamorphosis was reduced in the ASH environment for both populations; however the ASH population experienced a reduced cost in terms of survival than REF. Further, though plasticity for embryonic survival, GS25 size, and size at metamorphosis did not have slopes significantly different from zero and did not respond consistently to the rearing environments, plasticity for GS25 size and size at metamorphosis were heritable. This suggests that the environmental influence on size at these life stage transitions is partially controlled by the interaction between genetics and the environment; however the direction of that response was not consistent within population.

While we cannot determine if this divergence is directly due to selection for tolerance to high levels of TEs in the ASH population, indirectly due to the influence of those elements on resource quality and abundance, or other unmeasured environmental factors, the ASH population clearly experienced reduced costs associated with developing in their natal rearing environment impacted by environmental contaminants. Importantly, this potentially adaptive tolerance also appears to be costly in the absence of CCW stressors, which could negatively impact the fitness of populations in nearby, uncontaminated wetlands. By incorporating an evolutionary perspective, we found evidence that an anthropogenically degraded habitat has resulted in the

phenotypic divergence of an amphibian population that may come at some cost to fitness in uncontaminated environments. While estimates of dispersal rates to new breeding ponds are uncommon and depend on environmental factors, 80-95% of adults of a related bufonid may utilize their natal ponds for breeding (Reading et al. 1991). This high site fidelity could accelerate local adaptation by isolating populations under unique selection regimes. Although we saw no reduction in survival for the ASH population in the REF environment relative to the REF population, the delayed development and growth of those individuals could translate to reductions in survival outside of the experimental conditions that excluded predators and competitors. This pattern of elevated survival in the presence of TEs by the ASH population corresponding with delayed development and growth rate in the REF environment relative to the CCW-naïve REF population could be explained by the energetically costly constitutive expression of TE detoxification pathways observed in other systems (Roelofs et al. 2006, 2009). Future studies characterizing patterns of gene expression and trace element accumulation would provide further insight into the physiological mechanisms underlying the elevated tolerance of the ASH-derived population to the contaminated environment and phenotypic trade-offs in the absence of TE stressors.

Our study provides further evidence that natural populations can adapt to human impacted environments characterized by multiple chemical stressors and that such adaptation may be costly. Given the severity and extent of anthropogenically impacted environments can only be expected to increase in the near future, assessing how populations cope with such changes is crucial for understanding the conservation implications of past and continued environmental changes. As a group, amphibians are a globally imperiled taxon, but some species appear able to successfully cope and even thrive in the face of major environmental

perturbations. By incorporating an evolutionary perspective, we were able to determine that a series of ponds constructed to manage CCW appears to have resulted in a resident population of amphibians evolving tolerance to the chemical stressors present there that may have come with costs to fitness in the absence of those stressors. While our study was not designed to isolate the effect of metal tolerance from other indirect effects associated with a contaminated environment, future laboratory studies isolating the response of organisms to metals from other environmental factors could elucidate this question and provide insight into the specific mechanisms underlying adaptive responses to TE contaminants, common in human impacted environments.

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TABLES

Table 2.1. Proportion of phenotypic variance explained by causal variance components for embryonic and larval traits. Estimates obtained from population and environment specific posterior-mean estimates (95% HPDI). Initial number of embryos present in each experimental unit was included as a covariate in models of embryonic survival. Growth used mass at metamorphosis as the response variable with time to metamorphosis as a covariate. Early growth used the difference in total length of larvae between days 7 and 21of the field study. Estimates for larval size at day 21 were omitted for space and as they did not differ qualitatively from day 7 estimates. $h^2 = V_A/V_P$, mat = V_M/V_P, dom = V_D/V_P. ttm = developmental time in day to metamorphosis, svl = snout vent length of metamorphic toads.

		embryo survival			size GS25		
Pop.	Env.	h^2	mat	dom	h^2	mat	dom
REF	REF	0.11 (0.00-0.49)	0.38 (0.06-0.76)	0.06 (0.00-0.32)	0.56 (0.23-1.03)	0.23 (0.00-0.61)	0.49 (0.21-0.85)
	ASH	0.06 (0.00-0.81)	0.45 (0.15-0.81)	0.05 (0.00-0.25)	0.48 (0.18-0.93)	0.32 (0.01-0.70)	0.38 (0.15-0.72)
ASH	REF	0.05 (0.00-0.25)	0.51 (0.21-0.82)	0.05 (0.00-0.25)	0.44 (0.16-0.86)	0.35 (0.05-0.71)	0.33 (0.13-0.59)
	ASH	0.08 (0.00-0.39)	0.42 (0.14-0.76)	0.04 (0.00-0.20)	0.42 (0.17-0.76)	0.28 (0.01-0.63)	0.36 (0.15-0.62)

		size (7 d)			size (21 d)		
Pop.	Env.	h^2	mat	dom	h^2	mat	dom
REF	REF	0.33 (0.06-0.82)	0.23 (0.00-0.78)	0.23 (0.05-0.55)	0.27 (0.06-0.71)	0.156 (0.00-0.69)	0.32 (0.07-0.75)
	ASH	0.39 (0.09-0.93)	0.18 (0.00-0.73)	0.36 (0.08-0.80)	0.4340 (0.10-1.05)	0.15 (0.00-0.72)	0.56 (0.13-1.20)
ASH	REF	0.29 (0.11-0.56)	0.12 (0.00-0.40)	0.25 (0.10-0.47)	0.30 (0.10-0.64)	0.03 (0.00-0.22)	0.30 (0.10-0.60)
	ASH	0.29 (0.11-0.58)	0.08 (0.00-0.33)	0.27 (0.10-0.49)	0.27 (0.10-0.55)	0.08 (0.00-0.32)	0.27 (0.10-0.50)

		early growth			ttm		
Pop.	Env.	h^2	mat	dom	h^2	mat	dom
REF	REF	0.13 (0.02-0.43)	0.06 (0.00-0.40)	0.27 (0.04-0.78)	0.29 (0.07-0.74)	0.12 (0.00-0.64)	0.28 (0.07-0.65)
	ASH	0.23 (0.04-0.72)	0.06 (0.00-0.47)	0.48 (0.06-1.26)	0.27 (0.03-0.89)	0.06 (0.00-0.51)	0.24 (0.02-0.82)
ASH	REF	0.10 (0.01-0.31)	0.08 (0.00-0.29)	0.17 (0.00-0.50)	0.31 (0.10-0.67)	0.03 (0.00-0.23)	0.30 (0.10-0.63)
	ASH	0.13 (0.04-0.32)	0.04 (0.00-0.20)	0.16 (0.04-0.37)	0.11 (0.02-0.32)	0.02 (0.00-0.14)	0.17 (0.03-0.48)

		svl			growth rate		
Pop.	Env.	h^2	mat	dom	h^2	mat	dom
REF	REF	0.44 (0.08-1.04)	0.24 (0.00-0.80)	0.34 (0.07-0.76)	0.48 (0.07-1.16)	0.35 (0.00-0.88)	0.32 (0.05-0.74)
	ASH	0.44 (0.08-1.05)	0.24 (0.00-0.81)	0.37 (0.07-0.83)	0.49 (0.08-1.21)	0.29 (0.00-0.85)	0.38 (0.07-0.88)
ASH	REF	0.35 (0.12-0.68)	0.10 (0.00-0.39)	0.34 (0.13-0.63)	0.41 (0.13-0.83)	0.21 (0.00-0.58)	0.30 (0.10-0.59)
	ASH	0.40 (0.15-0.77)	0.09 (0.00-0.37)	0.51 (0.20-0.91)	0.40 (0.13-0.79)	0.17 (0.00-0.50)	0.34 (0.11-0.64)

		meta probability		
Pop.	Env.	h^2	mat	dom
REF	REF	0.20 (0.00-0.97)	0.04 (0.00-0.59)	0.20 (0.00-0.86)
	ASH	0.17 (0.00-0.68)	0.04 (0.00-0.49)	0.23 (0.00-0.89)
ASH	REF	0.14 (0.00-0.38)	0.02 (0.00-0.20)	0.30 (0.00-0.90)
	ASH	0.17 (0.00-0.67)	0.03 (0.00-0.27)	0.14 (0.00-0.57)

Table 2.2. Reaction norm slopes (β) and accompanying narrow-sense heritability (h^2_{pl}) MCMC-estimates obtained from population-specific plasticity models (see *Methods: statistical and quantitative genetic analyses*). Errors are given as 95% HDPIs, bolded β-estimates are those for which slope of reaction norms was significantly different from zero. β-estimates were obtained from population-specific 'environment' terms and given as trait values in the ASH environment relative to REF. ttm = developmental time in day to metamorphosis, svl = snout vent length of metamorphic toads.

trait	population	*N*	β-estimate	h^2_{pl}
embryo survival	REF	303	0.001 (-0.331-0.340)	0.03 (0.00-0.14)
	ASH	381	-0.105 (-0.386-0.198)	0.02 (0.00-0.10)
GS25-size	REF	894	0.005 (-0.015-0.028)	0.25 (0.10-0.45)
	ASH	1280	-0.003 (-0.023-0.019)	0.23 (0.10-0.40)
day7-size	REF	313	**0.066 (0.007-0.131)**	0.21 (0.05-0.47)
	ASH	681	**0.041 (0.005-0.075)**	0.21 (0.09-0.38)
day21-size	REF	273	0.048 (-0.017-0.113)	0.20 (0.05-0.45)
	ASH	557	**0.062 (0.015-0.107)**	0.19 (0.09-0.34)
early growth	REF	273	0.048 (-0.018-0.122)	0.20 (0.05-0.46)
	ASH	557	**0.063 (0.020-0.110)**	0.19 (0.09-0.35)
ttm	REF	234	**0.153 (0.010-0.300)**	0.19 (0.04-0.51)
	ASH	515	**0.111 (0.003-0.209)**	0.10 (0.03-0.21)
svl	REF	228	0.000 (-0.053-0.055)	0.23 (0.04-0.52)
	ASH	497	-0.001 (-0.031-0.031)	0.23 (0.11-0.41)
growth rate	REF	384	-0.062 (-0.205-0.05)	0.14 (0.02-0.42)
	ASH	768	-0.041 (-0166-0.065)	0.09 (0.02-0.26)
meta probability	REF	384	-0.559 (-1.193-0.132)	0.12 (0.00-0.50)
	ASH	768	-0.299 (-0.809-0.198)	0.07 (0.00-0.30)

FIGURES

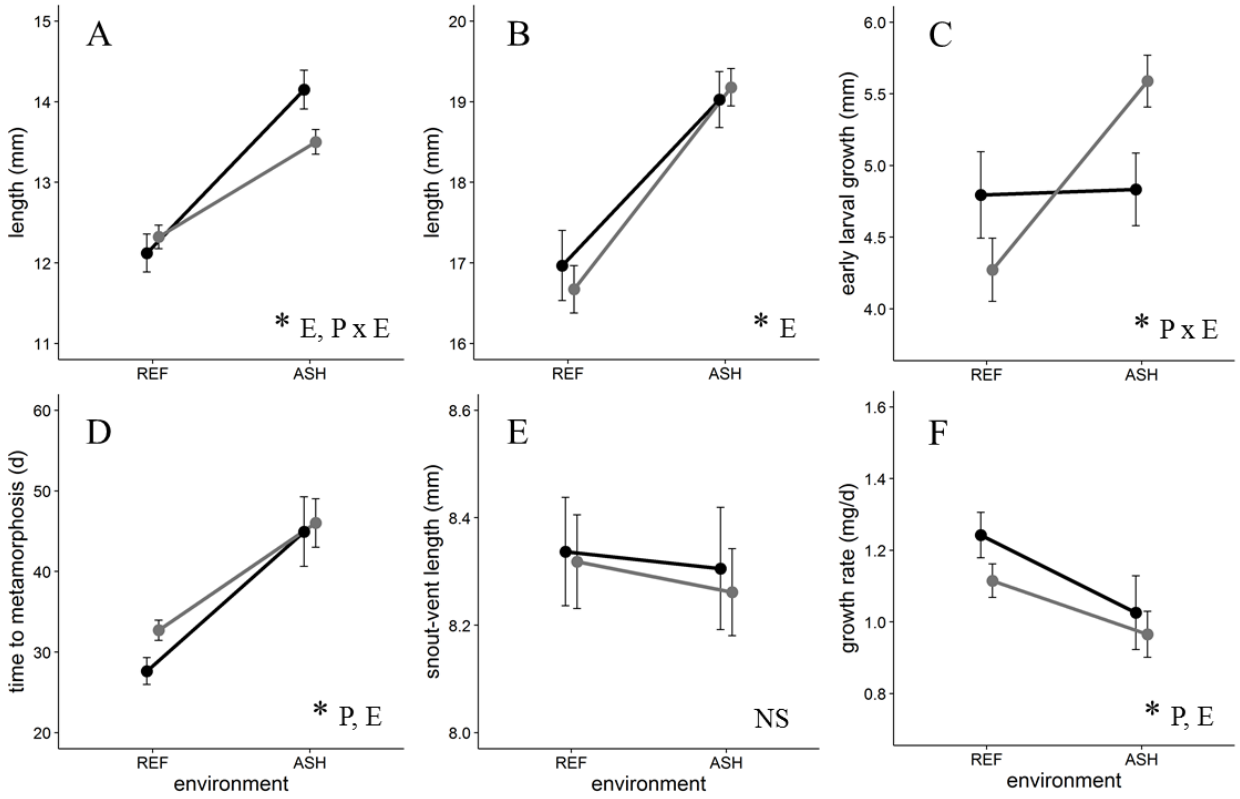

Figure 2.1. Plots of larval (A-C) and metamorphic (D-F) trait means (95% HPDIs) for REF (black) and ASH (gray) populations in each environment. Panels A and B are total larval length at 7 and 21 days of development respectively. Panel C shows growth over 14 days of early larval development (i.e. day 21 – day 7). Panels D-F show time required to reach metamorphosis (ttm), size at metamorphosis (svl), and growth rate over entire larval period (i.e. mass at metamorphosis/time to metamorphosis) respectively. The plot of mass at metamorphosis was similar to length at metamorphosis (E) and omitted for space. Asterisks and accompanying letters in bottom right of panels denote significant population (P), environment (E), and population x environment effects.

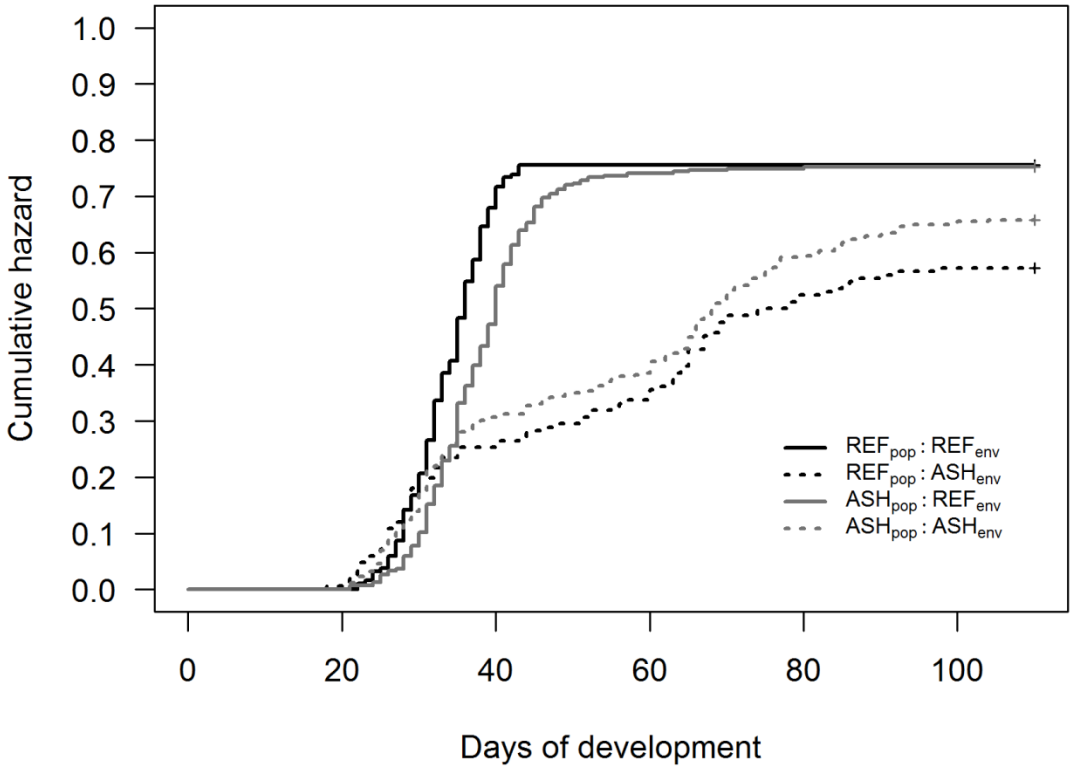

Figure 2.2. Cumulative hazard function for probability of successful metamorphosis over the course of the study. In the REF environment (solid lines) both populations had a similar proportions metamorphosing. The ASH environment reduced the probability of metamorphosis by 50%; however the extent of this reduction was dependent on the population of origin.

CHAPTER 3

ELEMENTAL ANALYSIS OF METAMORPHIC AMPHIBIANS PROVIDES INSIGHT INTO MECHANISMS OF CONTAMINANT TOLERANCE[2]

[2] R. Wesley Flynn, Allison M. Welch, John C. Seaman, & Stacey L. Lance. To be submitted to *Functional Ecology*.

ABSTRACT

Human activities have radically altered ecosystems globally. Many of these alterations result in degraded habitats that present stressful conditions to native wildlife. Amphibians are particularly susceptible to environmental changes, especially those affecting the quality of the water resources they rely on for reproduction and larval development. Chemical contamination of aquatic habitats is a particularly pervasive hazard that can negatively affect survival, and fitness-related traits amphibians. Under certain conditions some species may be able to rapidly adapt to cope with contaminants, which could mitigate the risks they pose to population persistence. However, little is known concerning the physiological mechanisms by which amphibians may achieve tolerance to chemical contaminants. To examine this, we characterized the accumulation of toxic trace elements and maintenance of essential elements in offspring from two populations of southern toad (*Anaxyrus terrestris*) differing in their tolerance to coal combustion wastes (CCWs). The more tolerant population maintained normal levels of essential elements when reared in the presence of CCWs, while the susceptible population experienced marked reductions in elements critical to normal physiological function. Further the tolerant population accumulated higher levels of some trace elements suggesting tolerance to CCW contaminants is not due to an enhanced ability to eliminate or reduce assimilation of toxic elements. These results suggest that tolerance to this complex mixture of toxic elements is primarily due to improved ability to maintain ionic- and osmotic regulation, though improved internal sequestration of toxic elements may also play a role. Further, characterizing relationships between the elemental composition of metamorphic toads reared in the contaminated environment and life history traits hint that the rate of development may serve to modulate exposure times, and thus toxicity to some extent. Given these observations were made on post-

metamorphic individuals, the effects associated with developing in a contaminated environment may persist into terrestrial life stages. Quantifying concentrations of essential major elements in individual carcasses, in addition to heavy metals and metalloids, provided a more comprehensive understanding of mechanisms of toxicity and tolerance in natural amphibian populations.

INTRODUCTION

The impacts of human activity on the environment are globally pervasive and often create conditions stressful to wildlife. The effects of these activities are of particular concern for freshwater ecosystems, especially the ephemeral wetlands that provide a host of ecosystem services (Constanza et al., 1997) and critical habitat for number of at risk taxa, such as amphibians. Since European colonization, the U.S. has lost over 50% of its wetland acreage (T. Dahl, 1990). While the rate of loss has declined in recent years (T. Dahl, 2009), human activities still impact the quality of the remaining. The main driver of amphibian population declines continues to be habitat loss (Stuart et al., 2004), but in areas retaining suitable habitat have more subtle environmental changes associated with human activity are contributing to declines (Collins & Storfer, 2003). Environmental contamination of wetland habitats is one factor associated with human activities that can negatively impact otherwise suitable habitat. In order to better predict how this global phenomenon may be contributing to current and future population declines, it is essential to understand how organisms cope with long-term exposures to environmental contaminants.

Trace elements (TEs) are a broad class of elements, including heavy metals and metalloids, that are generally found in biological systems at very low concentrations, relative to major elements (e.g. sodium, calcium, and phosphorous), and do not degrade in the environment.

Elevated levels of TEs in aquatic environments are exceedingly common and often tied to mining, agriculture, urbanization, and energy production activities (Nriagu & Pacyna, 1988). Amphibians, and other aquatic organisms, are susceptible to even slight elevations in a number of TEs (Flynn, Scott, Kuhne, Soteropoulos, & Lance, 2015; Gross, Chen, & Karasov, 2007; Lance, Flynn, Erickson, & Scott, 2013; Meador, 1991; Taylor, McGeer, Wood, & McDonald, 2000). Toxicity of TEs to aquatic organisms is determined by the ability of the organism to 1) maintain internal levels of essential elements disrupted by TE exposure and 2) regulate the bioavailability of internal levels of the TEs themselves.

A number of TEs elicit toxicity in part by perturbing osmoregulation (Cd (McGeer, Szebedinszky, McDonald, & Wood., 2000; Saglam, Atli, & Canli, 2013), Hg (Daoust, Wobeser, & Newstead, 1984; Jagoe, Shaw-Allen, & Brundage, 1996), Cu (Brooks & Mills, 2003; Grosell, Nielsen, & Bianchini, 2002; McGeer et al., 2000; Saglam et al., 2013), Zn (McGeer et al., 2000; Spicer, Morritt, & Maltby, 1998), Pb (Amado, Freire, & Souza, 2006)) by increasing the diffusivity of gill tissue or inhibiting the active uptake of sodium (Na) and other essential electrolytes across the gills (Grosell et al., 2002). Both of these mechanisms result in extracellular depletion of Na that leads to a physiological cascade culminating in death in the most severe cases (Grosell et al., 2002). Trace elements also induce toxicity via the negative effects associated with excessive concentrations within the body. High levels of TEs internally can result in the formation of reactive oxygen species leading to oxidative stress, interference with metabolic processes by binding to critical biological ligands or replacing essential elements due to structural similarities (Jaishankar, Tseten, Anbalagan, Mathew, & Beeregowda, 2014). Though TEs can be toxic at high levels, many are also essential for life within a narrow physiological range (Fraga, 2005; Goldhaber, 2003). As such, organisms have evolved complex

biological pathways to ensure internal levels of bioavailable TEs are kept in ranges that limit deleterious effects arising when levels of these elements are either too high or low (Bleackley & MacGillivray, 2011; Prévéral et al., 2009).

While organisms have mechanisms to cope with variation in levels of TEs in the environment, human activities can have created conditions outside which organisms have experienced in their evolutionary history (Sih, Ferrari, & Harris, 2011). Thus evolved mechanisms designed to maintain homeostasis can prove insufficient. These pathways under genetic control have the potential to evolve to accommodate new environments (Amiard, Amiard-Triquet, Barka, Pellerin, & Rainbow, 2006; Craig, Hogstrand, Wood, & McClelland, 2009), provided there is sufficient genetic variation on which selection can act. In fact, multigenerational exposure to environments with high levels of TEs has been shown to result in tolerance to TEs in both fish (Kolok & L'Etoile-Lopes, 2005; Xie & Klerks, 2004) and invertebrates (Agra, Soares, & Barata, 2011; Klerks & Levinton, 1989; Martinez & Levinton, 1996; van Straalen & Donker, 1994). Studies characterizing mechanisms of tolerance to TEs in aquatic organisms have primarily focused on the relationships tolerant organisms' ability to maintain internal concentrations of crucial electrolytes (e.g. Na and potassium) (Kolok & L'Etoile-Lopes, 2005; McGeer et al., 2000; Taylor et al., 2000; Taylor, Wood, & McDonald, 2003). However, there are also a number of diverse physiological mechanisms regulating assimilation, excretion, and sequestration of TEs that all serve to effectively reduce the bioavailability of internal TEs, thus reducing toxicity (Klerks & Weis, 1987; Leo Posthuma & Van Straalen, 1993). Interestingly, in most cases these observations have been limited to exposure to a single TE (though see (Klerks & Bartholomew, 1991; Klerks & Levinton, 1989; Martinez & Levinton, 1996) for exceptions in invertebrate systems) and some research has

indicated that the presence of multiple chemical stressors can hinder effective adaptation (Rolshausen et al., 2015). Surprisingly, we know little about the potential for amphibians, a group sensitive to changes in aquatic environments, to adapt to environments contaminated with one or more TEs.

We report the results of an *in situ* reciprocal transplant study in which we quantified the elemental composition of metamorphic southern toads from two populations, reared in both reference and TE contaminated environments through aquatic development. We had previously determined that these two populations were phenotypically divergent, in that offspring population derived from the contaminated environment had greater early larval growth and survival in the contaminated environment, but delayed development and growth in the reference environment relative to the reference population (Chapter 2). Our objectives were to 1) quantify how population of origin and rearing environment affect elemental composition to test adaptive hypotheses and 2) characterize relationships between aquatic life history traits and elemental composition of metamorphic toads.

METHODS

Study populations and sites

The southern toad (*Anaxyrus terrestris*) is an amphibian native to the southeastern U.S. and occurs across a variety of habitats. This pond breeding species can be extremely abundant in both natural and highly disturbed environments and will utilize a variety of aquatic environments for breeding (Bartlett & Bartlett, 1999). In juvenile and adult life-stages, this anuran is exceptionally terrestrial in its habit, relying on ponds and wetlands primarily for reproduction (Lannoo, 2005).

We collected adults in breeding condition from a natural wetland that periodically dries and has no known history of contamination (REF) and a site with multiple permanent ponds constructed to manage coal combustion waste (CCW) generated by a coal fired power plant (ASH). We used artificial fertilization methods to generate 64 full /half-sib families of offspring from each population. Briefly, we set up four breeding blocks in each population of eight male and two female adult toads. Unfertilized eggs were obtained from females, which were then divided into roughly eight equal parts. Sperm suspensions from each male within a breeding block were used to fertilize each of the divided sections of eggs, resulting in 16 unique families of offspring from each breeding block.

The two study sites are located ~3 km apart on the Department of Energy's Savannah River Site, near Aiken, SC. The REF site is a large (28 acre) Carolina bay that is characterized as a long hydroperiod wetland that experiences periodic drying, high levels of dissolved organic carbon (DOC), native vegetation, and no point-source anthropogenic inputs of contaminants (Roe, Hopkins, & Jackson, 2005; Sharitz & Gibbons, 1982). The ASH-site was constructed and put into use in the 1950's. It consists of a series of ponds that receive a slurry of coal fly ash (i.e. CCW) and water from the adjacent coal-fired power plant. The water chemistry of this site differs from that of natural wetlands, having lower DOC and higher pH, conductivity, and levels of TEs (Appendix Tables A1, A2). Trace elements (primarily heavy metals and metalloids) in the water in the ponds at the ASH site are elevated due to the abundance CCW sediments, which also results in high levels in the biofilms ingested by developing anurans (Roe et al., 2005; Rowe, Hopkins, & Coffman, 2001). *A. terrestris* use both of these sites for reproduction. In a separate paper, we demonstrate these two populations differ in their tolerance to the CCWs in the ASH environment, such that offspring from the ASH population have greater survival and

growth in the presence of CCWs relative to the REF population (Chapter 2). Further, the CCW-tolerant ASH population showed signs of trade-offs when reared in the REF environment, where they do not develop or grow as quickly as those from the CCW-naïve REF population.

Experimental Design

We used the offspring from full-sib/half-sib families in a reciprocal transplant design, such that each family from each population was reared in both their native (i.e. REF larvae in REF environment) and foreign environment (i.e. REF larvae in ASH environment). Post-fertilization, embryos were maintained in the laboratory under both environmental conditions by bringing in water collected from the respective sites to fill experimental units. We assessed embryonic survival using a full factorial design that incorporated 2 populations x 2 water treatments (i.e. water sourced from each environment) x 64 full/half-sib families x 3 replicates for a total of 768 experimental units. The number of embryos in each experimental unit varied considerably (5 – 45) due to variation in fertilization success. Experimental units were 0.5 L square plastic containers filled with 400 mL of water from either REF or ASH sites. We performed daily checks, counting and removing any dead embryos until five days post-fertilization, at which time all normally developing embryos had reached the free-swimming and feeding stage, Gosner stage 25 (GS25). Upon reaching GS25, we pooled all survivors within family x water treatment combinations and haphazardly selected six larvae to continue to the larval field study.

During the field study, larvae from both populations were individually reared in both environments in floating containers that allowed for the regular exchange of water and sediments with the surrounding environment. Observations were made for death, growth over 14 days of early larval development, completion of metamorphosis, and size at metamorphosis. Individuals

were brought into the lab upon the emergence of front limbs (i.e. GS42), where they were allowed to complete metamorphosis in individual containers containing a paper towel dampened with water from their field containers. Two days after completing metamorphosis (i.e. complete metamorphosis defined as <1.0 mm of tail remaining [GS 46]), we took measurements of mass and snout-vent length before euthanizing in 3% MS-222. We dissected out the liver, a 1 cm section of gut, and the back right limb of every individual before transferring the rest of the carcass to Whirl-Pak® to be used for elemental analysis. We analyzed a subset of these individuals for their elemental compositions (Table 3.1).

Sample preparation

Lyophilized samples and standards (TORT-3) were massed to the nearest 0.01 mg (Mettler Toledo XPE26 DeltaRange®) and transferred to individual 15 mL trace metal free tubes (VWR #89049). Samples were digested following a modified acid digest protocol for small masses. Briefly, we added 200 µL of trace metal free nitric acid (JT Baker #9598) to each tube before placing in a heat block at 80^{o}C for two hours with caps fit loosely on. Each sample was visually inspected to ensure full digestion of tissue. Samples were then diluted to 3.3% HNO_3 with 5.8 mL of ultra-pure water. As we were working with small sample masses and wanted to keep detection limits (DLs) for TEs as low as possible, we aliquoted 0.65 mL of sample digestates into fresh, metal-free tubes for further dilution and analysis of major elements (Ca, Fe, K, Mg, Na, and P), while the remaining 5.35 mL was used for analysis of TEs (As, Cd, Cu, Ni, Se, Sr, and Zn). For the aliquots to be diluted for major element analysis, we added 2.35 mL of 3.3% HNO_3 to achieve a final dilution of 1:100 while retaining the same acid concentration as in the TE analysis (i.e. 3.3% HNO_3).

Elemental Analysis

We analyzed the composition of individual, metamorphic toad carcasses, prepared as above, for a total of 13 elements (As, Ca, Cd, Cu, Fe, K, Mg, Na, Ni, P, Se, Sr, and Zn) using inductively coupled plasma-mass spectroscopy (Nexion 300X ICP-MS; Perkin Elmer, Norwalk, CT, USA) according to the QA/QC protocols outlined in USEPA Method 6020B (U.S. Environmental Protection Agency, 2014). We used external calibration standards (High-Purity Standards, Charleston, SC, USA encompassing a range of 0.5 -500 ug/L for all elements analyzed. Certified reference material (TORT-3; National Research Council, Ottowa, ON, Canada) and blanks were included after every 15 study samples for quality control and in calculation of element detection limits (N = 22). Trace element recoveries were generally within 20% of certified values for reported elements (exceptions of Fe and Ni which were within 22%). We did not correct data for percent recovery. Instrument minimum detection limits (MDLs) by element can be found in Table 3.2.

Statistical analyses

All statistical analyses were carried out in program R (Team, 2013). As the number of elements we examined was large (n = 13) and preliminary analysis determined strong correlations between a number of elements, we performed a principal component analysis (PCA, *prcomp*) to reduce the dimensionality of the dataset and obtain a set of linearly uncorrelated variables. For elements in which <50% of samples in a given environment were less than the MDL, values for values below MDL were replaced with ½ of the MDL for that element (Table 3.2). The PCA was performed using all elements that had >50% of values above the MDL for all population x environment combination, which excluded only Cd and Se (see Table 3.2). The PCA was done with centering and scaling of untransformed element values. We kept only the

first four PCs as subsequent eigenvalues were ≤1. We used a varimax rotation on the four retained PCA axes to more evenly distribute variation across the set of components and facilitate interpretation.

We used the saved factor scores as independent variables in linear mixed effect models (*lme4*, Bates et al. 2015) examining the fixed effects of population, environment, and their interaction on the elemental composition of metamorphic toads (i.e. PCs), while accounting for environment-specific spatial block and parentage in the random effects structure. As two physiologically important elements (i.e. Cd and Se) had too few samples with reliable values in the REF environment, we also examined population-level differences in the elemental composition of metamorphic toads in the ASH environment separately to specifically test how accumulation of these (log-transformed) elements differed between populations, using population as a fixed effect and the same random effects structure as the full models. Statistical significance of fixed effects was determined using Type III sums of squares with the *Anova* function (*car* package, Fox and Weisberg 2011), which is calculated using a Wald χ^2 test.

To examine relationships between individuals' life history trait values and their elemental composition in the contaminated ASH environment, we fit univariate linear mixed effect models with the life history trait of interest as response variables (i.e. early larval growth [mm], time to metamorphosis [d], size [mg] and body condition at metamorphosis [BCI], and daily averaged growth rate [mass at metamorphosis/time to metamorphosis]), with population, PCs 1-4, log(Cd), and log(Se) as fixed effects, and the same random effects structure specified in the above models. Body condition index used residuals derived from regressions of mass at metamorphosis on snout-vent length across the entire dataset (Schulte-Hostedde, Zinner, Millar, & Hickling, 2005). Early larval growth was determined by subtracting the total length (i.e. tip of rostrum to

tip of tail) of larvae on day 7 of the field study from total length at day 21 using scaled photographs taken in the field and measured using ImageJ software (Schneider, Rasband, & Eliceiri, 2012). Early larval growth, time to metamorphosis, and size at metamorphosis were log-transformed prior to analyses to normalize trait distributions. Initially, we included interactions between population and PC and element variables, as these populations responded differently to rearing in the ASH environment. However, because there was only one weak interaction present across all life history trait and element combinations (i.e. for time to metamorphosis between PC3 and population, $p = 0.01$), our final models included only main effects.

RESULTS

Effects of population and rearing environment on elemental composition

The first four PCs explained 69.3% of the variation in elemental body burdens we observed (Table 3.3). Principal components partitioned into groups of physiologically related elements (Table 3.3), such that PC1 largely describes TE concentrations, PC2 Na and K, PC3 Ca, Mg, and P, and PC4 Fe.

Overall, mean levels of TEs (PC1) and essential major elements (PC2-4) were similar between populations when reared in the REF environment (Figure 3.1); however, when reared in the ASH environment, offspring from the ASH population had higher levels of elements associated with PCs 2-4 and Se. Principle component analysis effectively identified and grouped elements that would be expected to vary considerably between the two habitats (i.e. PC1 TE variation primarily due to rearing environment) and major elements that are directly related to normal physiological function (i.e. PCs 2, 3, and 4).

In the full models incorporating both population of origin, rearing environment, and their interaction, the main effect of population was only significant for PC1 (i.e. TE concentrations), such that the ASH population had overall higher levels of TE (i.e. more negative PC1 values). However, rearing environment was by far the strongest influence on PC1 values, with individuals reared in the ASH environment having much lower PC1 scores and therefore higher levels of TEs (Table 3.4, Fig 3.1A). There was no significant interaction between population x environment, indicating that both populations were similarly influenced by rearing environment in terms of TE accumulation. While the TE Cd and Se were not included in the PCA because <50% of the individuals from the REF environment were BDL, levels in individuals reared in the ASH environment were virtually all above the detection limit making qualitative inference clear: Individuals reared in the ASH environment accumulated significantly more Cd and Se (Table 3.5, see Appendix Figure B1). When we fit models for the ASH environment data only, population did not have a significant effect on PC1 scores or Cd-levels, but toads from the ASH population had significantly higher burdens of Se (Table 3.5).

We saw a significant effect of rearing environment and population x environment interaction on PC2, which largely describes levels of major electrolytes (i.e. K and Na, Fig 3.1B, Table 4). Being reared in the ASH environment had a strong negative effect on the REF population's PC2 scores, but not the ASH population (Figure 3.1B). Looking at the ASH rearing environment separately, population of origin still had a significant effect on PC2 scores, where individuals from the ASH population maintained higher levels of electrolytes than the REF population.

Examination of PCs describing levels of other essential major elements found rearing environment (PC4: Fe) and a population x environment interaction (PC3: Ca, Mg, P)

significantly influenced levels in metamorphic toads (Fig 3.1C,D, see Appendix Figure B2 for response by element). Scores for each of these PCs were similar between populations in the REF environment, but in the ASH environment these scores were significantly elevated in the ASH population (Figure 3.1C, D).

Relationships between elemental composition and life history traits

The elemental composition of metamorphic toads explained a significant amount of variation in some larval and metamorphic life history traits. More rapid development and greater daily averaged growth over larval development (i.e. mg/d) were both associated with significantly lower levels of PC1 TEs (Figure 3.2B, D), PC3 elements, Cd, and Se, but elevated levels of PC2 (Fig. 3.3B, D) and PC4 elements (Table 3.6). Larger sizes at metamorphosis (i.e. mass) and more rapid early larval growth were related to lower PC1 TE levels at metamorphosis (i.e. higher PC1 scores, Fig. 3.2A ,C, Table 3.6), but we did not see any significant relationships with other PC factors. Rapid early larval growth was also tied to higher levels of Cd (Table 3.6). We saw no significant relationships between body condition at metamorphosis and any PC groups or elements; however, there was a trend towards individuals with better body conditions having reduced PC2 scores. The population term was not significant in models for any life history traits.

DISCUSSION

Our study provided a unique opportunity to examine potential mechanisms of adaptation to environmental contaminants in wild populations of a native amphibian. Analysis of the elemental composition of metamorphic southern toads reared *in situ* through the aquatic life stage revealed patterns supporting a hypothesis of adaptive divergence of these populations in

response to TE stressors. The ASH population, sourced from a habitat contaminated by high levels of TE for over 60 years, had previously been documented to exhibit greater survival and early larval growth than the REF population when reared in the ASH environment (Chapter 2). Because we had measures of life history traits and measures of the elemental breakdown of their bodies at the individual levels, we were able to examine unique relationships between the accumulation of potentially toxic TEs and retention of elements critical for physiological function.

Physiological divergence between populations

Comparing the elemental composition of southern toads from two populations, differing in their history of exposure to high levels of TEs, in both contaminated and uncontaminated environments we were able to infer some possible physiological mechanisms underlying contaminant tolerance. Most notably, individuals from the REF population experienced a reduction in levels of the electrolytes Na and K (i.e. PC2) when reared in the ASH environment, while those from the ASH population were able to maintain normal levels (i.e. not significantly lower than those in the absence of TE stressors). This pattern whereby populations susceptible to TE contaminants suffer major losses in whole body Na and/or K when exposed to high levels of TEs in the aquatic environment relative to more tolerant populations or species, has been observed in the laboratory in fish (G De Boeck et al., 2001; Kolok & L'Etoile-Lopes, 2005; McGeer et al., 2000; Taylor et al., 2000, 2003) and invertebrates (Spicer et al., 1998). This suggests that adaptive tolerance to aquatic TEs in amphibians occurs by similar mechanisms as found in other taxa. Interestingly, there is evidence that TE-induced gill damage, followed by cellular repair, may even be essential for acclimation to TEs (i.e. Cu, Tate-Boldt and Kolok 2008). Given our dataset was limited to only surviving individuals, under this hypothesis we

would have expected PC2 (i.e. Na and K) levels to return to normal following repair, which we did not observe in survivors from the REF population reared in the ASH environment. Another proposed mechanism regulating tolerance to aquatic TEs is Na turnover rate, which tends to increase with body size (Grosell et al., 2002). All freshwater organisms with gills must take up Na from the water in exchange for protons to maintain osmotic balance (Grosell, Nielsen, & Bianchini, 2002). Due to smaller organisms having larger surface area to body mass ratios, they experience greater diffusive loss of Na and hence must achieve higher rates of Na influx to maintain internal Na levels. Therefore, smaller organisms with higher Na turnover rates tend to be more sensitive to TEs that impede the uptake or increase the loss of Na. While this hypothesis can explain some among taxa variation in susceptibility to CCWs (i.e. amphibian species that are larger in larval stages appear to be more tolerant to CCWs [*Rana catesbeiana* (Rowe, Kinney, Nagle, & Congdon, 1998) > *Rana clamitans* (Snodgrass et al., 2004) > *Bufo terrestris* (Metts, Buhlmann, Scott, Tuberville, & Hopkins, 2012; Rowe et al., 2001)]), it does not appear to explain the variation in our data, considering that larvae from the REF population were overall larger than those from the ASH population at the start of the study and larvae and metamorphic toads from both populations in the ASH environment were of similar size (Chapter 2). In light of these hypotheses, our results suggest either levels of gill damaging TEs in the ASH environment were not high enough to induce the damage necessary for acclimation of REF individuals (Tate-Boldt & Kolok, 2008), the complex mixture of toxic elements present in that environment may have prevented their repair, or that there are other mechanisms co-determining tolerance to these contaminant mixtures.

The major elements Ca, Mg, and P on PC3 showed a unique pattern from all other elements examined. The REF population maintained similar PC3 levels across environments;

however the ASH population experienced a large increase in these elements when reared in the ASH environment. All three elements are critical for nerve transmission and energy metabolism, but the bulk of these elements in vertebrates are found in the skeleton (Reinhardt, Horst, & Goff, 1988). Calcium and P are generally found together (e.g. calcium phosphate in bone) and thus we would expect environmental perturbations affecting levels of one to affect the other. One of the most obvious reasons we might see higher concentrations of these elements is that individuals from the ASH population reared in the ASH environment were larger and thus had more developed skeletons than the REF population or either population in the REF environment. We saw no evidence of this. Considering levels were similar among the other three population x environment combinations, we would assume those to represent 'normal' levels of PC3 elements. In other animals, Ca homeostasis is maintained in part by the release of parathyroid hormone (Moe, 2013). Intriguingly, adult *A. terrestris* from the ASH population have been found to have altered regulation and response of the hypothalamo-pituitary –interrenal (HPI) axis, which is inherently tied to regulation of the parathyroid (Zoeller, Tan, & Tyl, 2007). Disruption of parathyroid signaling induced by stress can lead to depletion of PC3 elements in larval amphibians (Kirschman, Haslett, Fritz, Whiles, & Warne, 2016). Therefore, altered function of critical hormone pathways induced by exposure to the TE contaminants in the ASH system could have altered homeostasis of Ca, Mg, and P in larval and metamorphic individuals from the ASH population in our study.

Comparison of PC4 (i.e. Fe) values between populations and environment show a similar trend as PC2, with only the REF population experiencing a notable reduction when reared in the ASH environment. Iron is an essential element, critical for a number of physiological processes (Andrews, 1999; Muñoz, Villar, & García-Erce, 2009; Siah, Ombiga, Adams, Trinder, &

Olynyk, 2006). Reductions in Fe levels can have negative consequences for organisms, including anemia and tissue inflammation (Halliwell & MC, 1984). Though there are numerous factors that can result in Fe deficiency (Andrews, 1999), inadequate absorption due to competition for binding sites with other metals is especially relevant in this case. Thus the high levels of heavy metals in the ASH environment may have effectively competed with Fe in the REF population. Because the ASH population was able to maintain normal levels of Fe, it is possible the physiological mechanisms allowing them to maintain internal homeostasis of other essential elements maybe a conserved mechanism pertaining to other essential metals as well.

Although exposure to dissolved TEs at the gill surface is of great importance in determining toxicity, dietary exposure also plays a role though less studied (reviewed in Wang 2013). The TEs abundant in CCWs accumulate to high levels in primary producers in the ASH environment (Roe et al., 2005), in addition to the fine particulate CCW sediment unintentionally consumed by grazing feeders like southern toad larvae (personal observation during dissections and *pers.comm*. WA Hopkins). The nature of our experimental conditions did not allow us to tease apart the relative role of these exposure pathways; however there are some interesting trends that provide some insight. Though PC1 scores did not differ significantly between populations when reared in the ASH environment, the ASH population tended to have lower scores (i.e. higher levels), which is supported by results of *post hoc* univariate tests run on those elements (see Appendix Figure B1). Specifically, the ASH population had higher Cd, Se, and Zn levels when reared in the ASH environment compared to the REF population. Studies in terrestrial invertebrates have found two different mechanisms by which tolerance to TEs is associated: 1) enhanced excretion and 2) enhanced internal sequestration (Leo Posthuma & Van Straalen, 1993; van Straalen & Donker, 1994). These two mechanisms result in distinct patterns

of TE accumulation. Under the enhanced excretion hypothesis, we would expect to find lower TE accumulation in more tolerant populations, while enhanced sequestration would appear as either similar or higher levels of TEs (van Straalen & Donker, 1994). Our results support the hypothesis that the more tolerant ASH population has a greater capacity for effective internal sequestration of TEs.

Taken together, it appears that offspring from the ASH population have acquired mechanisms allowing them to maintain levels of essential elements when confronted with toxic levels of dissolved TEs and more effectively sequester TEs accumulated via aquatic and dietary routes that may allow them to survive better in the presence of CCWs. Future research will be needed to determine the mechanisms underlying these phenotypic differences, which are likely mediated by the expression of detoxification (Roelofs, Overhein, de Boer, Janssens, & van Straalen, 2006; van Straalen, Janssens, & Roelofs, 2011) and osmoregulatory (Craig, Hogstrand, Wood, & McClelland, 2009; Taylor et al., 2003) associated genes.

Relationships between elemental composition and life history traits

Relationships between the elemental breakdown of metamorphic toads and their development and morphology when reared in a CCW-contaminated environment provide insight into how exposure to TEs in that environment influences individual performance, or alternatively how performance influences exposure outcomes. More rapid development and daily averaged growth rate were generally associated with lower levels of TEs (i.e. PC1, Cd, Se). While the nature of our study does not allow us to determine which factor is driving the other, these relationships offer several interesting possibilities. First, a shorter larval developmental period may limit exposure to and accumulation of CCWs in the ASH environment. Development under natural conditions can be quite variable and individuals able to develop most rapidly are

effectively experiencing an abbreviated exposure to the toxic elements present. However, it is also possible that individuals less effective at eliminating toxic TEs accumulate higher levels of those elements, which impaired development (van Straalen & Donker, 1994). As growth rate is a composite metric of mass at metamorphosis, standardized by time to metamorphosis, the three measures are inherently correlated, such that more rapid development (and larger size at metamorphosis) generally conveyed the highest growth rates (Pearson product-moment $\rho = -0.90$ and 0.25 for time to metamorphosis and mass respectively). Therefore, while it is possible that higher accumulated levels of TEs impaired growth rate, it is also possible that this association was largely determined by the length of time spent in the aquatic environment. Interestingly, individuals that grew most rapidly in early development and achieved the largest sizes at metamorphosis tended to have the lowest levels of PC1 TEs. All else being equal, we might assume more rapid early larval growth and larger sizes at metamorphosis would be associated with higher levels of TEs at metamorphosis, as individuals may have to consume more food resources and thus more TEs to achieve this growth. The fact that this relationship is in the opposite direction suggests that some individuals may be more efficient at excreting TEs (L Posthuma, Hogervorst, & van Straalen, 1992) and thus suffer fewer of the negative effects associated with accumulating high levels of TEs.

Interestingly, other studies examining tolerance to TEs have found that populations evolving enhanced tolerance can have higher constitutive expression of detoxification genes, such as metallothionein (Mt, Roelofs et al. 2009). Expression of Mt and other such pathways is energetically costly (Pook, Lewis, & Galloway, 2009), thus constitutive expression in the absence of the contaminants whose presence is usually necessary for expression could negatively impact fitness by limiting the resources available for growth and development. In fact we

observed reduced growth and delayed development of the ASH population under reference conditions relative to a population with no history of exposure to TE contaminants (Chapter 2). Further, adult toads from the ASH population had been previously found to have constitutively higher levels of stress hormones, including cortisol (Hopkins, Mendonça, & Congdon, 1997, 1999), which has been associated with improving tolerance to aquatic TEs and reducing body burdens (Bury, Jie, Flik, Lock, & Bonga, 1998; G De Boeck et al., 2001; Gudrun De Boeck, De Wachter, Vlaeminck, & Blust, 2003) . Future studies examining expression of TE detoxification pathways and hormone levels in larvae from both population under reference and TE-contaminated conditions would be essential to determine the exact nature of these relationships and provide further insight into the mechanisms underlying adaptation.

Examining whole body levels of essential major elements provides further support for the idea that developmental period may play a large role in determining exposure outcomes. Levels of essential major elements on PC2 (i.e. Na and K) showed significant relationships with more rapid development and growth rate. As exposure to dissolved TEs impacts maintenance of essential electrolytes (i.e. Na and K), this fits with observations that longer exposure times to TE-stressors (i.e. slower development) decrease whole body electrolyte levels. Maintaining levels of these elements is critical to normal physiological function, so larvae able to develop more rapidly and thus receiving reduced exposures, can mitigate some of the detrimental reductions in PC2 elements associated with the ASH environment. Interestingly, there were no significant differences between populations in their developmental time ($p = 0.466$) in the subsample of individuals used in this study. This suggests that although developmental period clearly influences levels of PC2 elements, there must be inherent physiological differences between populations co-determining PC2 levels.

Unlike the essential major elements on PC2, metamorphic toads that developed more slowly and had lower growth rates maintained higher levels of PC3 elements. This axis was primarily describing levels of Ca, Mg, and P, which are critical to a number of physiological processes, but the bulk of which are stored in the skeletal system. Surprisingly, we did not see any significant relationship between high PC3 values and larger sizes at metamorphosis, which presumably would have had the most well developed skeletons. Rather, individuals that grew and developed more rapidly may have done so at the expense of bone mineralization. We note that PC3 is the one factor for which we saw population x environment interactions with respect to trait values. Post-hoc regressions of Ca, Mg, and P against developmental time and growth rate offer more insight into this relationship. While individuals from the ASH population continue to accrue higher Ca levels as developmental time increases, the REF population does not (Appendix Figure B3). Further, Mg levels in the ASH population remain steady with increasing developmental time, but levels steadily decline with developmental time in the REF population and there were no significant relation with P. Some TEs (e.g. Cd and Ni) that are elevated in the ASH environment can alter Ca and Mg metabolism (Abelson & Aldous, 1950; Pratap, Fu, Lock, & Wendelaar Bonga, 1989; Revis, Zinsmeister, & Bull, 1981). The fact that the REF population showed no benefit, in terms of extended developmental time, on Ca and Mg levels as the ASH population did provides another line of evidence of adaptive physiological processes in the ASH population providing some protection from the toxic effects of TEs. In humans, genetics play a strong role in determining bone mass (US Department of Health and Human Services, 2004), but further studies will be necessary to determine whether the observed differences are genetic or epigenetic, whether they are definitively due to changes in bone

mineralization, and how such changes could influence post-metamorphic foraging, dispersal, and predator avoidance.

Conclusions

Using a novel approach that incorporated the individual rearing and elemental quantification of offspring from two populations in a reciprocal transplant design provided us a unique opportunity to examine the physiological consequences of multigenerational and novel exposures to environmental contaminants in the aquatic environment. Our results demonstrate the potential for native amphibian populations to adapt to environments contaminated with complex mixtures of heavy metals and other TEs. We observed distinct divergence between populations in their ability to maintain internal homeostasis of these elements under more realistic exposure conditions than allowed by laboratory studies. While these apparent physiological adjustments have not completely mitigated the negative effects associated with aquatic development in a contaminated environment, they are associated with reduced mortality relative to a population naïve to CCWs. We also document that the negative physiological effects of TE exposure in larval southern toads are congruent with observations made in fish, suggesting many these taxa may share many similar mechanisms of toxicity and adaptation. Further, our observations were made in metamorphic amphibians, days after which they had emerged from the water and resorbed their gills, showing there is potential for contaminated aquatic environments to impact amphibian health even after dispersal into the terrestrial landscape.

While management strategies employing wetlands and other constructed ponds to remove contaminants from wastewater have been extremely effective in mitigating the flow of contaminants to the surrounding environment, it is critical to consider how the novel environments they create in the immediate area impact wildlife. The biotic and abiotic

complexity inherent in the field is often avoided in favor of controlled laboratory studies; however *in situ* studies embracing this complexity will ultimately be necessary to understand how organisms respond to different exposure scenarios under realistic conditions. We have determined that multigenerational exposure to these environments can result in enhanced tolerance to these degraded environments that seems to be driven by complex physiological responses. Future studies will be needed to ascertain specific mechanisms of toxicity, relative roles of aquatic vs. dietary exposure routes, causal relationships underlying associations between life history traits and accumulation of contaminants, and if our observations can be generalized across other populations, species, and TE exposure scenarios.

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TABLES

Table 3.1. Tabulation of the dams, sires, families, and total individual included in this study, by population and rearing environment.

population	environment	dams (*n*)	sires (*n*)	families (*n*)	individuals (*n*)
REF	REF	2	11	11	51
	ASH	4	16	29	86
ASH	REF	2	8	13	59
	ASH	4	16	29	99
	Total	**12**	**51**	**82**	**295**

Table 3.2. Elements analyzed in metamorphic toad bodies (n = 13), calculated detection limits (DL), the number of samples falling below the DL (BDL) by rearing environment, and percent of the total number of individuals analyzed that were BDL. Sample totals by rearing environment were REF = 110 and ASH = 185.

	As	Ca	Cd	Cu	Fe	K	Mg	Na	Ni	P	Se	Sr	Zn
DL (µg/g)	0.34	161.05	0.14	0.20	45.31	51.40	51.44	48.86	0.16	82.97	5.22	0.33	0.49
N_{BDL} (REF/ASH)	49/0	0/0	81/0	0/0	0/0	0/0	0/0	0/0	37/1	0/0	108/0	0/0	0/0
%BDL (REF/ASH)	44/0	0/0	**74/0**	0/0	0/0	0/0	0/0	0/0	34/<0.1	0/0	**98/0**	0/0	0/0

Table 3.3. Loading scores and summary statistics of principal component analysis of elemental composition of metamorphic toads. For clarity, only loadings >|0.25| are shown and bolded values are those for rotated PC axis that element had the greatest loading. Sums of squares loadings, proportion of variance explained by a given axis, and the cumulative proportion of variance explained by each factor are shown at bottom of table.

Variable	Rotated PC axis			
	1	2	3	4
As	**-0.82**			
Sr	**-0.76**		0.42	
Ni	**-0.70**			0.45
Cu	**-0.58**	-0.26		
Zn	**-0.47**			
K		**0.94**		
Na		**0.93**		
Ca		-0.28	**0.76**	
Mg			**0.70**	0.41
P	-0.55		**0.70**	
Fe				**0.81**
SS loadings	2.62	2.02	1.77	1.20
variance	24%	18%	16%	11%
cumulative variance	24%	42%	58%	69%

Table 3.4. Results of univariate linear mixed effect models testing effects of population, rearing environment, and their interaction on the elemental composition of metamorphic toads using the first four PCs. Below each PC response variable are the individual variables that contributed most to that component. Reported estimates are for ASH relative to REF and estimates with p-values <0.05 bolded.

	term	estimate	*p*
PC1	pop	**-0.33**	**0.027**
trace elements	env	**-1.86**	**< 0.001**
	pop:env	0.15	0.237
PC2	pop	-0.13	0.513
Na, K	env	**-0.43**	**0.026**
	pop:env	**0.58**	**0.015**
PC3	pop	-0.17	0.553
Ca, P, Mg	env	-0.06	0.777
	pop:env	**0.74**	**0.003**
PC4	pop	0.30	0.255
Fe	env	**-0.49**	**0.022**
	pop:env	0.26	0.298

Table 3.5. Results of univariate models testing the effect of population on the elemental composition of metamorphic toads within the ASH environment. Reported estimates are for the ASH population relative to REF and estimates with p-values <0.05 bolded.

	estimate	*p*
PC1 *trace elements*	-0.19	0.181
PC2 *Na, K*	**0.47**	**0.019**
PC3 *Ca, P, Mg*	**0.57**	**0.016**
PC4 *Fe*	**0.55**	**0.002**
Cd	0.12	0.260
Se	**0.11**	**<0.001**

Table 3.6. Summary of results of linear mixed effect models examining relationships between the elemental compositions of individual metamorphic southern toads. Though not shown for brevity, the fixed effect of population was also included in these models and was only significant for early larval growth. Note that all trace elements on PC1 had negative loadings, thus positive model estimates denote reductions in trace element levels.

	factor	estimate	*p*
early growth	PC1	**0.11**	**0.019**
	PC2	0.02	0.316
	PC3	0.02	0.354
	PC4	0.03	0.220
	Cd	0.06	0.101
	Se	-0.19	0.069
ttm			
	PC1	**-0.27**	**<0.001**
	PC2	**-0.09**	**<0.001**
	PC3	**0.09**	**<0.001**
	PC4	**-0.18**	**<0.001**
	Cd	**0.12**	**0.003**
	Se	**0.73**	**<0.001**
mass			
	PC1	**0.06**	**0.017**
	PC2	-0.01	0.474
	PC3	-0.01	0.586
	PC4	0.00	0.824
	Cd	-0.03	0.147
	Se	0.01	0.741
bci			
	PC1	-6.43	0.315
	PC2	-4.87	0.095
	PC3	-0.01	0.998
	PC4	0.00	0.824
	Cd	-0.03	0.147
	Se	0.04	0.531
growth rate	PC1	**0.33**	**<0.001**
	PC2	**0.09**	**<0.001**
	PC3	**-0.09**	**<0.001**
	PC4	**0.17**	**<0.001**
	Cd	**-0.17**	**<0.001**
	Se	**-0.64**	**<0.001**

FIGURES

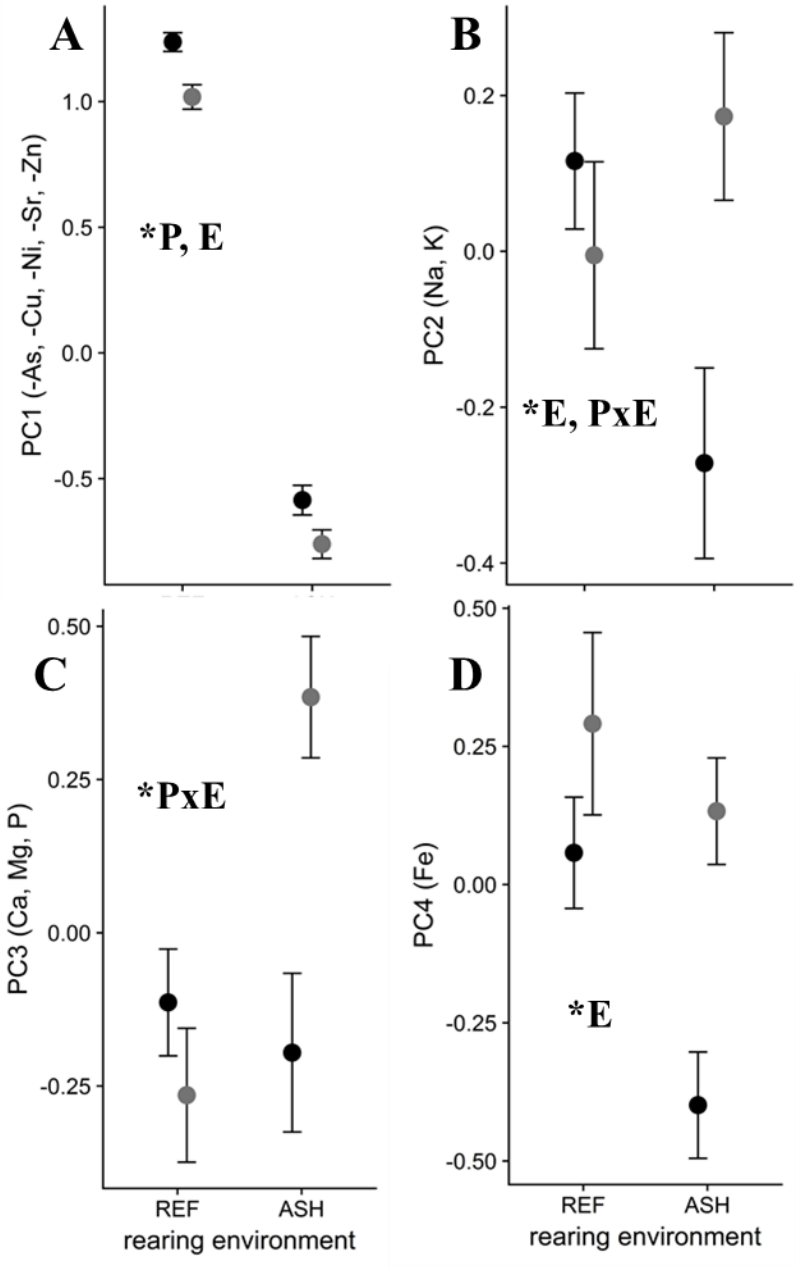

Figure 3.1. Plots of mean (+/- SE) PC scores of metamorphic toad body burdens by population (REF = black, ASH = gray) and rearing environment. On each y-axis we denote the variables that were most heavily loaded on a given axis. Asterisks with letters denote significant effects of population (P), rearing environment (E), and their interaction (PxE). Note that all element

loadings on PC1 are negative, hence higher PC1 scores are associated with lower levels of trace elements.

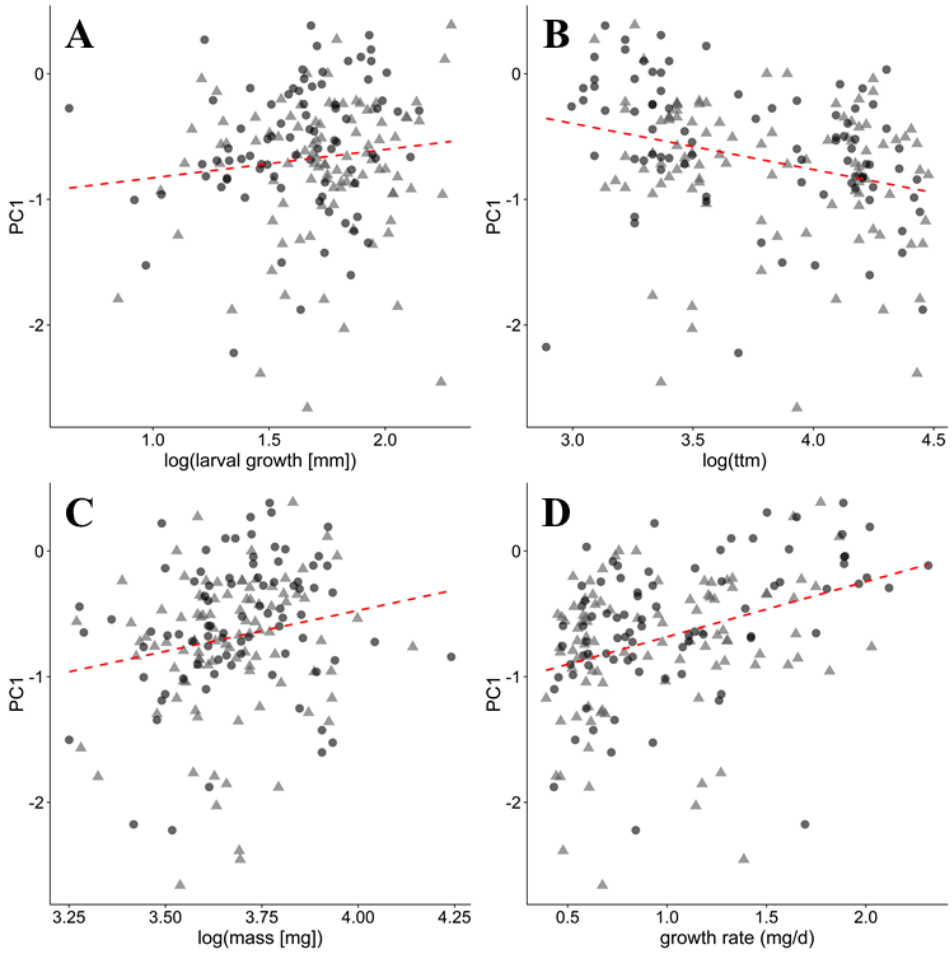

Figure 3.2. Scatter plots showing relationships between PC1 factor scores and early larval growth (A), developmental time (B), size at metamorphosis (C), and growth rate (D) in the ASH environment. Individuals from the REF population are shown as black circles and those from the ASH population as gray triangles. Red dotted lines represent simple regressions of traits on PC1 scores.

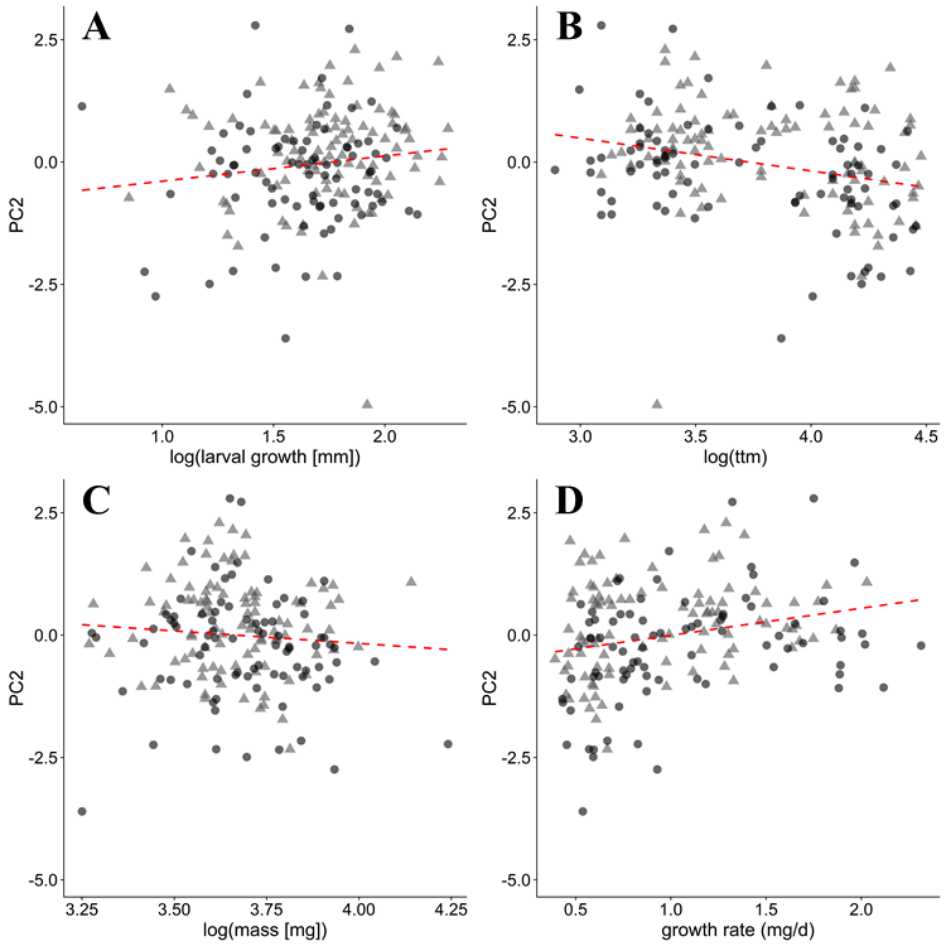

Figure 3.3. Scatter plots showing relationships between PC2 factor scores and early larval growth (A), developmental time (B), size at metamorphosis (C), and growth rate (D) in the ASH environment. Individuals from the REF population are shown as black circles and those from the ASH population as gray triangles. Red dotted lines represent simple regressions of traits on PC2 scores.

CHAPTER 4

RELATIONSHIPS BETWEEN HOST LIFE HISTORY, REARING ENVIRONMENT AND GUT MICROBIOME[3]

[3] R. Wesley Flynn, Kelsey N. Thompson, Gregory D. Mayer, & Stacey L. Lance. To be submitted to *Applied and Environmental Microbiology*.

ABSTRACT

Human activities have rapidly altered environments globally, creating stressful novel conditions to which wildlife must cope. Recent recognition of the role host-associated microbial communities play in mediating the response of organisms to environmental stressors has shed light on the natural variation in outcomes of stressor exposure. Exposure to chemical contaminants is exceedingly common for aquatic organisms and is a scenario likely to be mediated by microbial communities. Numerous microbial species regularly colonizing the digestive tracts of wildlife have been found to rapidly adapt to environmental contaminants. This adaptation often involves metabolizing toxic compounds to less bioavailable forms, thus reducing toxicity to themselves and potentially the hosts with which they are associated. We designed our study to examine associations between amphibian gut microbial communities, rearing environment, and host phenotype under field conditions in a wild outbred population of amphibians. Rearing environment significantly influenced the structure of gut communities, such that development in the environment contaminated with trace elements resulted in greater abundance of potentially pathogenic bacteria. Both overall microbial community richness and structure were also associated with phenotypic variation in life history traits and accumulation of toxic trace elements; however these relationships were dependent on the environmental context in which they occurred. Further research will be needed to determine whether the composition of gut communities is driving host phenotypic variation or vice versa. Our results suggest that maintaining diverse gut microbial communities has positive effects on fitness related traits and that degraded environments could put developing amphibians at greater risk of opportunistic infection.

INTRODUCTION

Organismal development is dependent not only on an individual's own genetic architecture and abiotic environment, but also on the microbial communities to which they are inextricably tied (McFall-Ngai 2002; Fraune and Bosch 2010; Pradeu 2011; McFall-Ngai et al. 2013). Gut microbiota (GM) in particular are critical to normal development and overall health of the hosts with which they are associated (Cox et al. 2014; Round and Mazmanian 2014; Coon et al. 2016). Interactions between hosts and GM are inherently complex and influenced by host genetics (Spor et al. 2011) and environmental factors including diet (Duncan et al. 2007; Zhang et al. 2009; Kohl et al. 2014), life stage (Kohl et al. 2013), temperature (Kohl and Yahn 2016), water chemistry (Sylvain et al. 2016), and contaminant exposure (Kohl et al. 2015; Jin et al. 2017). Anthropogenic activities have altered environments globally, often creating novel conditions that impact not only wildlife, but the microbial communities they are associated with.

Human-induced environmental changes often create stressful, degraded habitats that threaten population persistence and shift community structures by altering species interactions (Godinho and Ferreira 1998; Johnson et al. 2013), patterns of dispersal (Riley et al. 2006), and life histories (Palumbi 2001a; Allendorf and Hard 2009). Habitat degradation resulting from chemical contamination is one of the most common features of anthropogenic activities and can negatively affect the overall biodiversity (Johnston and Roberts 2009) and health of ecosystems (Diaz and Rosenberg 2008; Acevedo-Whitehouse and Duffus 2009; Kelishadi and Poursafa 2010). While the direct effects of contaminant exposure on wildlife and bacterial communities have been extensively studied (Braunbeck et al. 1998; Vos et al. 2003; Sparling et al. 2010), it is less clear how these chemical perturbations can alter host-associated microbial communities (Jin et al. 2017) and how those effects could translate into effects on host health.

Contaminants can affect the diversity and structure of microbial communities in the environment (Hemme et al. 2010; Sutton et al. 2013; Singh et al. 2014). However, studies investigating the effects of contaminants on host-associated GM communities in field settings have been mixed, with exposure resulting in either reduced, elevated, or similar diversity (reviewed in Jin et al. 2017). For example, some studies using murine models found dietary exposure to heavy metals (arsenic [As] and cadmium [Cd]) and persistent organic pollutants reduced ratios of *Firmicutes*/*Bacteroidetes* in the gut (Lu et al. 2014; Zhang et al. 2015a,b), but other systems effects were observed in the opposite direction (lead [Pb] and polychlorinated biphenyls; J. Wu et al. 2016; Kohl et al. 2015). While characterizing effects of contaminant exposure on host-associated microbial communities at the phylum-level can be useful in attempts to identify patterns of such exposure across species and stressors, such characterizations are likely over simplistic to offer much insight into how such shifts could translate into effects on host health. Each phylum encompasses an abundance of genetically and functionally distinct genera; thus we would not expect for all the taxa within a given phyla to respond the same to environmental contaminants. Maintaining GM diversity increases the number of metabolic pathways available to their associated hosts for not only energy, but mitigating the negative effects of toxic compounds (Karasov and Carey 2009) and inhibiting colonization of pathogenic bacteria (Dillon and Charnley 2002). Characterizing the responses of these bacterial communities at multiple taxonomic levels is a critical step that allows us to determine not only how they respond to environmental perturbations, but how those community changes may interact with the host to determine phenotypic and toxicological outcomes.

Microbial communities have the capacity to adapt more rapidly to changing environmental conditions than the hosts they are associated with, and thus may play a critical

role in the establishment and persistence of wildlife in contaminated environments. In fact, a number of environmentally derived bacterial taxa that associate with animal hosts are not only tolerant to high levels of toxic trace elements (Burton et al. 1987; Clausen 2000; Belimov et al. 2005), but capable of metabolizing such elements to less bioavailable forms that are less toxic to wildlife (Combs et al. 1996; Anderson and Cook 2004; Madhaiyan et al. 2007). Considering dietary exposure to contaminants can be a significant route of exposure (Deforest and Meyer 2015), GM communities could influence the toxicity of ingested contaminants in host organisms. It has been suggested that 'internal exposure' (i.e. assimilated contaminants) may be a more accurate and biologically relevant approach to understanding toxicity (Escher and Hermens 2004). Assimilation efficiency of heavy metals in the diet can influence toxicity (see Posthuma and Van Straalen 1993), but generally attributed to host physiology, rather than the metabolism of gut microbial communities.

Internal exposure, or dose, of environmental contaminants is mediated by host physiology and metabolism, which are both influenced by GM communities. One way to determine how GMs may affect toxicity is to rear individuals in a common garden while controlling for genetic background. By examining associations between host body burdens of contaminants, which are determined in part by elemental speciation and bioavailability (Eggleton and Thomas 2004; Slaveykova and Wilkinson 2005), and GM structure we can begin to characterize bacterial taxa over-represented in the guts of individuals with lower relative body burdens. However, the extent to which GMs impact host health is not limited to their interactions with contaminants, but virtually every aspect of organismal health and development.

Acknowledgment of these essential and complex roles of microbial communities on hosts in the modern view of life, forces us to accept organisms not as autonomous individuals, but as

holobionts comprised of biomolecular networks among host and symbiotic microbial cells (Bordenstein and Theis 2015). This is especially pertinent for microbial symbionts living in the digestive tracts of organisms that are critical for the normal development, growth, and immune function of their associated hosts (Round and Mazmanian 2014). Chemical perturbation of GM early in development can have lasting impacts on host health into adulthood, long after the exposure inducing the perturbation has ended (Cox et al. 2014). Controlled laboratory studies have been necessary to determine causal relationships between GM and host phenotype in settings that reduce 'environmental noise' in data. However, ultimately it is essential to test these relationships in non-model organisms in the field to determine how universal these observations are and the strength of their influence relative to other factors at play in natural systems.

In this study, we characterize GM communities in a wild amphibian population, reared *in situ* in both an environment characterized by high levels of potentially toxic trace elements and a reference site with no history of contamination. This approach, combined with controlled breeding allowed us to control for the influence of host genetics on GM community structure (Spor et al. 2011), while limiting the influence of artificial, laboratory conditions known to affect GM communities (Dhanasiri et al. 2011; Kohl and Dearing 2014). Our unique approach provides insight into how GM respond to environmental contaminants and relationships between GM communities and host life history traits. Our objectives were to 1) characterize the gut microbial communities in a wild population of amphibians, 2) quantify how development in a contaminated environment alters gut communities, and 3) examine relationships between microbial community metrics, host contaminant uptake, and life history traits. Addressing these questions will provide empirical evidence to better understand how GMs may mediate

interactions between hosts and the environment and even facilitate host persistence in degraded environments.

METHODS

Study Species

The southern toad (*Anaxyrus terrestris*, hereafter 'toad') is a largely terrestrial habitat generalist common throughout the southeastern United States that will utilize heavily disturbed environments. They are considered indiscriminant breeders, using ephemeral wetlands, roadside ditches, and constructed surface impoundments. Females lay single clutches of 2500 – 4000 eggs in strings around emergent vegetation near the water's surface in the spring. Eggs hatch in 2-4 days (Jensen 2008) and larvae graze on algae, biofilms, and decaying organic matter and develop rapidly, reaching metamorphic climax in 30 – 63 days (Beck and Congdon 1999).

Study Sites

The study sites we used for our *in situ* study consisted of a large ephemeral wetland with no history of contaminants (REF) and a nearby surface impoundment used to manage coal combustion waste (CCW) generated by the adjacent coal fired power plant (ASH). The aquatic environment in REF is typical of wetlands in the area with acidic conditions and high levels of organic matter (Sharitz and Gibbons 1982), while the ASH-site consists of a series of surface impoundments that received regular inputs of CCWs since the 1950's. The conditions at ASH differ considerably from that of REF, having low levels of organic matter (Hopkins et al. 2004), a neutral pH, high levels of trace elements (Figure 4.1, see *Statistical Analyses* for description of principal component analysis), and permanent water. Toads use both of these sites for reproduction.

Experimental Design

Briefly, adults were collected from the ASH-site, brought back to the lab, and bred in a quantitative genetic breeding design with two females mated to eight males in each breeding block. The individuals in this study represent the offspring from one of these breeding blocks (i.e. 2 females and 8 males). Fertilized eggs from each family were divided among two treatment groups, half developing in water obtained from a natural ephemeral wetland (REF) and half developing in water taken directly from the CCW-disposal site (ASH). Clutches developed in our climate controlled animal care facility until reaching Gosner stage 25 (GS25) at which point they were pooled within family x treatment groups and six random offspring from each group were selected to be included in the field study. Individuals in this study represent a subset of families from a larger study (Chapter 2).

We reared larvae individually through metamorphosis in 1 L plastic containers, nested within larger screened field enclosures that allowed for the regular exchange of water and sediments with the surrounding environment. At each site, enclosures were grouped into six spatial blocks, such that one larva from each family was represented in each block. Individual tadpoles were assigned to enclosures using a random number generator. Initially, observations were made every other day until we observed larvae developing rear legs at which point observations were made daily until the end of the study. At each observation, we checked for survival and the emergence of front legs (GS 42) before fully exchanging the enclosure water by lifting. We measured early larval size and growth from scaled photos of individual larvae taken at seven and 21 days after the start of the field trial (Schneider et al. 2012). We also measured developmental time (i.e. time to metamorphosis in days), size at metamorphosis (i.e. snout vent

length and mass), and growth rate of the entire period of larval development (i.e. mass at metamorphosis/time to metamorphosis).

Water quality measurements (pH and specific conductance) and water samples (for major and trace element analyses) were taken from every other spatial block at each rearing environment at two time points throughout the study (05/02/14 and 06/09/14) to characterize the aquatic environment. We also place sealed iButton temperature loggers in every other enclosure at both sites, recording a data point every hour, for the duration of the study.

Sample Processing

At metamorphosis, animals were placed in ventilated plastic containers containing an unbleached paper towel and a small amount of water taken from the container in which they developed, and held in the lab until they achieved complete tail resorption (i.e. < 1 mm of tail remaining). Two days after tail resorption, we took mass (nearest mg) and snout-vent length measurements before euthanizing animals in a 3% solution of MS-222. Euthanized animals were dissected under a microscope using sterile tools within three minutes of death. We removed ~5 mm of digestive tract from the anterior portion of the stomach to the posterior end of the intestine (hereafter referred to as 'gut') and stored all samples in 1.5 mL microcentrifuge tubes containing RNAlater. Samples were incubated at room temperature for at least 1 h to ensure entire sample was saturated with RNAlater before storing to -80^{o}C.

Gut tissue samples were digested following a modified Beadbeater protocol (see Appendix Text C1). Briefly, samples were transferred to sterile 2.0 mL polypropylene microvials (Biospec Products, #522S). We added 350 µL of lysis buffer (Appendix Text C1) and one sterilized 3.5 mm stainless steel bead to each sample tube. Tubes were run on a mini-beadbeater 24 (Biospec Products, Bartlesville, OK, USA) for three minutes at 2,500 oscillations/minute and

checked to ensure samples were fully lysed. After centrifugation for 3 minutes (all centrifugation steps were done at 14,000 rcf), the supernatant was then pipetted onto spin columns set in 2 mL collection tubes (Epoch®, #1920-050) and centrifuged for one minute to bind DNA. We then performed three washes (Appendix Text C1) before eluting DNA in 50uL of ultra-pure DEPC-treated water. DNA concentration (ng/µL) and quality were assessed using a Nanodrop 1000 Spectrophotometer (ThermoFisher, Waltham, MA, USA) with 2 µL of sample DNA.

16S rRNA Amplicon Diversity Sequencing

Extracted DNA was amplified and partial 16S rDNA was sequenced using an Illumina miSeq . Briefly, the sequencing workflow follows a two-step amplification process followed by product purification before sequencing. The first round of amplification utilizes forward primer constructs (5'-3') of Illumina i5 sequencing primer (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and 28F primer (GAGTTTGATCNTGGCTCAG). The reverse primer for the first round is a combination of the Illumina i7 primer (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) and the 519R primer (GTNTTACNGCGGCKGCTG). These primer sets target the V1-V3 region of the bacterial 16S DNA. PCR amplification was conducted using 25µL reactions of the Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, CA, USA), 1ul of each 5 µM primer, and 1ul of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, CA, USA) with the following thermal profile: 95°C for 5 min, then 25 cycles of 94°C for 30 sec, 54°C for 40 sec, 72°C for 1 min, followed by a final elongation step of one cycle at 72°C for 10 min then a 4°C hold.

Resulting 16S DNA products from the first round of amplification were run on a 1% agarose eGel (Life Technologies, Grand Island, NY, USA). The second round of amplification

was used qualitatively determine concentrations of the products. The primer set for second PCR were designed based on Illumina Nextera PCR primers and are as follows: Forward-AATGATACGGCGACCACCGAGATCTACAC[i5index]TCGTCGGCAGCGTC and Reverse - CAAGCAGAAGACGGCATACGAGAT[i7index]GTCTCGTGGGCTCGG. The thermal profile of the second PCR was the same as the first, except it ran for 10 cycles. The products were also visualized with an eGel to check for amplification.

Products from different samples were then pooled and size selected using SPRIselect protocol (BeckmanCoulter, Indianapolis, IN, USA) using a 0.7 ratio in both rounds of the protocol. DNA concentrations were quantified using Quibit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and loaded on an Illumina MiSeq (Illumina Inc., San Diego, CA, USA) 2x300 flow cell at 10 pM and sequenced at RTLGenomics.

Bioinformatic Analysis

Sequencing results were analyzed using a standard microbial diversity analysis pipeline. The pipeline consists of two major steps; denoising and chimera removal stage and the microbial diversity analysis stage. Denoising is conducted by removing short sequencing, singleton sequences and noisy (poor quality) reads. Once low quality reads are removed, chimera detection is performed to remove chimeric sequences. Finally, any remaining sequences are analyzed and corrected base by base to remove any remaining noise from the dataset. Stage two begins by clustering the reads into Operational Taxonomic Units (OTUs) based on 97% sequence similarities. OTUs are then run through an in-house, curated database at RTL Genomics for taxonomic classification or bacterial identification. Data from the microbial identification pathway is further sorted removing any reads that did not identify as kingdom Bacteria. Then a rarefaction curve is created to check for sufficient sampling and sequencing depth. For each

sample in the set relative abundance of each bacterial taxon was determined by dividing each OTU by the total number of reads. Ecological diversity of the bacterial communities was assessed using alpha and beta diversity metrics. Alpha diversity was measured in two ways; overall sample richness and then sample richness and evenness. To determine overall sample richness either the total number of observed OTUs per sample (i.e. observed richness) or Chao1 Richness (i.e. Chao1) estimates were calculated. Sample richness and evenness were measured using Shannon-Weiner Species Diversity.

Trace element analysis

As amphibian larvae and bacterial communities are highly susceptible to chemical changes in the aquatic environment, we measured water quality and chemistry parameters at two time points over the course of the study (5/2/14 and 6/9/14) in every other spatial block in each environment over the course of the study. We quantified pH and specific conductance (spc, µS/cm) in the field and collected water samples to determine levels of major (Ca, Mg, Na, K) and trace elements (Al, As, Ba, Be, Cd, Co, Cu, Ni, Sb, Se, Sr, Th, U, V, and Zn). Samples were acidified to 1% HNO_3 using trace metal grade HNO_3 (JT Baker #9598), before running on an inductively coupled mass spectrometer (ICP-MS, Nexion 300X ICP-MS; Perkin Elmer, Norwalk, CT, USA).

To examine how GM communities may affect the accumulation of the potentially toxic trace elements associated with the ASH environment, we quantified individual body burdens for all but four individuals (two REF and two ASH) from samples consisting of whole carcasses minus guts and livers. Carcasses were freeze-dried and weighed to the nearest 0.01 mg (Mettler Toledo XPE26 DeltaRange®) before transferring to a metal-free 15 mL conical tubes (VWR® #89049). Additionally, we included samples for standards (TORT-3, National Research Council

of Canada) and acid blanks. We added 200 µL of HNO_3 to each tube and incubated samples in a heat block at 80^{o}C for two hours. Following digestion, we added 1800 µL of ultrapure water. We transferred 1 mL of this digestate to a second metal-free 15 mL conical containing 2 mL of ultrapure water to achieve a final concentration of 3.3% HNO_3. These diluted samples were then run on the ICP-MS for trace elements known to be toxic to aquatic organisms and elevated in the ASH environment (i.e. As, Cd, Cu, Ni, Se, Sr, and Zn).

Statistical Analysis

We examined differences in water quality (pH and spc) and trace element concentrations (Al, As, Ba, Cu, Fe, Ni, Se, Sr, V, Zn) between environments using principal components analysis (PCA; *prcomp*; R Core Team, 2013) with scaling and centering. Using scree plots and axis eigenvalues, we determined the first two axes sufficiently explained the variation in the dataset as later components all had eigenvalues <1.0. We performed a varimax rotation on these two axes to facilitate interpretation and retained the sample scores obtained for plotting and general site descriptions.

Though the phenotypic results of the larger dataset from which these samples were derived is reported elsewhere (Chapter 2), we analyzed how life history traits differed between rearing environments in this subset of the data. We used univariate linear mixed effect models (*lme4*, Bates et al. 2015) with the trait of interest as the independent response variable, rearing environment as a fixed predictor, and parentage (sire, dam, sire x dam) and site-specific spatial block as random effects.

Differences in alpha diversity metrics between rearing environments were analyzed using linear mixed effect models that included rearing environment as a fixed effect and site specific parentage (sire, dam, sire x dam) and rearing block as random effects. Permutational Multivariate

Analysis of Variance Using Distance Matrices (PERMANOVA) function (*ADONIS*, Oksanen et al. 2015) used Unifrac distances, weighted or unweighted, to scan for differences in the beta diversities of the samples or groups (ASH vs. REF) using a ANOVA-like simulation. Principal Coordinate Ordination Analyses (PCOA), stack bar plots, and heatmaps were plotted to aid in visualization of data. Analyses and plotting were conducted in R (Team 2013), using *vegan* (Oksanen et al. 2015), *phyloseq* (McMurdie and Holmes 2013), and *ggplot2* (Wickham 2009) packages.

We also tested how the relative abundance of specific bacterial taxa changed between environments to investigate in more detail the OTUs that were consistently affected by rearing environment that underlie changes in community membership and structure. To accomplish this, we converted OTU counts to integers that were then used as response variables in generalized linear models with negative binomial distributions and a log-link functions in (*DESeq2*, Love, Anders, and Huber 2014). Rearing environment was included as a fixed effect and stratified by within environment spatial block.

Rearing environment influenced distributions of both gut microbial community metrics and phenotypic traits of hosts, thus we examined relationships between these measures within each environment separately. This allowed us to more effectively assess associations between microbial communities and other host traits, while avoiding the confounding effect of the rearing environment. First, we tested for relationships between gut microbial alpha-diversity metrics and host life history traits using linear mixed effect models including a fixed effect for microbial diversity and random effects of sire, dam, and spatial block. Though we cannot truly determine whether variation in host traits were driving microbial diversity or vise-versa, we opted for these

models over correlations as they allowed incorporation of the critical components of the experimental design (i.e. spatial block and parentage).

We examined relationships between beta-diversity measures (weighted- and unweighted-Unifrac distances) and host life history traits using PERMANOVAs where beta-diversity was the response variable, the trait of interest as the predictor variable, and within rearing environment spatial block as a covariate. As these models only test whether overall beta-diversity estimates are associated with the traits of interest, we also performed analyses using the DESeq2 package to more closely examine relationships between specific OTU abundances related to phenotypic traits. As DESeq2 requires categorical variables to examine differential abundance of OTUs among groups, prior to analysis, we assigned host phenotypic values to three classes. For full models examining relationships across environments, classes were determined across the entire dataset using trait means and standard deviations (SD). Trait values falling within +/- 1 SD of the mean were assigned to the 'average' class, while those falling above and below 1 SD were categorized as fast/small/low and slow/large/high respectively. For trace element body burden phenotypes, there were cases where values for a given element (generally those from the REF environment) fell below the instruments detection limit. When >50% of samples from a given rearing environment had samples above the detection limit an element, we assigned that sample a values of ½ the detection limit; however if <50% of samples were above detection limit we excluded the element from formal analysis (i.e. Cd and Se in REF). We also examined relationships between OTU abundances and host traits within each rearing environment, as models incorporating both environments need cautious interpretation due to the confounding effect of rearing environment on host phenotypes and GM structure. For this set of models we used within environment means and standard deviations (SDs) of each trait to determine

phenotypic classes (i.e. slow/small/low, average, and fast/large/high). DESeq analyses were run on paired categories as follows: average vs. low, high vs. low, and high vs. average.

RESULTS

Our sequencing efforts yielded a total of 1,057,327 high-quality reads across all samples after QC and filtering that resulted in 9,700 ± 865 reads per sample. We found no significant differences in sequencing depth between environments (*F-stat*: 1.904, $p = 0.171$) or among spatial blocks (*F-stat*: 1.214, $p = 0.292$) that would influence comparison of microbial composition between rearing environments.

The two rearing environments differed markedly along PC1, indicating the ASH environment had a less acidic pH, higher specific conductance (µS/cm), selenium (Se), strontium (Sr), arsenic (As), barium (Ba), and nickel (Ni), and lower zinc (Zn) and iron (Fe) (Fig. 4.1). Within environment variation among spatial blocks was explained primarily by PC2 (i.e. Cu, Al, V) and the ASH environment was overall more variable in these elements than REF (Fig. 4.1). We also found the ASH environment was significantly warmer than REF (Appendix Figure C1), but daily mean temperatures were fairly consistent within each environment. Because distributions of temperatures did not overlap between sites and were autocorrelated with spatial block, we opted to use spatial block in place of temperature as it accounted for other spatial differences among blocks in addition to temperature.

The effects of rearing environment on host life history traits were comparable to those seen in the full dataset (Chapter 2). Briefly, individuals developing in the ASH environment experienced significantly greater early larval growth, better body condition at metamorphosis, and delayed development (Appendix Table C2). Rearing environment did not have a significant

effect on size at metamorphosis or growth rate averaged over the entire developmental period. Body burdens of all trace elements examined (As, Cd, Cu, Ni, Se, Sr, Zn) were consistently higher in metamorphic toads reared in the ASH environment (Appendix Table C3). As more than 50% of toads reared in the REF environment had Cd and Se levels below the instruments detection limit, we did not perform statistical tests on these comparisons; however it is clear concentrations were much greater in toads reared in ASH.

Effects of rearing environment on gut microbial communities

We did not find any statistically significant effects of rearing environment or spatial block within environment on total observed diversity, Shannon, or Chao1 diversity indices (Fig. 4.2). However, rearing environment did influence community membership (unweighted-Unifrac: $p < 0.001$) and community structure (weighted-Unifrac: $p < 0.001$) (Fig. 4.3). While community structure did not differ significantly among spatial blocks within rearing environment, community membership varied slightly, but only in the ASH environment ($p = 0.047$).

Gut microbial communities in both rearing environments were dominated by *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* which together made up ~ 85% of all bacterial OTUs present (Fig. 4.4A).We found over 50 OTUs differentially represented in GM communities between rearing environments (see Table 1 for subset). At the phyla-level, GM communities in the ASH environment had greater abundances of *Bacteroidetes* and *Tenericutes* and reduced abundances of *Actinobacteria* and *Spirochetes* (Fig. 4.4B). Interestingly, overall abundance of *Proteobacteria* did not change between rearing environments even though this phylum had the most OTU abundances influenced by rearing environment, which is likely due to a roughly equal number of OTUs being over- and under-represented within the group. Comparison of bacterial OTUs between sites revealed a number of taxa over- ($n = 33$) and under-represented ($n = 24$) in

the ASH environment relative to REF (Table 4.1). Notably, *Aeromonas*, *Cellvibrio*, *Mycoplasma*, *Pseudomonas*, and *Shewanella* species were overrepresented in ASH, while *Methylobacterium*, *Sphingomonas*, and *Polynucleobacter* were more abundant in the guts of individuals reared in REF. The genus *Sphingomonas* stood out from other genera, by having considerable variation among OTUs in response to rearing environment with some OTUs being overrepresented and others underrepresented in each environment (Fig. 4.5).

Relationships between microbial diversity and host phenotype

Life history traits

We found significant relationships between increased gut microbial diversity and developmental time, greater growth rate, and body condition at metamorphosis. However, these relationships were dependent on the environmental context in which they occurred. Full models that incorporated both rearing environments found shorter developmental times and greater average daily growth were associated with greater GM diversity. Examining the relationships in each environment separately revealed these associations were primarily driven by interactions in the ASH environment and were never significant in the REF environment (Table 4.2). On the other hand, there was no significant relationship between GM diversity and body condition in the full models; however environment specific models uncovered that this was likely due to the opposing directions of this association depending on the environment in which they occurred. In the REF environment, greater GM diversity was related to better body condition at metamorphosis, while in the ASH environment it was associated with lower body conditions.

Trace element body burdens

Relationships between GM alpha-diversity and trace element concentrations in metamorphic toad carcasses appeared to be dependent on rearing environment (Table 4.3). We

did not detect any significant association between trace element levels and alpha diversity measures in individuals reared in the REF environment. Higher levels of GM Shannon diversity in the ASH environment were associated with reduced body burdens of Se, but higher levels of Ni (Table 4.3). Further, these relationships were also dependent on the diversity measure being considered, as we did not see these associations for Se and Ni with observed OTU richness or Chao1 diversity. Body burdens of Cd were trending in a direction such that higher observed OTU richness tended to be associated with lower Cd burdens; however this relationship was not statistically significant ($p = 0.093$).

Relationships with community structure and host phenotype

Life history traits

Microbial community membership and structure (unweighted- and weighted-Unifrac distances respectively) were associated with variation in host life history traits (Table 4.4). When considering individuals across both rearing environments, beta-diversity showed significant relationships with early growth, developmental time, and growth rate over larval development (Table 4.4). However, as beta-diversity and host trait distributions were both influenced by rearing environment, interpreting relationships across both rearing environments is challenging. Thus, we also examined relationships within rearing environments to draw stronger inferences and more directly assess how GM membership and structure may explain variation in host phenotype. Developmental time was related to community membership and structure only in the ASH environment, while early growth, mass at metamorphosis, and mean growth rate only showed relationships in the REF environment (Table 4.4).

To better understand how GM structure may be influencing or influenced by host phenotype, we examined changes in the abundances of GM community members related to host

phenotypic class. Development (days to metamorphosis) was generally associated with elevated abundances of OTUs in both environments (i.e. positive log_2-fold changes); however the genera associated with more rapid development were unique to each rearing environment (Table 4.5, Fig. 4.6). These results support the results of PERMANOVA analyses, with developmental time as the only trait in the ASH environment with significant ties community structure; however in REF we identified several significant relationships between host traits and OTU abundances. In the REF environments, larger sizes at metamorphosis were associated with elevated abundances of OTUs across *Actinobacteria*, *Bacteriodetes*, and *Proteobacteria* phyla. Notably *Chryseobacterium aquaticum*, *Elizabethkingia miricola,* and *Nevskia sp* abundance increased going from small to average to large size classes (Fig. 4.7). Further, comparison between individuals from fast and slow growth rate classes identified six OTUs differentially represented. Interestingly, what distinguished the fast growth rate class from the slow one was an overall reduction in the abundance of six OTUs from *Bacteroidetes* and *Proteobacteria*.

Trace element body burden

Accumulation of trace elements in metamorphic toads was related to GM community structure. Assessment of these relationships across both rearing environments produced significant test results for all trace elements under consideration (Table 4.6). However, as with host-life history traits these relationships need be interpreted with caution as environmental levels of trace elements and GM beta-diversity both differed between rearing environments, which confounds clear interpretation. Examining each rearing environment separately revealed that these associations were largely driven by individuals reared in the ASH environment and the only test approaching significance in the REF environment was between As body burden and community structure ($p = 0.072$). Microbial beta-diversity measures in the ASH environment

were significantly related to body burdens of Cd, Cu, Se, and Sr; however these patterns were not consistent between beta-diversity measures (Table 4.6).

Trace element levels were only elevated to potentially toxic levels in the ASH environment and because our primary goal was to assess how the abundance of microbial OTUs could influence trace element accumulation, we focused on relationships within this environment. With one exception, lower body burdens of As, Ni, Sr, and Zn were associated with greater abundances of OTUs in the gut than individuals with higher burdens (Table 4.7). The exception to this rule was between groups having low vs. higher burdens of Sr, where the only OTU differentially represented was *Mycoplasma microti*, which was less abundant in the low-Sr group. Only two OTUs showing elevated abundances in groups with lower body burdens of trace elements were observed to be common across elements. Greater numbers of *Chelatococcus asaccharovorans* were tied to lower body burdens of both Ni and Sr, while a similar pattern was seen for *Sphingomonas sp* in relation to As and Zn burdens (Table 4.7). Otherwise, OTUs more abundant in digestive tracts of individuals with lower body burdens were unique among the trace elements examined.

DISCUSSION

Environments contaminated by elevated levels of toxic elements are often expected to decrease biodiversity at all levels, by selecting for species inherently more tolerant to the chemical stressors and against susceptible species. Unexpectedly, individuals reared in the highly contaminated ASH environment did not have less diverse gut communities than those from the REF environment; however rearing environment did impact GM community membership and structure. In general, GMs in the contaminated environment were characterized by an

overabundance of potentially pathogenic OTUs from the genera *Aeromonas*, *Mycoplasma*, *Pseudomonas,* and *Shewanella*, while development in the REF environment resulted in higher levels of typically more symbiotic genera *Methylobacterium*, *Microbacterium*, and *Polynucleobacter*. Interestingly, *Aeromonas* and *Pseudomonas* species are abundant in contaminated aquatic environments globally and both are capable of metabolizing toxic trace elements found in the ASH environment to less bioavailable forms (Oremland et al. 1989; Combs et al. 1996; Anderson and Cook 2004; Madhaiyan et al. 2007; Drewniak et al. 2008). Further, methanogens, like *Methylobacterium*, can facilitate more efficient energy extraction from carbohydrates (Backhed 2005) and were more abundant in individuals in the REF environment where development was more rapid. Thus, while unsurprising that these methanogens that thrive in low oxygen environments with high levels of organic matter were more abundant in the REF environment, it suggests that their absence in the ASH environment could contribute to poorer resource utilization and thus development. Controlling for host genetic background allowed us to determine rearing environment, not host genetic variation, was responsible for the observed differences in GM communities; however we cannot conclude whether this environmental effect was driven by distinct environmental pools of microbes in each environment, direct effects of CCWs on the hosts and gut communities, or some combination of the two. Importantly, our study was able to document significant relationships between GM communities and host phenotypes in a wild outbred population of amphibians reared in the field for duration of aquatic development.

The diversity and structure of host-associated microbial communities has been repeatedly shown to influence host health (Le Chatelier et al. 2013; Cox et al. 2014; Hanning and Diaz-Sanchez 2015; Shreiner et al. 2015); however the majority of these studies have relied on inbred

laboratory strains of model organisms and/or artificial laboratory rearing environment. Controlled laboratory studies have been critical in elucidating causal associations between hosts and their microbial communities, but present challenges when trying to extrapolate such results to interactions in the field. Interestingly, our results both support and refute several important hypotheses generated from controlled laboratory studies. We found hosts with more diverse GM communities tended to develop more quickly, have more rapid growth rates, and greater body conditions at metamorphosis. The direction of these relationships aligns with the paradigm that more diverse gut communities somehow convey an advantage to the host, which is often attributed to increases in the diversity of metabolic pathways afforded by such communities (Hemme et al. 2010; McFall-Ngai et al. 2013) and resistance against opportunistic pathogens (Dillon and Charnley 2002; Piovia-Scott et al. 2017). However, the composition and structure of GM communities is likely even more critical in determining host health. We also found relationships between microbial beta-diversity and early larval growth, developmental time, size and body condition at metamorphosis, and growth rate. Individuals with phenotypes associated with greater fitness generally had elevated abundances of symbiotic OTUs, which aligns with our observations in the same direction for alpha-diversity and changes community structure. Interestingly, all of these relationships were unique to the environment in which development occurred, which suggests cautious extrapolation of interactions between GM communities and their associated hosts beyond the environment in which observations occurred.

Differential representation of OTUs among trait classes was only observed for developmental time in the ASH environment, but in the REF environment we saw such relationships for mass at metamorphosis and growth rate over aquatic development. Larger size classes at metamorphosis were tied to increases in the abundance of 11 OTUs. Of particular

interest was *Chryseobacterium*, which was less abundant in the guts of the smallest individuals at metamorphosis and those with the slowest growth rates. This genus is capable of inhibiting the growth of the amphibian pathogen *Batrachochyritum dendrobatidis* (Bd, Piovia-Scott et al. 2017). Though we did not quantify Bd presence in animals in our study, Bd is a prevalent pathogen in our study area. Given the ability of *Chyrseobacterium* to reduce infection risk, it is possible that individuals with greater abundances of this taxon may have to divert fewer resources away from growth and development to fight infection.

Environmental contaminants present challenges to not only aquatic animals, but bacteria as well. Bacteria, however, are capable of responding much more rapidly to changing environmental conditions due to their diverse metabolisms, rapid generation times, and potential for horizontal transfer of genetic information. In contaminated environments, dietary exposure through the food web or direct consumption of contaminated sediments can contribute substantially to accumulation of toxic elements in aquatic organisms (Wu et al. 2009; Wang 2011). In fact, a number of bacteria known to associate with animal hosts have been found in heavily contaminated environments and are capable of metabolizing contaminants in such a way to reduce their bioavailability (Oremland et al. 1989; Combs et al. 1996; Anderson and Cook 2004; Madhaiyan et al. 2007; Drewniak et al. 2008). While we found GM diversity associated with accumulation of Cd, Ni, and Se, this appears to be less important than the actual composition of GM communities. In the trace element contaminated ASH environment GM composition was tied to the extent to which Cd, Cu, Se, Sr, and to a lesser extent As accumulated in amphibian hosts. *Sphingomonas*, *Agrobacterium*, and *Chelatococcus* were more abundant in the digestive tracts of individuals with lower body burdens of As, Ni, Sr, and Zn. All three of these genera have been shown to be capable of not only surviving in environments with high

levels of chemical stressors, but degrading or binding compounds and thereby reducing their bioavailability (Robinson et al. 2001; Cheng et al. 2013; Li et al. 2016). However, only *Chelatococcus* has been experimentally demonstrated to reduce the bioavailability of trace element stressors specifically (Li et al. 2016). It is important to note that other bacteria in this analysis may be capable of metabolizing or internalizing trace elements, but those studies have not been performed. Interestingly, several of the OTUs more abundant in the ASH environment have been definitively shown to reduce the availability of metals and metalloids. In particular, *Pseudomonas* and *Aeromonas*, whose abundances did not seem to explain within environment variation in trace element body burdens. This may be due to the fact there was little variation in their abundances across individuals and that trace element accumulation is determined in part by uptake from the water. Regardless, our results demonstrate the potential for gut microbial communities to mediate the accumulation of toxic trace elements and detrimental effects associated with their accumulation in contaminated environments.

Our study highlights the importance of GM communities for the health of amphibian hosts developing in contaminated and uncontaminated environments. While relationships such as these have been documented in other systems, most have relied on laboratory conditions. Considering associations between host and their associated microbial communities was highly dependent on the environmental context in which they occurred, extrapolations from laboratory conditions to natural systems should be interpreted cautiously. However, some broader relationships do appear to be consistent among laboratory and field observations, namely that greater GM diversity appears to confer advantages to the hosts with which they are associated. This suggests the diversity of microbiomes is important to host health regardless of the system, though the relative impact of that diversity may be more or less pronounced depending on

environmental conditions. Future research quantifying pathogen prevalence, other environments, species, and populations in the field will be necessary to determine which effects of host-associated microbial communities are broadly applicable to wildlife health. Given the challenges of predicting which species and populations will be most impacted by emerging infectious diseases and widespread environmental contamination, closer examinations of interactions between the environment, microbiomes, and disease may provide crucial insight into often enigmatic population declines.

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TABLES

Table 4.1. DESeq2 results of OTUs with significantly different relative abundances in the guts of toads reared in the ASH-environment relative to REF. OTUs more abundant in the ASH environment are highlighted in gray. For clarity, we only included OTUs in table that met the following criteria: baseMean > 1 and padj < 0.0001.

base-mean	log_2-fold change	lfcSE	Phylum	Species
3.50	-6.48	1.07	Bacteroidetes	*Parabacteroides goldsteinii*
32.43	-6.19	0.66	Proteobacteria	*Polynucleobacter necessarius*
5.85	-4.89	1.06	Proteobacteria	*Nevskia sp*
6.94	-4.36	0.90	Proteobacteria	*Sphingomonas sp*
2.82	-4.19	0.88	Proteobacteria	*Limnobacter sp*
1.16	-4.10	0.85	Proteobacteria	*Methylobacterium sp*
2.24	-3.64	0.74	Proteobacteria	*Novosphingobium taihuense*
6.30	3.22	0.69	Proteobacteria	*Rheinheimera sp*
49.33	3.46	0.55	Proteobacteria	*Pseudomonas sp*
7.34	3.59	0.65	Proteobacteria	*Vogesella sp*
6.91	3.68	0.82	Proteobacteria	*Aeromonas sp*
2.17	5.13	1.11	Proteobacteria	*Leptothrix mobilis*
3.14	5.22	0.96	Proteobacteria	*Cellvibrio sp*
2.42	5.33	0.93	Proteobacteria	*Shewanella sp*
17.64	6.11	0.90	Proteobacteria	*Aeromonas sp*
4.16	6.53	0.98	Proteobacteria	*Shewanella sp*
5.78	6.62	1.04	Tenericutes	*Mycoplasma microti*

Table 4.2. Result summary for linear mixed effect models testing relationships between GM alpha-diversity and host life history traits. POOL = entire dataset (i.e. individuals from both environments), ASH = individuals reared in ASH, REF = individuals reared in the REF. Bolded values tests significant at $p \leq 0.05$, italicized values those showing trends (i.e. $0.05 < p \leq 0.1$), and the sign of the estimate denotes the direction of the relationship.

		observed richness		*Chao1 diversity*		*Shannon diversity*	
trait	environment	estimate	*p*	estimate	*p*	estimate	*p*
len.grow	POOL	0.004	0.382	0.059	0.459	0.020	0.662
	ASH	0.004	0.393	0.037	0.629	0.020	0.657
	REF	0.006	0.555	0.108	0.478	0.020	0.821
ttm	POOL	**-0.009**	**0.041**	-0.089	0.179	*-0.075*	*0.051*
	ASH	*-0.013*	*0.070*	-0.158	0.170	*-0.120*	*0.070*
	REF	-0.004	0.928	0.008	0.891	-0.003	0.928
mass	POOL	0.004	0.149	*0.066*	*0.096*	0.009	0.695
	ASH	0.001	0.615	0.047	0.284	-0.009	0.729
	REF	*0.009*	*0.052*	0.109	0.116	0.041	0.315
svl	POOL	0.002	0.124	0.030	0.123	0.008	0.503
	ASH	0.002	0.212	0.031	0.138	0.012	0.362
	REF	0.003	0.222	0.033	0.331	0.003	0.898
bci	POOL	-0.194	0.726	-0.237	0.978	-1.116	0.828
	ASH	-0.799	0.265	-8.338	0.486	*-12.536*	*0.078*
	REF	1.255	0.178	14.180	0.289	**16.805**	**0.024**
mass.grow	POOL	**0.014**	**0.009**	**0.181**	**0.033**	*0.089*	*0.073*
	ASH	**0.015**	**0.033**	*0.220*	*0.062*	*0.113*	*0.097*
	REF	0.012	0.174	0.122	0.342	0.053	0.479

Table 4.3. Results of linear mixed effect models testing relationships between GM alpha-diversity and trace element uptake in the amphibian host. POOL = entire dataset (i.e. individuals from both environments), ASH = individuals reared in ASH, REF = individuals reared in the REF. Bolded values indicate significant relationships ($p \leq 0.05$), italicized values those that were trending ($0.05 < p \leq 0.1$), and the sign of the estimate denotes the direction of the relationship.

		observed richness		*Chao1 diversity*		*Shannon diversity*	
trait	environment	estimate	*p*	estimate	*p*	estimate	*p*
As	POOL	-0.001	0.177	-0.100	0.311	-0.043	0.431
	ASH	-0.001	0.247	-0.098	0.410	-0.053	0.446
	REF	-0.001	0.621	-0.120	0.516	-0.010	0.902
Cd	POOL	**-0.003**	**0.047**	*-0.310*	*0.076*	-0.090	0.355
	ASH	*-0.002*	*0.093*	-0.268	0.148	-0.139	0.193
	REF	NA	NA	NA	NA	NA	NA
Cu	POOL	-0.001	0.235	-0.055	0.535	-0.016	0.753
	ASH	-0.001	0.482	-0.051	0.725	-0.037	0.665
	REF	-0.001	0.314	-0.046	0.643	0.029	0.615
Ni	POOL	0.001	0.189	0.144	0.284	**0.224**	**0.003**
	ASH	0.002	0.165	0.218	0.221	**0.279**	**0.006**
	REF	-0.001	0.774	-0.055	0.797	0.083	0.447
Se	POOL	-0.001	0.210	0.069	0.352	**-0.101**	**0.016**
	ASH	-0.001	0.276	-0.056	0.444	**-0.093**	**0.024**
	REF	NA	NA	NA	NA	NA	NA
Sr	POOL	-0.001	0.117	-0.079	0.223	*-0.063*	*0.095*
	ASH	-0.001	0.136	-0.140	0.138	-0.078	0.162
	REF	0.000	0.885	0.028	0.754	-0.034	0.498
Zn	POOL	0.000	0.850	0.012	0.857	-0.022	0.578
	ASH	0.000	0.904	-0.011	0.913	-0.029	0.630
	REF	0.000	0.841	0.045	0.628	-0.011	0.832

Table 4.4. Relationships between gut microbial beta-diversity and host phenotype for unweighted and weighted-UniFrac distances for all samples (POOL) and those reared in the ASH and REF environments separately. For clarity, models where $p < 0.05$ have been bolded and those where $0.05 < p < 0.10$ are italicized.

		unweighted-Unifrac			*weighted-Unifrac*		
trait	environment	F-stat	*p*	R^2	F-stat	*p*	R^2
early growth	POOL	**2.402**	**0.001**	**0.02**	**2.028**	**0.008**	**0.02**
	ASH	0.928	0.606	0.04	1.224	0.145	0.05
	REF	**1.516**	**0.036**	**0.03**	0.913	0.506	0.02
ttm	POOL	**3.137**	**0.001**	**0.03**	**4.202**	**0.001**	**0.04**
	ASH	**2.691**	**0.003**	**0.05**	**2.636**	**0.003**	**0.05**
	REF	1.162	0.196	0.02	1.264	0.206	0.02
svl	POOL	*1.342*	*0.090*	*0.01*	1.117	0.286	0.01
	ASH	0.941	0.490	0.02	0.902	0.537	0.02
	REF	1.271	0.120	0.02	0.944	0.487	0.02
mass	POOL	*1.313*	*0.080*	*0.01*	1.189	0.216	0.01
	ASH	0.655	0.951	0.01	1.077	0.360	0.02
	REF	**1.450**	**0.042**	**0.02**	1.090	0.332	0.02
bci	POOL	1.227	0.174	0.01	**2.033**	**0.007**	**0.02**
	ASH	1.057	0.313	0.02	*1.623*	*0.063*	*0.03*
	REF	1.239	0.136	0.02	1.451	0.118	0.02
growth rate	POOL	**2.402**	**0.002**	**0.02**	**2.028**	**0.011**	**0.02**
	ASH	0.849	0.794	0.03	1.160	0.216	0.05
	REF	**1.516**	**0.032**	**0.03**	0.913	0.537	0.02

Table 4.5. Results of DESeq2 analysis of microbial OTUs differentially represented in individuals in the average developmental class relative to those in the slow class and those in the slow class relative to the fast class by rearing environment. There were no OTUs differentially represented between slow and fast developmental classes in the REF environment or fast and average in either environment and thus are not shown.

Comparison	environment	baseMean	log2-fold change	padj	Phylum	Species
Average vs. Slow						
	REF	2.69	4.98	0.042	Actinobacteria	*Microbacterium sp*
		3.90	3.75	0.012	Proteobacteria	*Acinetobacter junii*
		11.47	5.57	0.012	Proteobacteria	*Nevskia sp*
		5.32	4.70	0.024	Proteobacteria	*Limnobacter sp*
		74.82	3.19	0.012	Actinobacteria	*Microbacterium sp*
		3.82	4.87	0.029	Proteobacteria	*Nevskia ramosa*
		2.84	5.31	0.015	Bacteroidetes	*Spirosoma panaciterrae*
		12.38	5.43	0.000	Proteobacteria	*Agrobacterium tumefaciens*
		4.34	5.59	0.012	Proteobacteria	*Pelomonas saccharophila*
	ASH	13.86	5.29	0.025	Proteobacteria	*Rhizobium sp*
		8.98	5.11	0.017	Proteobacteria	*Azospirillum sp*
		17.43	4.83	0.017	Proteobacteria	*Ideonella sp*
Fast vs. Slow						
	ASH	17.43	-7.53	0.008	Proteobacteria	*Ideonella sp*
		14.90	7.76	0.011	Tenericutes	*Mycoplasma microti*

Table 4.6. Relationships between gut microbial beta-diversity and host body burdens of trace elements for unweighted- and weighted-UniFrac distances for all samples (POOL) and those reared in the ASH and REF environments separately. For clarity, models where $p < 0.05$ have been bolded and those where $0.05 < p < 0.10$ are italicized.

		unweighted-Unifrac			*weighted-Unifrac*		
element	environment	F-stat	*p*	R^2	F-stat	*p*	R^2
As	POOL	**4.317**	**0.001**	**0.040**	**5.434**	**0.001**	**0.050**
	ASH	1.274	0.125	0.027	*1.672*	*0.063*	*0.035*
	REF	1.225	0.153	0.022	*1.632*	*0.072*	*0.029*
Cd	POOL	**3.790**	**0.001**	**0.035**	**4.057**	**0.001**	**0.038**
	ASH	**1.590**	**0.028**	**0.033**	0.965	0.465	0.021
	REF	NA	NA	NA	NA	NA	NA
Cu	POOL	**2.445**	**0.003**	**0.023**	**3.867**	**0.001**	**0.036**
	ASH	1.105	0.266	0.023	**1.914**	**0.029**	**0.040**
	REF	1.006	0.457	0.018	1.063	0.368	0.019
Ni	POOL	**2.194**	**0.001**	**0.021**	**2.742**	**0.001**	**0.026**
	ASH	0.999	0.392	0.021	1.271	0.200	0.027
	REF	0.924	0.576	0.017	0.983	0.432	0.018
Se	POOL	**6.520**	**0.001**	**0.060**	**5.059**	**0.001**	**0.047**
	ASH	**1.498**	**0.043**	**0.032**	**2.710**	**0.005**	**0.056**
	REF	NA	NA	NA	NA	NA	NA
Sr	POOL	**4.947**	**0.001**	**0.046**	**5.452**	**0.001**	**0.050**
	ASH	**1.703**	**0.022**	**0.036**	1.217	0.243	0.026
	REF	0.831	0.773	0.015	1.220	0.201	0.022
Zn	POOL	**2.306**	**0.002**	**0.022**	**1.937**	**0.004**	**0.018**
	ASH	0.752	0.722	0.016	0.898	0.636	0.019
	REF	1.233	0.208	0.022	1.217	0.178	0.022

Table 4.7. Results of DESeq2 analysis of microbial OTUs differentially represented between trace element body burdens (Low, Average, High) of individuals reared in the ASH environment. Only elements that had OTUs differentially represented between two groups are included.

Comparison	Element	baseMean	log_2-fold change	padj	Phylum	Species
Average vs. High	*As*	6.33	5.57	0.042	Proteobacteria	*Blastomonas natatoria*
		5.81	5.09	0.049	Proteobacteria	*Sphingomonas sp*
		14.60	5.07	0.036	Proteobacteria	*Rhizobium sp*
		1.71	5.07	0.036	Proteobacteria	*Methylobacterium sp*
		1.91	4.42	0.042	Proteobacteria	*Pseudomonas nitroreducens*
		4.32	3.97	0.036	Proteobacteria	*Agrobacterium sp*
		27.95	3.29	0.036	Actinobacteria	*Microbacterium sp*
	Ni	66.01	7.43	0.004	Proteobacteria	*Chelatococcus asaccharovorans*
	Zn	7.17	5.66	0.007	Proteobacteria	*Ideonella sp*
		4.35	5.44	0.024	Proteobacteria	*Pseudoxanthomonas mexicana*
		4.90	5.28	0.031	Proteobacteria	*Xylophilus ampelinus*
		3.78	5.28	0.024	Proteobacteria	*Reyranella massiliensis*
		9.50	5.08	0.024	Proteobacteria	*Azospirillum sp*
		10.89	4.48	0.037	Proteobacteria	*Sphingomonas sp*
		12.36	4.39	0.025	Proteobacteria	*Agrobacterium tumefaciens*
		16.22	4.25	0.022	Proteobacteria	*Pseudacidovorax intermedius*
		18.53	3.31	0.025	Proteobacteria	*Nevskia ramosa*
Low vs. High	*Sr*	5.80	-6.96	0.007	Tenericutes	*Mycoplasma microti*
Low vs Average	*Sr*	303.99	7.49	<0.001	Proteobacteria	*Chelatococcus asaccharovorans*

FIGURES

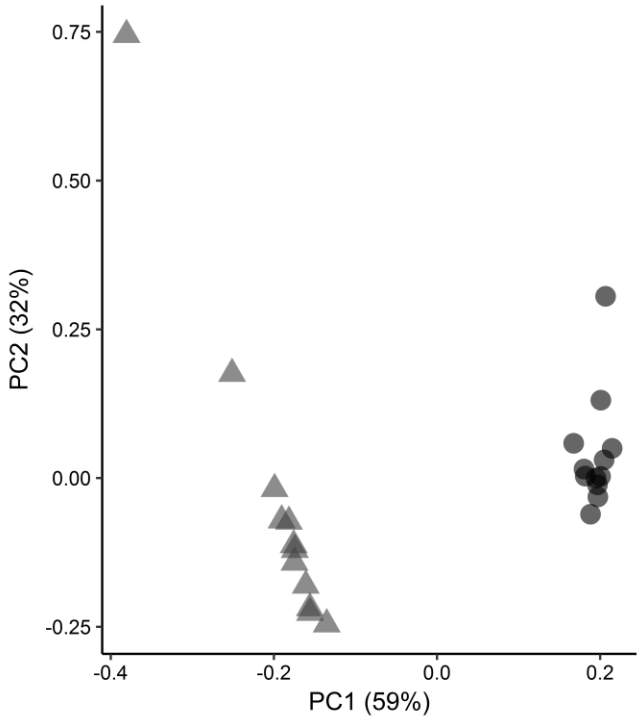

Figure 4.1. Plot of PC scores from environmental water samples taken from the REF (black circles) and ASH (gray triangles) rearing environments. PC1 primarily describes between environment differences, while PC2 accounts for mostly within environment variation.

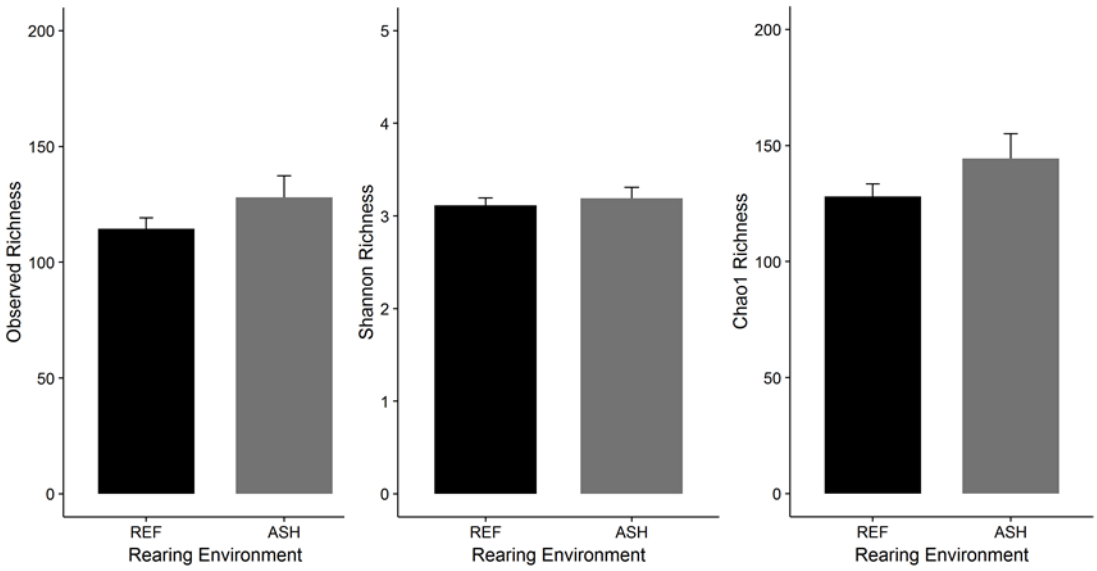

Figure 4.2. Plots of metamorphic toad gut microbial community mean (± SE) alpha diversity metrics by site. Black bars represent individuals reared in the REF environment and those in gray individuals reared in the ASH environment.

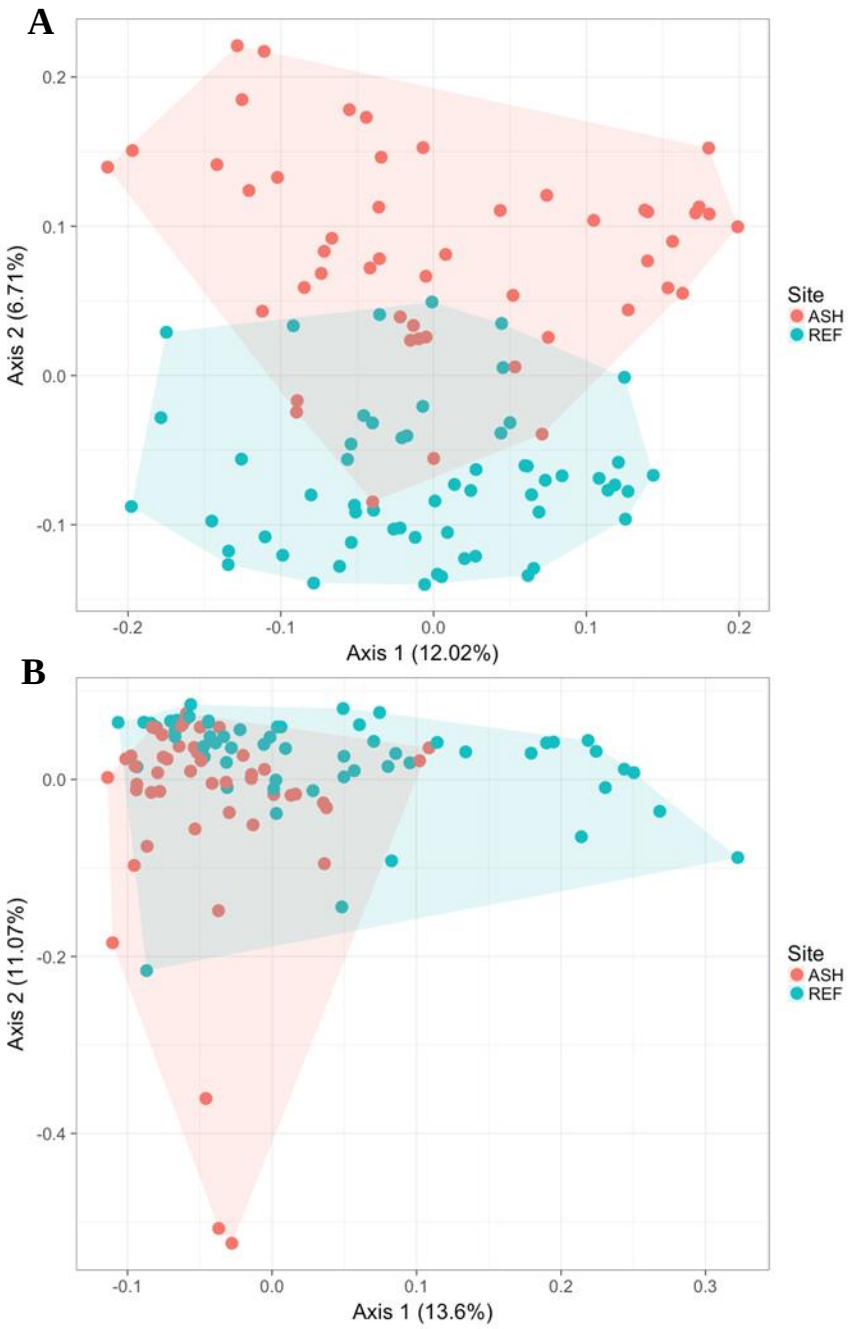

Figure 4.3. Principal coordinates analysis (PCOA) plots using unweighted- (A) and weighted-UniFrac (B) distance measures of gut microbial community similarity. Blue data points and polygons represent individuals reared in the REF environment and those in red individuals reared in the ASH environment. Percentages listed on x- and y-axes are the % variance explained by principal coordinate axes 1 and 2 respectively.

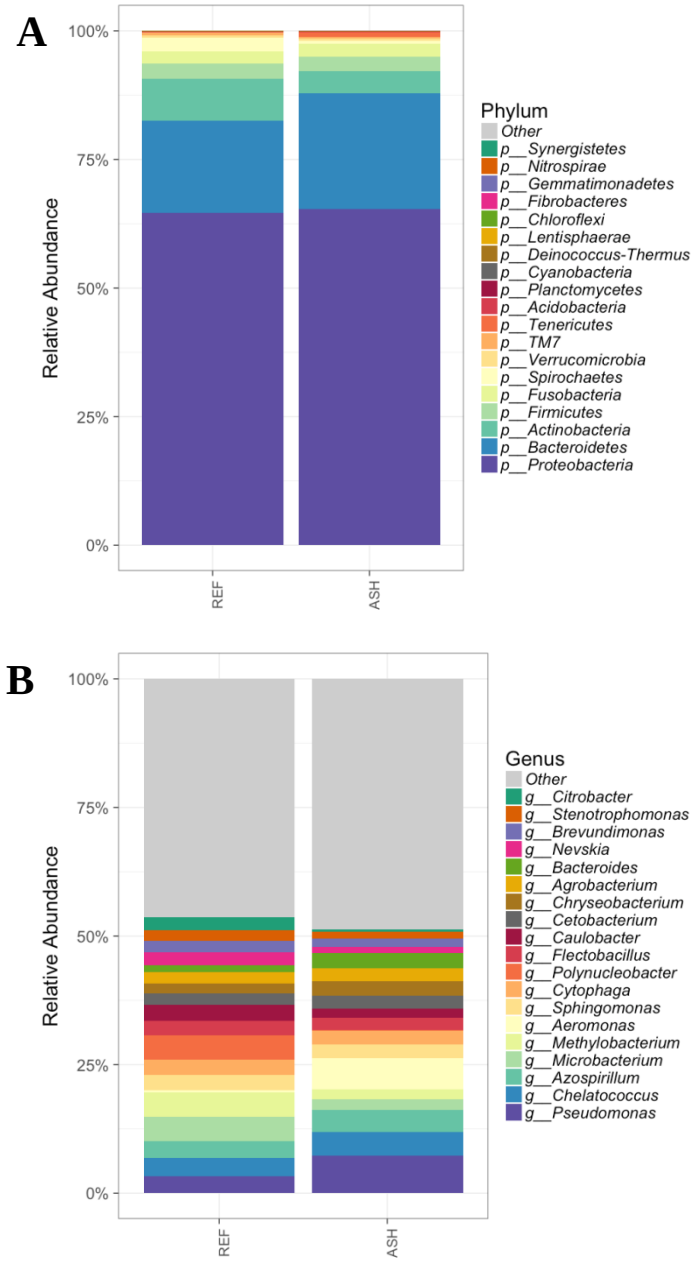

Figure 4.4. Relative abundances of the 19 most abundant gut microbial A) phyla and B) genera in metamorphic toads pooled by rearing environment (REF_N = 59, ASH_N = 50).

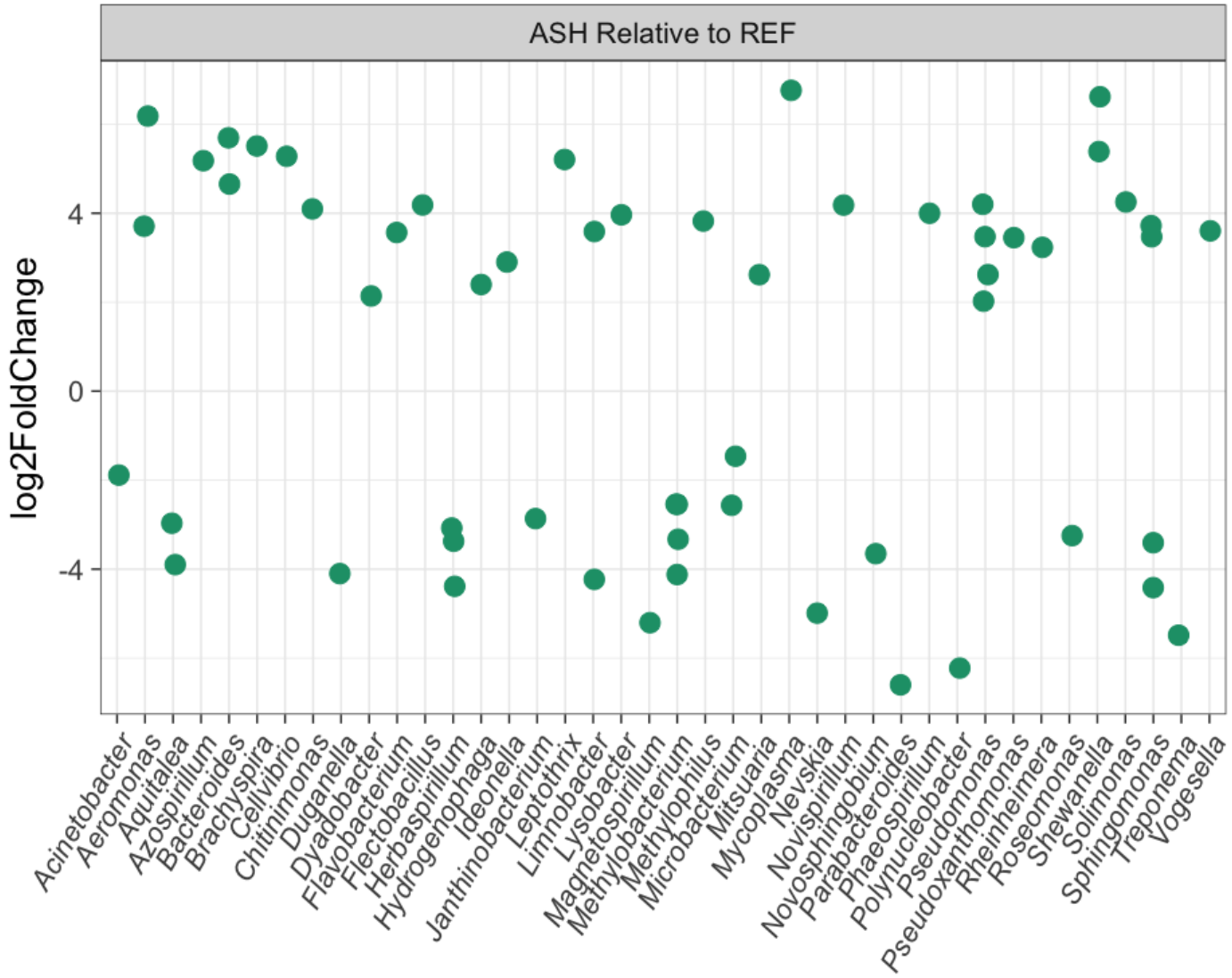

Figure 4.5. Plot of OTUs differentially represented between rearing environments, grouped by genera, based on DESeq2 results.

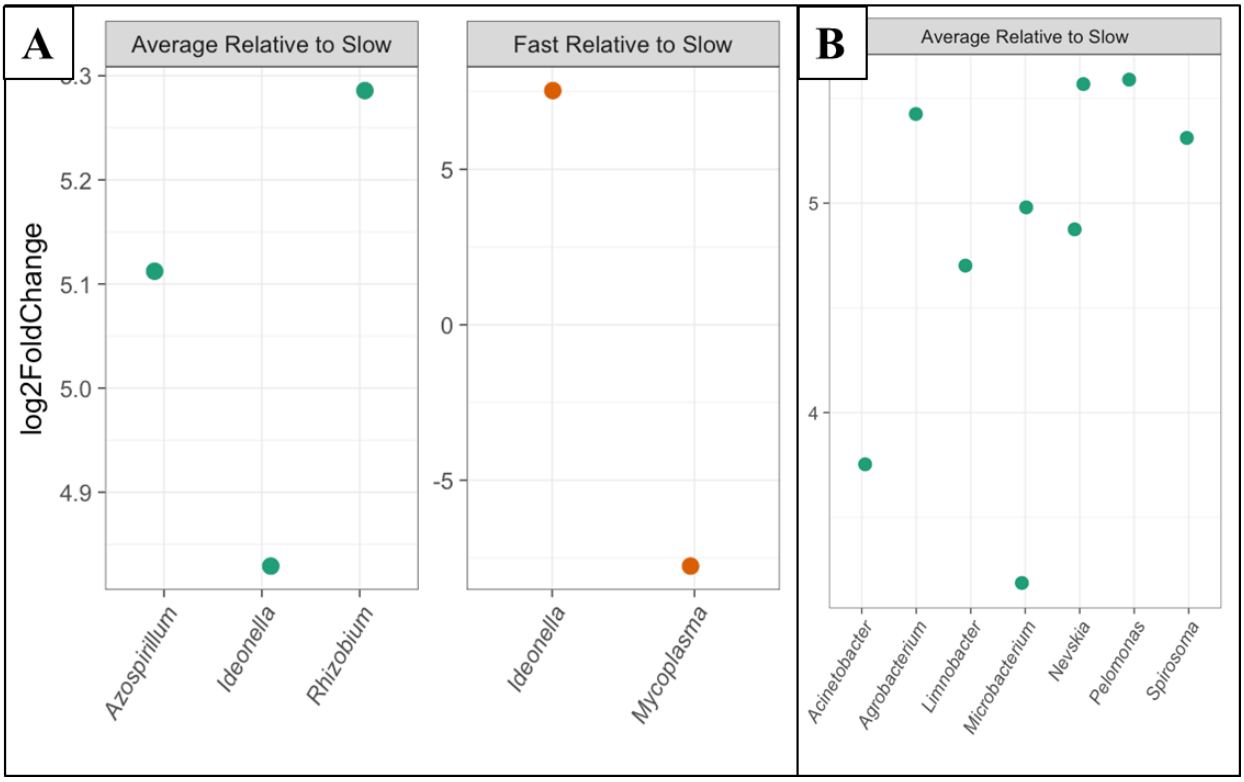

Figure 4.6. Log2-fold change in relative abundance of bacterial OTUs between developmental rate classes of toads reared in ASH (A) and REF (B).

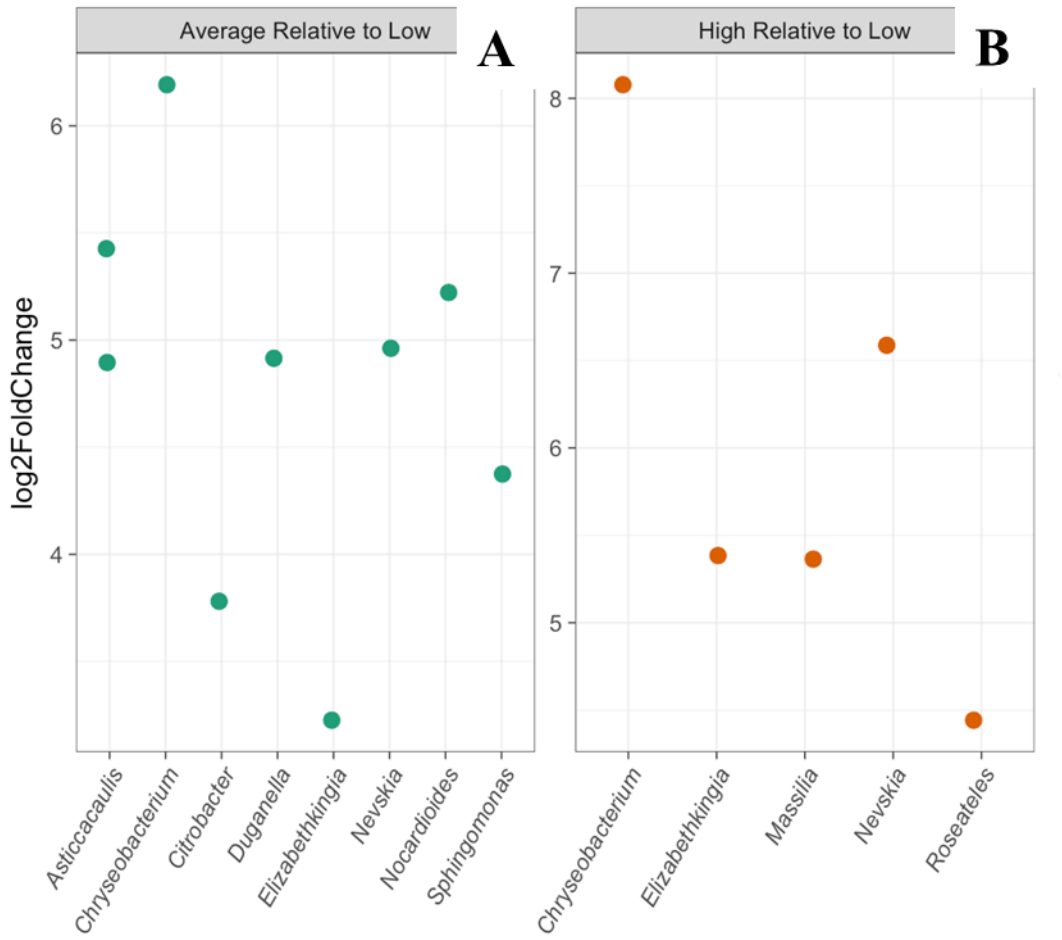

Figure 4.7. Log2-fold change in abundances of OTUs differing significantly in their abundance between toads reared in the REF environment varying by A) average vs. low and B) high vs. low mass at metamorphosis classes.

CHAPTER 5

PATTERNS OF METAL TOLERANCE AMONG AMPHIBIAN POPULATIONS IN A HETEROGENEOUS LANDSCAPE[4]

[4] R. Wesley Flynn, Cara N. Love, Austin Coleman, & Stacey L. Lance. To be submitted to *Environmental Pollution*

ABSTRACT

Human activities have radically shaped the global landscape, affecting the structure and function of ecosystems. Habitat loss is one of the most visible changes to the landscape and a primary driver of species declines; however anthropogenic environmental contamination also negatively affects population persistence, but is not readily observed. Aquatic organisms are especially susceptible to chemical perturbations, which can negatively impact survival and fitness related traits. Some populations have evolved tolerance to chemical stressors, which could mitigate the consequences associated with contamination. Amphibians are a group at particular risk to aquatic chemical stressors due to their reliance on wetlands for reproduction and larval development. We designed our study to examine whether amphibian offspring from ponds containing elevated levels of heavy metals are more tolerant to heavy metal exposure than those from ponds with no history of contamination. Considering many of the most common trace elements elicit acute toxicity by disrupting osmotic- and ionic-regulation, we hypothesized that alterations to these aspects of physiology resulting from multigenerational exposure to trace element mixtures would be the most likely routes by which tolerance would evolve. We used copper (Cu) as a proxy for heavy metal exposure because it is a widely distributed aquatic stressor known to cause osmotic stress that can also cause mortality at levels commonly encountered in the environment. We found considerable within and among population variation in Cu tolerance, as measured by time to death. Larvae from populations exposed to trace element mixtures, comparable to those in an environment occupied by toads previously established to be more tolerant to a mixture of trace elements (Chapter 2), were not more tolerant to Cu than those from reference sites. However, larvae from a population inhabiting a constructed wetland complex with exceptionally high Cu levels were significantly more tolerant. This wetland

complex has only been in place for <20 years, thus if elevated Cu tolerance in this population is due to selection in the aquatic habitat, such adaptation may occur rapidly (i.e. ~10 generation). Our results provide evidence that amphibians may be able to evolve tolerance in response to trace element contamination, though such tolerance may be specific to the combination of contaminants present.

INTRODUCTION

Environmental contaminants are widespread in aquatic habitats and pose a significant threat to aquatic life, including amphibians (Sparling, Linder, Bishop, & Krest, Sherry, 2010). These chemical stressors can negatively impact growth, development, and survival (Relyea & Hoverman, 2006; Sparling et al., 2010) and could lead to population declines (Salice, Rowe, Pechmann, & Hopkins, 2011; Todd, Bergeron, Hepner, & Hopkins, 2011). While aquatically respiring organisms have also evolved complex physiological pathways to cope with natural variation in water chemistry, such as altered salinity or pH (Krogh, 1939; Whitehead, Roach, Zhang, & Galvez, 2011), chemical perturbations associated with anthropogenic contamination can create novel chemical conditions organisms have not experienced in their evolutionary history (Sih, Ferrari, & Harris, 2011). Although contaminants represent relatively recent aquatic stressors, some species have been able to evolve tolerance (Hua, Morehouse, & Relyea, 2013; Klerks & Levinton, 1989; Klerks & Weis, 1987; Meyer & Di Giulio, 2003; Xie & Klerks, 2004) or acclimate (Herkovits & Pérez-Coll, 2007; Hua, Morehouse, et al., 2013; Tate-Boldt & Kolok, 2008) to these perturbations. Both acclimation and adaptation provide mechanisms by which populations can reduce the negative effects associated with elevated levels of contaminants;

however the conditions under which these mechanisms can ameliorate toxicity are not well established.

Trace elements (TEs, i.e. heavy metals and metalloids) are environmental contaminants of particular concern to aquatic life and are commonly associated with human activities such as agricultural practices, urban runoff, mining, and energy production (Gimeno-García, Andreu, & Boluda, 1996; Rowe, Hopkins, & Congdon, 2002; Scholes, Shutes, Revitt, Forshaw, & Purchase, 1998). Management strategies are often employed to reduce the risk of TE contamination of natural waterways. One common strategy is the construction of artificial wetlands capable of removing significant quantities of metals before the water is discharged into surrounding waterways. However, these strategies also create localized hotspots containing high levels of the very elements being managed. Though many TEs are essential for normal biological function within a narrow range of concentrations, they can quickly become toxic at slightly elevated levels (Goldhaber, 2003). Thus, these bodies of water provide a permanent source of freshwater that is attractive to wildlife, which are put in direct contact with the potentially toxic levels of contaminants (Bryan Jr, Hopkins, Parikh, Jackson, & Unrine, 2012; Haskins et al., 2017).

Trace elements are diverse and elicit toxicity via several different mechanisms (Ercal, Gurer-Orhan, & Aykin-Burns, 2001; Fragou, Fragou, Kouidou, Njau, & Kovatsi, 2011; Hall, 2002). In aquatic organisms, one of the primary mechanisms by which TEs induce acute toxicity is by impairing osmoregulation (Brooks & Mills, 2003; Grosell, Nielsen, & Bianchini, 2002; McGeer, Szebedinszky, McDonald, & Wood., 2000; Saglam, Atli, & Canli, 2013; Wendelaar Bonga & Lock, 1992). Tolerance to high levels of TE stressors in aquatic organisms is often associated with maintenance of internal sodium levels (Brown, Dobbs, Snodgrass, & Ownby, 2012; Grosell et al., 2002; Lauren & McDonald, 1987a; Tate-Boldt & Kolok, 2008), which is

critical for neural transmission, muscle action and other critical processes (Catterall, 1992). Thus, an ability to maintain internal sodium levels may confer tolerance to multiple TE contaminants, even without prior exposure to specific elements. The majority of studies examining mechanisms of TE toxicity and tolerance have been conducted in fish; however these mechanisms are expected to apply to developing amphibian larvae as well (Brown et al., 2012).

Trace elements are regularly found in amphibian habitats at levels that can elicit toxicity (Fedorenkova et al., 2012) and thus have the potential to act as potent agents of selection. A number of studies have documented considerable variation, within and among populations, in TE tolerance (Flynn, Scott, Kuhne, Soteropoulos, & Lance, 2015; Lance et al., 2012; Lance, Flynn, Erickson, & Scott, 2013). Additionally, our recent work (Flynn et al. Chapter 2) demonstrates that some of that variation is heritable and that amphibians can adapt to heavy metal contaminants. This is not surprising given that adaptive evolution in amphibians has been observed in response to pesticides (Cothran, Brown, & Relyea, 2013; Hua, Morehouse, et al., 2013) and anthropogenic acidification (Räsänen, Laurila, & Merilä, 2003). However, TEs often occur in mixtures that may constrain adaptation. In our study the population appeared to adapt to a mixture of TEs by maintaining normal internal sodium and potassium levels (Chapter 3). Such adaptation to contaminants may mitigate costs associated with exposure, but the consequences of such acquired tolerance are mixed. For example, adaptation to chemical stressors can be associated with reductions in fitness in the absence of those stressors (Chapter 2; Groeters, Tabashnik, Finson, & Johnson, 1994; Shirley & Sibly, 1999; Xie & Klerks, 2004). On the other hand, in some cases adaptive tolerance to one stressor can also confer tolerance to other environmental stressors (Hua, Cothran, Stoler, & Relyea, 2013; Karel AC De Schamphelaere &

Jana Asselman, Marlies Messiaen, Dieter De Coninck, Colin R Janssen, John K Colbourne, 2011) which may essentially pre-adapt populations to future stressors.

In addition to adaptation, phenotypic plasticity, or acclimation, may play a role in determining susceptibility to contaminants. Exposure to low levels of chemical stressors early in development has been repeatedly shown to increase tolerance when confronted with exposure later in life (Herkovits & Pérez-Coll, 2007; Hua, Morehouse, et al., 2013; Lauren & McDonald, 1987b; Wu, Yang, Lee, Gomez-Mestre, & Kam, 2014). Maternal transfer of contaminants to amphibian egg masses has been associated with reduced hatching success (Metts, Buhlmann, Tuberville, Scott, & Hopkins, 2013; Steen, Van Dyke, Jackson, & Hopkins, 2015) and an increased incidence of developmental malformations (Hopkins, DuRant, Staub, Rowe, & Jackson, 2006; Todd, Bergeron, Hepner, Burke, & Hopkins, 2011). However, it may also serve as a source of early exposure to contaminants and result in enhanced tolerance later in life. This aspect of maternal transfer has not been examined and could confound the relative roles of acclimation and genetic adaptation. Given that amphibians are a group of conservation concern whose global population declines have been contributed to by environmental contaminants (Collins & Storfer, 2003; Fedorenkova et al., 2012) it is critical to determine their potential for adaptation and/or acclimation to contaminants.

In our previous work we demonstrated that a population of amphibians with a history of exposure to a suite of TEs had diverged phenotypically from a nearby population with no history of exposure (Chapter 2). The exposed population appeared adapted to TEs and demonstrated an ability to maintain normal sodium and potassium levels when exposed to the suite of contaminants found in their natal pond (Chapter 3). Adults from this population transfer TEs they have accumulated to their egg masses (Metts, Buhlmann, Scott, Tuberville, & Hopkins,

2012; Metts et al., 2013) and thus tolerance may result from early exposure and acclimation. Female body burdens and subsequent transfer to egg masses varies significantly within this population (Metts et al., 2013). Thus, if tolerance to TEs is solely due to early exposure and acclimation we would predict tolerance to be tightly linked to maternal transfer. Alternatively, if the population is genetically adapted to TEs then maternal transfer may not be related to tolerance. Either acclimation or adaptation could have resulted in the ability to maintain sodium and potassium levels in the presence of TEs. Thus, we hypothesized that the population would display elevated tolerance to a common TE stressor (Fedorenkova et al., 2012), copper (Cu) to which it had not been previously exposed. We tested this hypothesis by assessing tolerance to acute metal stress within and among populations inhabiting sites varying in their history of TE contamination. Our objectives were to 1) test whether offspring from environments containing elevated levels of TE stressors are more tolerant to aquatic metal exposure and 2) determine whether variation in maternal transfer of TEs influences offspring susceptibility to metal stress.

Study populations and sites

We collected adult toads in breeding condition and egg masses laid within 24 h from wetlands on the United States Department of Energy's Savannah River Site near Aiken, SC from April – June of 2016 and 2017 (Figure 5.1). Three wetlands had histories of contamination with TEs: the ash plume wetland (APW, (Metts, Buhlmann, Scott, Tuberville, & Hopkins, 2012), Dunbarton bay (DUN), and the A-01 constructed wetland complex (A01). Both APW and DUN received untreated coal combustion wastes (APW: 1970s, DUN: 2000s), which have resulted in elevated levels of TEs in the sediments and water column (notably As, Cu, and Se, Hopkins et al. 2000; Lance et al. 2012). A01 is a wetland complex constructed in 2000 to remediate high levels of heavy metals (i.e. Cu, Zn, and Pb) from industrial wastewater (Knox et al., 2006). During the

collection of adult toads for breeding, some were caught on a road 250m from the A01 complex while moving in the direction of the wetlands. We assumed these individuals were migrating to A01 which had the only breeding chorus at the time; however we assigned them to a separate population (ROAD) and tested that survival in Cu did not differ between A01 and ROAD (see *Statistical analysis*). Sites with no known history of contamination were Rainbow bay (RB), Flamingo bay (FB), and Linda's pond (LP).

Adult toads were brought into the laboratory and induced to breed by injecting adult males and females with 250 and 1,000 IU of human chorionic gonadotropin (hCG, Sigma-Aldrich #CG10) respectively. We paired male and female toads from each collection haphazardly and placed them in plastic bins containing ~3 L of laboratory prepared soft water (US Environmental Protection Agency, 2002). We left them overnight to breed and collected any resulting eggs the next morning. When we collected egg masses directly from a wetland, we rinsed them thoroughly in soft water to remove any debris prior to any further processing. Additionally, in 2016 we clipped the two toes of each adult for TEs analyses. We photographed a subsample of 10 eggs from each clutch to determine mean egg size (i.e. diameter in mm), to account for possible maternal effects on survival associated with provisioning (Dziminski & Roberts, 2006; Laugen, Laurila, & Merilä, 2002; Loman, 2002). Another subset of eggs (~200) from each clutch was rinsed in ultra-pure water, and transferred to Whirl-Paks® for analysis of major and trace elements. After processing eggs, we measured (snout-vent length, SVL) and weighed adults.

Experimental design

All animals were held in the University of Georgia's Savannah River Ecology Laboratory's (SREL), climate controlled, animal care facility. We maintained a 16:8 light:dark

cycle and constant temperature of 24^{o}C (+/- 1.5). We divided clutches into small sections (<20 eggs/section) and distributed them among 1 L containers holding soft water (US Environmental Protection Agency, 2002) to standardize densities of ~ 200/L. Eggs were allowed to develop in these containers for five days, until all normally developing animals had reached the free-swimming and –feeding stage (Gosner stage 25 [GS 25]). We then pooled larvae from a given clutch prior to allocation to experimental units. Larvae from clutches were initially distributed among several containers to maintain a high rate of successful hatching and survival through GS 25, but pooled prior to allocation to experimental units to ensure our sampling was as representative as possible of the entire clutch.

To examine within- and among-population variation in susceptibility to Cu, we kept all clutches separate and exposed them to two treatments: a control treatment (0 ppb Cu) and a 50 ppb Cu treatment. We determined from previous studies (Lance, Flynn, Erickson, & Scott, 2013) and preliminary tests that 50 ppb Cu was the optimal dose of Cu to elicit a distribution of times-to-death over 48 h. To assess Cu tolerance, we haphazardly distributed 10 normally developing GS 25 larvae from each clutch into experimental units. Each population x clutch x treatment combination was replicated 4 (2016) or 5 (2017) times. All experimental units fit on the same rack in our animal care facility. We stratified replicates vertically to account for any spatial variation in temperature, with replicates 1-2, 3-4, and 5 grouped on shelves.

The exposures lasted a total of 48 hours and after 24 h we performed 100% water changes with renewed Cu solution. We made observations every two (2-12 and 26-32h) or four (16-24 and 36-48 h) hours, during which we counted surviving individuals and counted and removed any dead individuals. To determine how actual Cu concentrations varied over the course of exposures relative to the nominal concentrations prepared, we collected samples of

treatment water at the beginning, the end of 24 h, the new solutions refilled at 24 h, and at the conclusion of each trial.

Tissue and sample preparation and analysis

Water samples taken from experimental Cu trials, were acidified to 1% HNO_3 (JT Baker #9598) to determine exact Cu concentrations over the course of exposures. We quantified trace element levels in subsets of each egg mass by lyophilizing them and transferring 12-18 mg to trace metal free 15 mL conical tubes (VWR® #89049) and adding 400 µL of HNO_3, before digesting on a heat block at 80^{o}C for 2 h. In addition to egg mass samples, we also included standard reference material (TORT-3, Canadian National Resource Council) and blanks for quality control and to determine minimum detection limits. All samples were checked to ensure they had fully digested prior to diluting with 5.6 mL of ultra-pure water to achieve a final concentration of 6.67% HNO_3. We analyzed diluted samples using inductively coupled plasma-mass spectroscopy (Nexion 300X ICP-MS; Perkin Elmer, Norwalk, CT, USA). We used external calibration standards (High-Purity Standards, Charleston, SC, USA) encompassing a range of 0.5 -500 µg/L for all elements analyzed.

Statistical analysis

To determine how tolerance to Cu differed among populations and population-types (with [METAL] or without history of contamination [REF]), we used a combination of linear mixed effect models (*lmer*, Bates, Machler, Bolker, & Walker, 2015) and Cox proportional hazard analyses (*coxph* Therneau, 2011). The response variables were time to death (in hours) and a binary value indicating whether an individual survived through the study (0) or died (1). We included population and clutch identity as fixed predictors and used a clustering term for each experimental unit to account for the fact that individuals sampled from within an

experimental unit were not independent. We ran an initial model including only offspring from the A01 and ROAD sites to test for differences in survival. Survival models indicated there were no significant differences between offspring from these two sites ($p = 0.215$) and thus offspring from both were included in the full models as A01.

As the health and size of adult female toads from which offspring were obtained could influence offspring tolerance to aquatic stressors (Räsänen, Laurila, & Merilä, 2003b, 2005), we tested for differences among populations of adult mass and snout vent length and mean egg size of each clutch and relationships of these traits to survival probability and linear and Cox proportional hazard models respectively. Because egg size has been found to be associated with some amphibian populations that have adapted to chemically stressful environments (Räsänen et al., 2005), we initially included this as a covariate in linear models of probability of mortality and time-to-death; however, we found no relationships approaching significance in any models and thus dropped it from the final analyses reported above.

RESULTS

Phenotypic variation in parental animals

Overall tolerance to environmental stressors can be influenced by the provisioning of resources to offspring, which is determined in part by the size of females (Räsänen, Laurila, & Merilä, 2003b, 2005). Sizes of adult females used to generate offspring for the study did not differ between years ($p = 0.496$) and were generally similar among populations with the exception that females from FB were significantly smaller than those from other populations ($p = 0.034$). Embryo size was not associated with female size, but differed between populations ($p =$

0.003) such that embryos from APW were smaller than those from A01 ($p = 0.001$), but there were no differences among the other populations.

Trace element levels in egg masses among populations and site types

Levels of arsenic (As), Cu, nickel (Ni), selenium (Se), strontium (Sr) and zinc (Zn) in toad egg masses varied within and among sites. The highest levels of As were found in egg masses from APW and A01 females, while RB and LP had the lowest levels (Fig 5.1A). Egg masses from the A01 site had the highest Cu levels (Fig. 5.1B), suggesting the Cu the wetlands were designed to remediate is accumulating in the terrestrial food web. Nickel levels were similar across all sites except for APW, which had a mean concentration 250-500% greater than egg masses from the other sites (Fig. 5.1C). Concentrations of Se varied within and among reference (REF) and contaminated (METAL) site types. The APW and DUN females produced egg masses with the highest Se concentrations, while those from A01 and reference sites had similarly low levels (Fig. 5.1D). Strontium in egg masses was by far the greatest in eggs from the APW site and lowest from RB, while those from all other sites were similar (Fig. 5.1E). Eggs from APW stood out as having the lowest Zn concentrations and concentrations among all other sites were similar (Fig. 5.1F).

Metal tolerance among populations and site types

Acute metal tolerance was assessed by examining variation in probability of mortality. We used both linear models including individual mortality or time-to-death, as well as survival analyses incorporating both metrics. We observed >99% survivorship in all of the controls (0 ppb Cu) indicating there were no inherent differences between clutches or populations in innate ability to survive over the 48-h exposure period. In all cases, mortality was greater in the Cu treatment than the Cu-free control for all populations and clutches.

Comparisons of Cu tolerance between individuals from populations with and without environmental exposure to TEs revealed differences between exposure history and tolerance. Probability of mortality was nearly 10% greater and time-to death was 4 h earlier in offspring derived from sites with no history of TEs contamination (Fig. 5.2A). Time-to-death varied among populations, however the effect was only bordering on statistical significance (Fig. 5.2B, $p = 0.052$). Pair-wise comparison of time-to-death among populations revealed no significant differences, but mean time-to-death was greatest in APW, DUN, and A-01 populations and lowest in two of the REF populations (LP and FB). Time-to-death and survivorship were not significantly influenced by concentrations of any of the TEs we examined in egg masses or the size or body condition of female toads.

Survival analysis generally supported the results of linear mixed effect models and further, took into account the probability of mortality at the individual level after accounting for whether death occurred and the time to death. The hazard of mortality for offspring derived from TE contaminated sites in 50 ppb Cu was 75% that of those derived from sites with no history of contamination (Fig. 5.3, exp[coef] = 0.76, $p = 0.007$). This demonstrates that population exposure history significantly affects larval survival probability in the presence of a metal stressor. This was supported by models examining individual populations. Using RB for the baseline hazard, only the A-01 population had a significantly lower hazard of mortality (exp[coef] = 0.66, $p = 0.004$; Fig. 5.4). No other populations differed significantly from RB; however, mean hazard ratios were less than 1 for all TE contaminated sites.

We should note that sample sizes between populations differed considerably among populations, but was overall similar between population types (see Appendix Table D1). Notably, RB and APW had the most within population replication (22 and 30 respectively),

while DUN, FB, and LP had only minimal replication (3, 5, and 3 respectively). Considering the extensive within population variation observed in the well replicated RB and APW populations, Cu tolerance estimates among populations should be interpreted cautiously. However, reduced sample sizes are also accounted for with more uncertainty in confidence intervals, thus increasing sample sizes would likely produce more population differences.

DISCUSSION

Adaptation to anthropogenic contamination has been repeatedly documented (Hangartner, Laurila, & Räsänen, 2011; Harrison, Ceri, & Turner, 2007; Jasieniuk, Brûlé-babel, & Morrison, 1996; Klerks & Weis, 1987); however, the potential for amphibians, a globally imperiled taxa, to evolve tolerance to such chemical disturbances is unclear. Prior to our study (Chapter 2), cases where adaptive tolerance to chemical stressors has been observed or suggested in amphibians had been limited to single stressor systems (i.e. NaCl, Gomez-Mestre and Tejedo 2003; Brady 2012) and organic pesticides (Cothran, Brown, & Relyea, 2013). Trace element contaminants, including heavy metals and metalloids, are common contaminants in aquatic habitats globally and toxic to amphibians at low concentrations (Fedorenkova et al., 2012; Sparling, Linder, Bishop, & Krest, Sherry, 2010). Unlike other studies, which have found acquired tolerance to one stressor can confer tolerance to other stressors (Hochmuth, De Meester, Pereira, Janssen, & De Schamphelaere, 2015; Hua, Cothran, Stoler, & Relyea, 2013; Plautz, Guest, Funkhouser, & Salice, 2013), our results suggest that adaptation to TE contaminated environments may be more dependent on the specific levels and mixtures of elements present.

Though, a number of TEs elicit toxicity to aquatic organisms via similar mechanisms, the patterns of tolerance we observed suggest that adaptive tolerance is unique to specific

contaminants. Offspring from A01 were the most Cu tolerant of all the populations we examined. The A-01 constructed wetlands from which this population was sampled, is characterized by Cu levels 10x greater than any of the other sites we sampled (Hopkins, Congdon, & Ray, 2000; A. S. Knox et al., 2006; Lance, Seaman, Scott, Bryan, & Singer, 2012; Roe, Hopkins, Durant, & Unrine, 2006; Soteropoulos, Lance, Flynn, & Scott, 2014). The fact this site was constructed in the last 20 years suggests that exposure to very high Cu levels during susceptible aquatic life stages could lead to the development of tolerance in as few as ten generations (assuming conservative estimate of two years to first reproduction; D. Scott *pers comm*).Though levels of dissolved Cu in the A-01 wetlands (25.8 ug/L) is above that which has been shown to cause mortality in larval southern toads in laboratory toxicity studies (Lance, Flynn, Erickson, & Scott, 2013), toxicity is dependent on dissolved organic carbon (DOC) levels (Di Toro et al., 2001), which would be expected to mitigate toxicity by decreasing the bioavailability of Cu and other TEs. Levels of DOC, however are considerably lower than those common in natural wetlands in the area (i.e. 3.9 vs. 24 – 97 mg/L; Unrine et al. 2005; Knox et al. 2006b) and thus may be insufficient in reducing the toxicity of the high levels of Cu in the system.

Interestingly we did not see higher Cu tolerance in the APW and DUN populations. Unlike A01, these sites are characterized by complex mixtures of trace element metals and metalloids. Though these systems are not especially elevated in Cu, several of the constituents in these systems have been shown to impair ionic- or osmoregulation in aquatic organisms (e.g., Ag, Al, Cd, Cr, Hg, Ni, Zn ; Lignot et al. 2000). The APW site is within 500 m of the ash basins from which we previously sampled a population of toads (Chapter 2) that were more tolerant to the high levels of TEs in the basins than a reference population. Further, we were able to determine that larvae from the ash basins achieved tolerance, at least in part, to maintaining

normal sodium and potassium levels when exposed to coal combustion wastes (Chapter 3). Yet, this ability did not confer tolerance to Cu. We have not examined the elemental makeup of larvae from this study and thus cannot determine whether they were able to maintain ionic balance in the presence of Cu. The lack of elevated tolerance to Cu relative to other populations we tested could suggest either adaptive tolerance is specific to each trace element (i.e. APW and DUN have Cu levels closer to reference sites) or that tolerance to chronic exposure in the field may not be indicative of tolerance to acute exposure. Future studies could achieve a greater understanding of the conditions under which adaptive tolerance is possible in the field by incorporating a range of native species varying in their life history traits, quantifying physiological responses to TEs exposure between tolerant and susceptible populations, and testing tolerance over the full course of aquatic development.

While we did not find maternal effects significantly influenced tolerance in our study, egg masses from the Cu tolerant A01 population did have the highest observed Cu concentrations. Exposure to chemical stressors early in development can provide protection against those stressors later in life (Herkovits & Pérez-Coll, 2007; Hua, Morehouse, et al., 2013; Tate-Boldt & Kolok, 2008). The fact we did not observe a significant relationship between Cu transferred to eggs and tolerance lends evidence to the idea that the tolerance we observed in the A01 population is the result of multigenerational selection for Cu tolerance in this system. However, given the Cu levels in the eggs of this population were overall greater than those from less Cu-tolerant populations, the possibility remains that maternally derived trace element exposure early in development could contribute to larval tolerance.

Time-to-death studies are commonly applied to obtain estimates of tolerance to acute chemical stressors (Newman, 2013) and have been useful to rapidly test multiple populations due

to their short duration and simplified experimental design. However, some studies examining the effects of aquatic TEs on larval amphibians have found mortality and sublethal effects do not always manifest until later in development (Gutleb, Appelman, Bronkhorst, Berg, & Murk, 1999). Thus, while these techniques can provide insight into general patterns of tolerance to acute contaminant exposure, they do not account for ecologically relevant effects that may not emerge until later in development. Our study adds to the growing literature suggesting evolutionary rescue could facilitate population persistence in contaminated environments, but further work will be needed to determine whether acute tolerance translates into improved survival to metamorphosis in the field and how adaptation to chemical stressors could affect population persistence.

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FIGURES

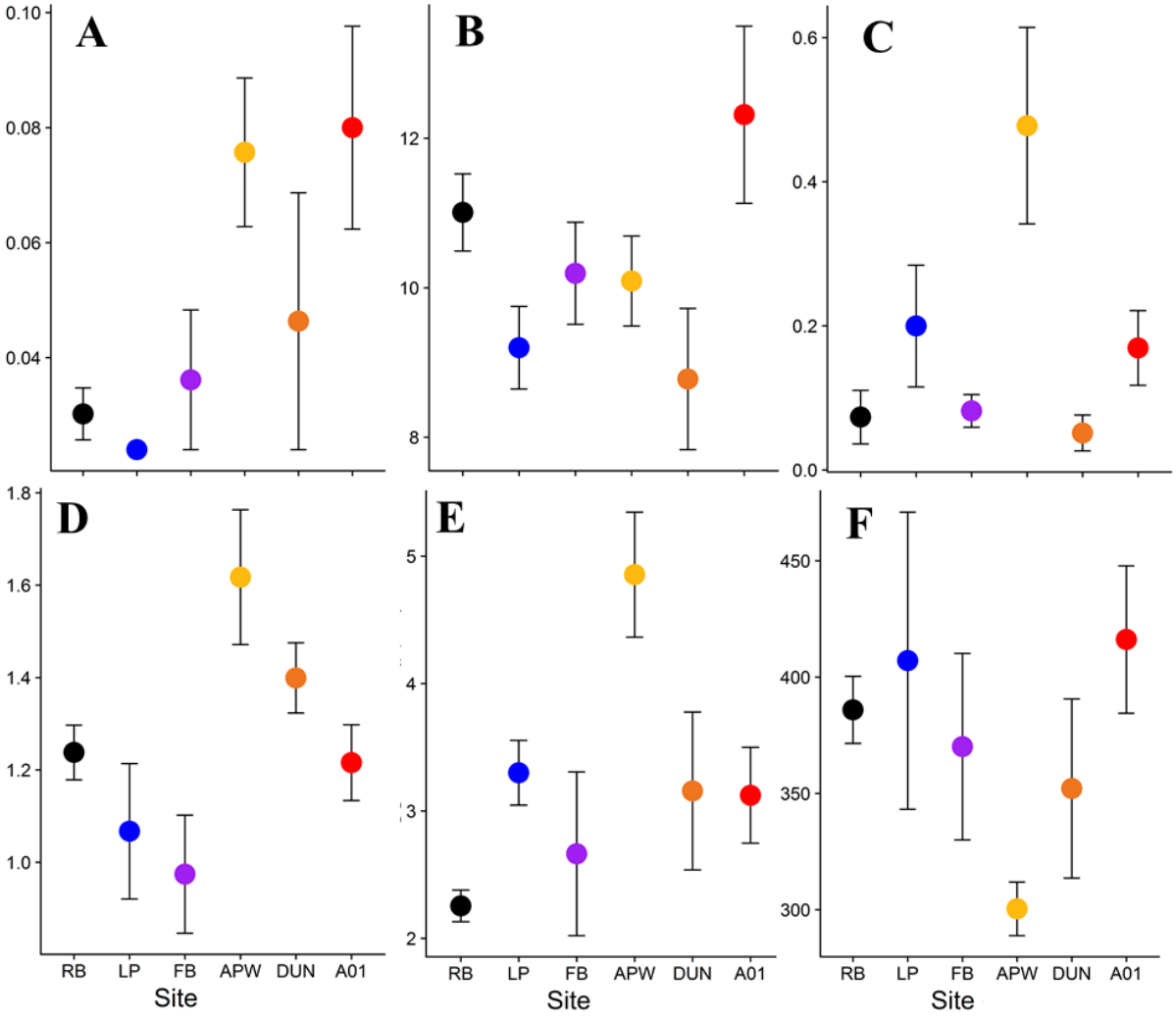

Figure 5.1. Plots of mean (ppm +/- SE) concentrations of A) As, B) Cu, C) Ni, D) Se, E) Sr, and F) Zn in egg masses across study sites.

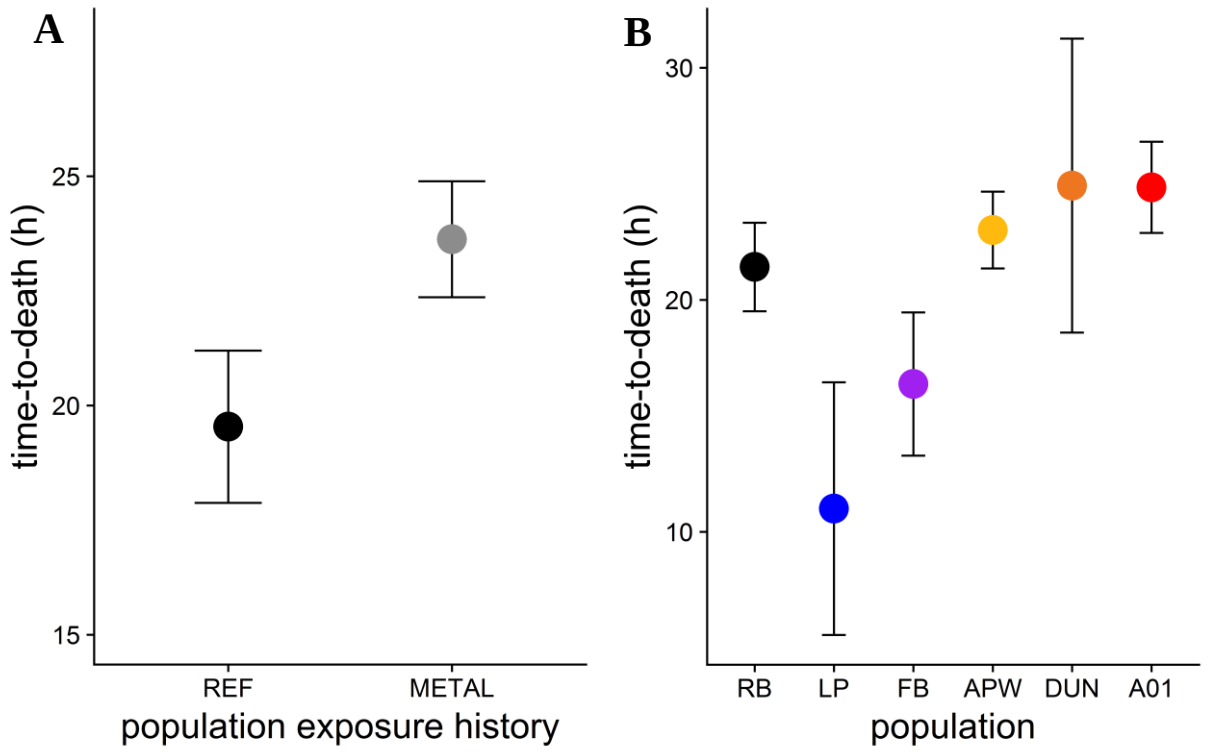

Figure 5.2. Plots of mean time-to-death (days +/- SE) over 48 h in 50 ppb Cu across population exposure history types (A) and populations (B).

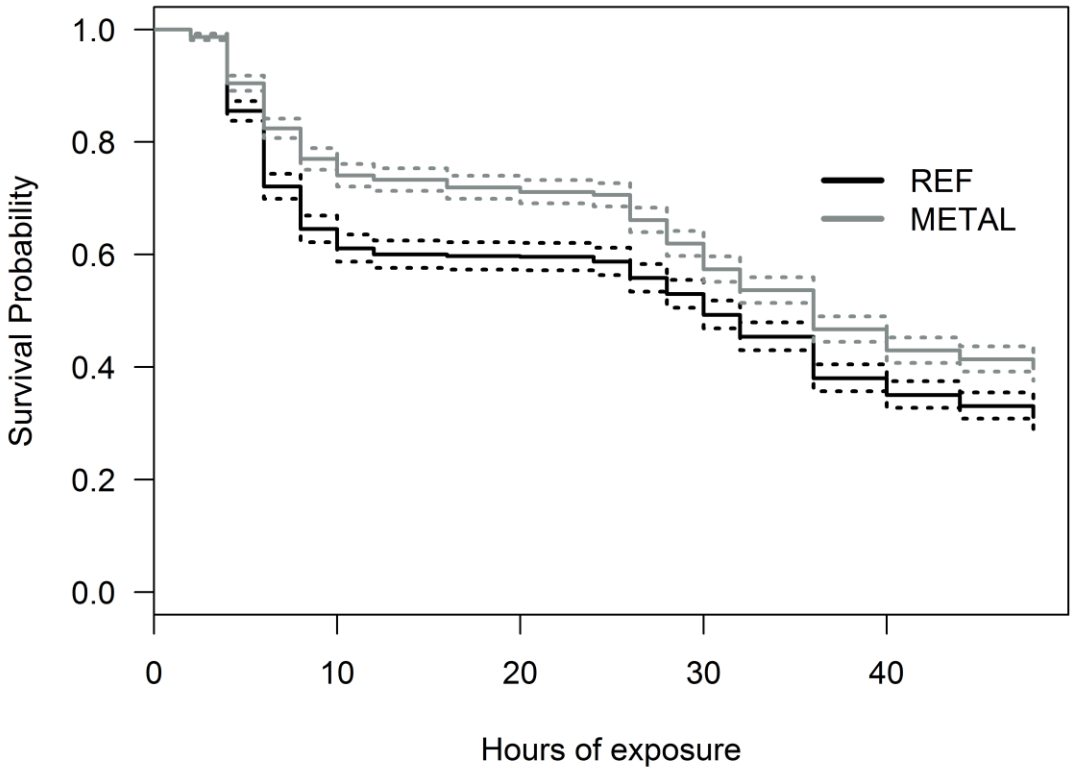

Figure 5.3. Survival curves for offspring pooled into sites with no history of contamination (REF) and those with histories of contamination with trace elements (METAL). Dotted lines depict 95% confidence intervals.

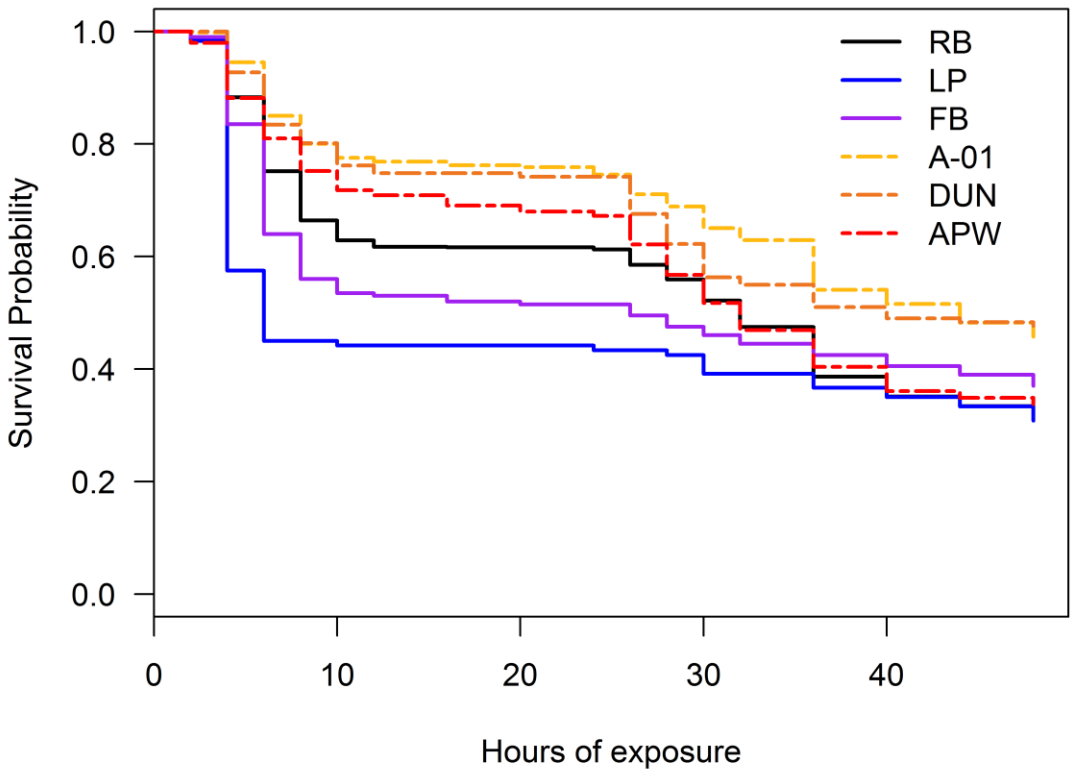

Figure 5.4. Larval survival curves in 50 ppb Cu across sampled populations. Solid lines are used to represent survival curves of populations with no history of environmental exposure to trace elements and dashed lines those with a history of exposure.

CHAPTER 6

CONCLUSIONS

This dissertation outlines a holistic approach to understanding how amphibians cope with environments contaminated with complex mixtures of toxic elements. In Chapter 2, I provide the first empirical evidence of an amphibian adapting to an environment contaminated with multiple heavy metal stressors. The potential for evolutionary rescue to mitigate some of the fitness costs incurred by amphibian larvae in contaminated environments is promising for this globally threatened group, but such adaptation comes at the cost of reduced growth and development in the absence of chemical stressors. Interestingly, I saw no evidence that selection in the contaminated environment had reduced the additive genetic variation in life history traits that is critical in maintaining a population's ability to respond to future environmental change.

Based on the results of Chapter 2, I focused my next two chapters on examining the mechanisms of tolerance to chemical contaminants. In Chapter 3 I investigate the physiological mechanisms underlying the adaptive tolerance to heavy metal. I assessed the elemental compositions of individuals from the putatively adapted and naïve populations reared under contaminated and uncontaminated conditions. My results provide further evidence of adaptation and suggest the mechanism underlying tolerance is the ability to maintain internal homeostasis when faced with environmental contaminants. This crucial function is performed largely by ATPase ion pumps, which are a highly conserved present in virtually all organisms and cell types. These proteins are critical to numerous physiological processes and require energy inputs to function; thus while selection can act to increase their efficacy, it is likely costly to the

organism and could alter other aspects of physiology that could impact development and growth. Understanding the mechanisms by which selection can act to enhance tolerance to contaminants provides insight into trade-offs that may be inherent to adaptation to anthropogenic stressors and add to our understanding of how human impacted environments can influence the evolutionary process.

In Chapter 4 I expand beyond amphibian physiology to determine what role the gut microbiota may play in tolerance to heavy metals. Though development in a heavy metal contaminated environment did not reduce the diversity of gut microbial communities, it did dramatically alter their community structure. The guts of individuals reared in the contaminated environment had significantly greater abundances of potentially pathogenic bacteria compared to individuals from the same families reared in a reference site. I also found support for the idea that maintaining diverse gut microbial communities confers advantages to hosts, as amphibians with more diverse gut communities tended to grow and develop more rapidly. Further, microbial diversity correlated with accumulation of trace elements in developing amphibians, with more diverse communities having lower body burdens of some potentially toxic elements (i.e., Cd, Se). Community composition and structure were also significantly associated with variation in life history traits and metal accumulation. These data provide crucial information that largely support hypotheses regarding the role of gut microbial communities on host health. These results illustrate the importance of the microbiome in determining outcomes of exposures to contaminants. Yet toxicology studies typically occur in laboratory settings where the microbiome is known to be altered. Thus, my results may help explain why field and laboratory studies of toxicity can lead to significantly different conclusions. In addition, they emphasize how alterations of the microbiome can lead to changes in fitness related traits of the host.

As Chapters 2-4 were relied upon observations between two populations, in Chapter 5 I follow up on those studies with an examination of tolerance to a common heavy metal stressor, copper, across populations varying in their exposure history to heavy metal contaminants. I examined six populations including three reference wetlands and three with a history of trace element contamination. Two populations were from sites contaminated with trace elements associated with coal combustion wastes, but not with copper. Because I had shown in Chapters 2-4 that one of these populations was adapted to trace elements I hypothesized that they would demonstrate cross tolerance to copper. However, the only population exhibiting elevated tolerance to copper was one that came from a wetland complex constructed to remediate wastewater high in copper. These results suggest either that trace element tolerance is unique to elements with shared mechanisms of action or that exposure to multiple chemical stressors may hinder adaptive tolerance to an one stressor (e.g. copper). Our observations in Chapter 3 of physiological divergence in the tolerant population suggested an ability to maintain homeostasis when exposed to trace elements. Thus, the lack of tolerance to copper suggests that survival in the field also depends on factors that were not accounted for under laboratory exposures to a single stressor (e.g. microbial communities, chronic effects).

Together, these chapters highlight how incorporating an interdisciplinary approach to examining the long-term impacts of environmental contaminants on amphibians can shed light on the ecological and evolutionary consequences of aquatic pollution. In particular, these results suggest that even management practices designed to mitigate the environmental consequences of human activities can impact amphibian populations.

APPENDIX A

Table A1. Summary of water quality and major element concentrations in aquatic environment at study sites. Bolded values are those that are significantly greater in that environment ($p \leq 0.05$).

	site	N	mean	sd	median	min	max
pH	REF	12	4.7	0.1	4.7	4.6	4.9
	ASH	12	**7.5**	0.2	7.5	7.3	7.8
conductivity	REF	12	83.3	5.4	85.4	71.7	86.5
(µS/cm)	ASH	12	**729.5**	41.1	737.0	663.0	772.0
Na (mg/L)	REF	12	0.73	0.31	0.62	0.45	1.62
	ASH	12	**69.24**	1.88	70.01	66.46	71.79
Ca (mg/L)	REF	12	1.23	0.22	1.15	0.98	1.80
	ASH	12	**17.54**	0.27	17.61	17.04	17.99
Mg (mg/L)	REF	12	0.40	0.06	0.39	0.36	0.59
	ASH	12	**4.31**	0.18	4.32	4.12	4.76
K (mg/L)	REF	12	0.78	0.12	0.78	0.59	1.02
	ASH	12	**8.96**	0.61	8.93	8.31	10.61

Table A2. Summary of trace element concentrations (μg/L) in aquatic environment at study sites. Bolded values are those that are significantly greater in that environment ($p \leq 0.05$). BDL = below instrument detection limit.

	site	N	mean	sd	median	min	max
Al	REF	12	226.08	80.76	239.25	116.50	389.50
	ASH	12	1265.71	1795.74	783.25	151.00	6759.50
As	REF	12	1.51	0.38	1.42	1.21	2.62
	ASH	12	**23.74**	2.12	23.83	21.34	26.92
Ba	REF	12	18.65	2.96	18.02	14.08	24.01
	ASH	12	**92.67**	15.32	89.20	79.77	134.01
Be	REF	12	0.04	0.01	0.04	0.02	0.06
	ASH	12	**0.24**	0.21	0.19	0.06	0.80
Cd	REF	12	0.06	0.06	0.04	0.02	0.22
	ASH	12	0.08	0.03	0.07	0.04	0.16
Co	REF	12	1.05	0.34	1.03	0.56	1.67
	ASH	12	2.17	1.49	1.87	0.86	6.72
Cu	REF	12	2.03	0.97	1.57	1.12	4.25
	ASH	12	3.63	1.88	3.08	1.95	8.62
Ni	REF	12	0.82	0.15	0.79	0.65	1.11
	ASH	12	**9.32**	2.65	8.91	6.24	16.41
Sb	REF	12	0.98	0.83	0.62	0.26	2.76
	ASH	12	0.98	0.20	0.91	0.80	1.51
Se	REF	12	0.25	0.04	0.27	0.17	0.30
	ASH	12	**12.06**	0.98	12.09	10.80	13.21
Sr	REF	12	9.16	0.90	9.01	7.98	11.28
	ASH	12	**903.16**	22.27	897.54	885.58	968.03
Th	REF	12	BDL	BDL	BDL	BDL	BDL
	ASH	12	**0.15**	0.18	0.08	0.08	0.70
U	REF	12	0.02	0.01	0.02	0.01	0.04
	ASH	12	**0.15**	0.12	0.12	0.05	0.46
V	REF	12	0.53	0.20	0.49	0.25	0.90
	ASH	12	**6.98**	3.46	6.03	4.14	15.80
Zn	REF	12	**54.86**	61.92	31.17	21.60	226.82
	ASH	12	7.39	3.97	6.10	4.06	16.68

Table A3. Table summarizing clutch x treatment combinations from embryonic study that did not have full set of replicates for the field study (i.e. < 6). The 'reps' column shows number of replicates for a given clutch x treatment combination that were included in the study.

pop	tx	dam	sire	reps
ASH	ASH	68	11	2
ASH	REF	68	11	2
REF	ASH	74	35	1
REF	REF	74	35	2
REF	REF	74	47	0
REF	ASH	76	46	2
REF	REF	77	56	2
REF	ASH	77	57	2
REF	REF	77	57	2
REF	ASH	77	58	0
REF	REF	77	58	0
REF	ASH	77	59	0
REF	REF	77	59	0
REF	ASH	77	62	2
REF	REF	77	62	2
REF	ASH	77	63	0
REF	REF	77	63	0
REF	ASH	78	51	0
REF	REF	78	50	0
REF	ASH	78	50	0

Table A4. Major cation and trace element levels in water samples collected from both REF and ASH environments used in laboratory embryonic study. Samples were taken from each carboy, containing field collected water, used to fill experimental units at the start of the trial (start) and a random subset of experimental units at the end of the trial (final). All elemental values are reported in μg/L. BDL = values below the instrument detection limit.

treatment water	rep	time	Ca	Fe	K	Mg	Na	Al	As	B	Ba	Be	Cd	Co	Cr	Cu	Mn
REF	1	start	1141	1899	1075	355	571	161	1.18	12.72	23.08	0.04	0.02	0.73	0.58	1.92	519.84
REF	2	start	1062	1968	1038	331	734	136	1.11	BDL	15.58	0.02	0.09	0.54	0.55	1.26	426.90
REF	3	start	1127	1894	1023	340	444	134	1.11	11.68	25.35	0.04	BDL	0.70	0.57	1.38	513.01
REF	1	final	1719	575	1744	618	1028	88	1.49	22.01	10.20	0.02	0.04	0.16	0.93	1.59	56.09
REF	2	final	4302	1915	3536	1453	2116	186	3.47	52.87	18.94	0.05	0.05	0.36	1.49	22.18	75.52
REF	3	final	2075	1047	447	768	95	104	1.93	15.50	19.90	0.03	0.25	0.46	0.94	2.12	394.59
REF	4	final	1873	968	834	580	10	121	1.69	21.05	23.78	0.04	0.02	0.52	0.98	5.77	503.19
REF	5	final	1753	1248	1227	584	463	110	1.73	13.04	22.89	0.03	0.02	0.75	0.72	2.22	638.49
REF	6	final	2092	1491	1373	694	1171	224	1.48	BDL	18.56	0.02	0.01	0.61	0.63	8.25	524.60
REF	7	final	2256	1211	705	589	560	105	1.67	14.45	20.64	0.02	0.03	0.71	0.67	11.55	578.59
ASH	1	start	17560	147	8252	4174	69861	262	21.44	296.85	77.18	0.12	0.08	2.18	0.47	4.05	114.84
ASH	2	start	17822	484	8354	4216	66928	785	26.43	299.52	91.02	0.38	0.24	6.61	1.08	5.21	222.75
ASH	3	start	17364	220	8197	4095	69273	458	22.26	292.94	78.77	0.17	0.10	3.22	0.59	3.62	147.79
ASH	1	final	27677	104	12371	6670	106427	210	29.08	496.22	114.26	0.11	0.15	1.69	1.08	5.62	53.68
ASH	2	final	47225	97	24213	11963	198659	204	54.99	744.10	188.69	0.08	0.16	1.75	1.14	5.91	60.03
ASH	3	final	30018	110	14059	7348	124133	248	33.47	498.41	122.13	0.05	0.24	0.83	0.95	4.29	20.37
ASH	4	final	31602	512	14862	7672	126594	2476	35.96	543.10	146.51	0.26	0.12	4.23	1.85	11.85	143.32
ASH	5	final	26405	388	12079	6465	107119	102	30.23	456.33	105.42	0.02	0.20	0.37	0.58	8.68	6.20

Table A4 (continued)

treatment water	rep	time	Ni	Pb	Sb	Se	Sr	Th	U	V	Zn
REF	1	start	0.57	1.22	0.27	0.42	8.82	BDL	0.014	0.36	36.66
REF	2	start	0.57	1.00	0.23	0.26	8.19	BDL	0.011	0.35	21.72
REF	3	start	0.66	0.84	0.17	0.32	8.57	BDL	0.011	0.29	24.13
REF	1	final	0.85	0.33	0.12	0.52	11.91	BDL	BDL	0.17	39.57
REF	2	final	2.15	1.36	0.68	1.15	27.70	BDL	0.022	0.51	177.47
REF	3	final	1.01	0.60	0.40	0.32	15.06	BDL	BDL	0.22	31.96
REF	4	final	0.98	0.56	0.39	0.43	13.28	BDL	BDL	0.20	51.68
REF	5	final	0.92	0.76	0.36	0.31	13.42	BDL	BDL	0.26	34.70
REF	6	final	0.89	0.62	0.29	0.28	11.14	BDL	BDL	0.21	31.76
REF	7	final	1.16	0.82	0.32	0.52	13.60	BDL	BDL	0.26	32.61
ASH	1	start	11.25	0.13	1.07	18.19	875.90	BDL	0.067	4.63	6.16
ASH	2	start	15.37	0.92	1.11	27.30	880.20	BDL	0.198	9.48	14.96
ASH	3	start	11.72	0.27	1.04	18.05	863.21	BDL	0.092	5.54	7.09
ASH	1	final	12.64	0.18	1.02	40.89	1402.00	BDL	0.107	7.22	7.95
ASH	2	final	16.00	0.13	2.52	41.12	2446.33	BDL	0.117	8.23	13.35
ASH	3	final	11.84	0.12	1.75	31.24	1554.40	BDL	0.101	6.22	15.92
ASH	4	final	16.25	0.71	1.72	29.61	1574.69	BDL	0.226	10.56	13.38
ASH	5	final	10.83	0.09	0.61	26.53	1356.49	BDL	0.060	5.10	34.41

Table A5. Water quality, major cation, and trace element levels at sampling locations (spatial blocks) within ASH and REF field sites. On each sampling date, two measurements were made at each spatial block, one in a random experimental enclosure (BIN) and one outside the enclosure. All element values reported in μg/L. spc = specific conductance reported in μS/cm.

Site	Block	Date	pH	spc	Ca	Fe	K	Mg	Na	Al	As	B	Ba	Be	Cd	Co	Cr
REF	A	5/2/2014	4.85	87	1105	1665	775	382	479	145	1.29	BDL	19.03	0.03	0.02	0.69	0.83
REF	A	6/9/2014	4.85	87	1145	3486	655	372	591	244	1.51	BDL	17.74	0.04	0.08	1.67	0.90
REF	A-BIN	5/2/2014	4.74	86	1317	1762	1015	416	895	196	2.62	BDL	16.05	0.02	0.05	1.18	0.95
REF	A-BIN	6/9/2014	4.74	86	1062	1692	802	391	675	333	1.75	BDL	18.26	0.06	0.22	0.88	0.93
REF	C	5/2/2014	4.68	86	1103	2432	653	376	454	247	1.44	BDL	24.01	0.05	0.02	1.38	0.78
REF	C	6/9/2014	4.68	86	978	1616	727	363	542	117	1.23	BDL	14.08	0.02	0.03	0.83	0.70
REF	C-BIN	5/2/2014	4.71	85	1800	2271	942	590	1618	390	1.34	BDL	17.77	0.03	0.02	1.17	0.88
REF	C-BIN	6/9/2014	4.71	85	1165	2849	782	411	559	235	1.39	BDL	21.17	0.03	0.03	1.36	0.94
REF	E	5/2/2014	4.57	85	1292	1608	760	361	860	126	1.21	BDL	16.21	0.02	0.08	0.56	0.91
REF	E	6/9/2014	4.57	85	1140	2418	594	372	628	254	1.53	BDL	23.49	0.04	0.04	0.87	0.97
REF	E-BIN	5/2/2014	4.65	72	1239	2136	825	400	609	249	1.35	BDL	17.14	0.04	0.02	1.28	0.97
REF	E-BIN	6/9/2014	4.65	72	1461	1728	780	410	811	182	1.45	BDL	18.89	0.04	0.12	0.69	1.09
ASH	A	5/2/2014	7.55	760	17177	160	8383	4146	67488	354	21.34	296.24	79.77	0.09	0.05	1.29	0.66
ASH	A	6/9/2014	7.81	681	17699	258	9016	4338	70548	804	25.28	300.16	90.29	0.21	0.05	1.80	1.08
ASH	A-BIN	5/2/2014	7.41	759	17468	306	8898	4219	68088	1194	21.55	296.82	88.11	0.19	0.08	2.00	1.17
ASH	A-BIN	6/9/2014	7.60	663	17581	63	8878	4302	70760	151	22.98	311.28	81.85	0.06	0.04	0.86	0.67
ASH	C	5/2/2014	7.38	771	17045	366	8314	4116	66472	470	21.41	298.05	83.69	0.13	0.05	1.56	0.97
ASH	C	6/9/2014	7.70	706	17761	208	8989	4367	71788	674	24.67	318.57	91.51	0.16	0.07	1.63	1.18
ASH	C-BIN	5/2/2014	7.30	767	17506	279	8661	4178	67719	763	22.01	290.49	86.18	0.19	0.06	1.68	0.89
ASH	C-BIN	6/9/2014	7.79	687	17662	270	9195	4397	71011	967	25.40	309.53	92.58	0.19	0.12	2.41	1.14
ASH	E	5/2/2014	7.36	771	17219	125	8369	4131	66461	256	21.64	300.86	80.37	0.11	0.06	1.93	0.65
ASH	E	6/9/2014	7.35	702	17688	268	8963	4339	70569	844	25.33	302.23	94.13	0.21	0.07	1.97	1.22
ASH	E-BIN	5/2/2014	7.29	772	17987	1329	10610	4761	69591	6760	26.34	301.84	134.01	0.80	0.16	6.72	3.66
ASH	E-BIN	6/9/2014	7.52	715	17643	578	9195	4404	70432	1954	26.92	308.60	109.52	0.52	0.09	2.13	1.97

Table A5 (continued)

Site	Block	Date	Cu	Mn	Ni	Pb	Sb	Se	Sr	Th	U	V	Zn
REF	A	5/2/2014	1.12	551.11	0.67	0.64	0.74	0.27	8.67	BDL	0.014	0.39	30.02
REF	A	6/9/2014	1.52	1368.40	0.87	1.50	0.28	0.30	8.97	BDL	0.022	0.73	32.68
REF	A-BIN	5/2/2014	2.40	852.57	0.65	0.91	0.52	0.23	8.44	BDL	0.019	0.38	32.32
REF	A-BIN	6/9/2014	3.00	782.07	1.11	0.81	0.72	0.20	11.28	BDL	0.036	0.90	29.81
REF	C	5/2/2014	1.28	824.69	0.69	1.37	0.36	0.27	8.63	BDL	0.027	0.66	25.00
REF	C	6/9/2014	2.46	755.37	0.73	0.69	0.26	0.18	7.98	BDL	0.005	0.25	26.70
REF	C-BIN	5/2/2014	1.44	903.97	0.92	0.79	2.13	0.27	8.89	BDL	0.017	0.45	27.89
REF	C-BIN	6/9/2014	1.61	1007.83	0.90	1.27	1.11	0.17	9.05	BDL	0.021	0.51	34.48
REF	E	5/2/2014	2.85	499.34	0.84	1.16	2.76	0.27	9.22	BDL	0.013	0.29	133.21
REF	E	6/9/2014	1.25	756.26	0.72	1.33	0.50	0.27	9.25	BDL	0.030	0.76	37.77
REF	E-BIN	5/2/2014	1.20	855.96	0.73	0.90	0.46	0.25	9.06	BDL	0.022	0.52	21.60
REF	E-BIN	6/9/2014	4.25	522.89	1.03	2.02	1.93	0.29	10.50	BDL	0.024	0.46	226.82
ASH	A	5/2/2014	2.25	108.13	8.89	0.16	0.83	13.16	891.90	BDL	0.060	4.14	6.18
ASH	A	6/9/2014	2.91	126.30	7.55	0.61	0.84	10.80	888.40	BDL	0.152	6.76	7.24
ASH	A-BIN	5/2/2014	4.09	134.64	12.18	0.44	0.91	12.60	898.17	BDL	0.116	5.76	16.68
ASH	A-BIN	6/9/2014	1.95	67.27	6.24	0.13	0.80	11.04	896.91	BDL	0.053	4.60	4.13
ASH	C	5/2/2014	2.54	116.73	8.92	0.26	0.84	13.21	908.35	BDL	0.084	4.75	4.06
ASH	C	6/9/2014	2.89	114.15	7.47	0.46	0.90	11.54	907.43	BDL	0.113	6.30	4.88
ASH	C-BIN	5/2/2014	3.24	114.99	9.30	0.46	1.11	12.79	891.14	BDL	0.116	5.61	6.01
ASH	C-BIN	6/9/2014	3.57	172.75	8.97	0.60	1.13	11.10	885.58	BDL	0.133	6.79	7.48
ASH	E	5/2/2014	2.19	150.25	9.20	0.14	0.91	13.18	887.35	BDL	0.059	4.20	5.23
ASH	E	6/9/2014	3.57	119.13	7.86	0.76	0.98	10.90	902.16	BDL	0.144	7.12	5.59
ASH	E-BIN	5/2/2014	8.62	360.54	16.41	1.97	1.51	12.84	968.03	0.70	0.464	15.80	14.28
ASH	E-BIN	6/9/2014	5.78	98.61	8.83	2.08	1.03	11.57	912.47	0.26	0.327	11.87	6.94

Table A6. Quantitative genetic variance component means (HPDI) derived from population and environment-specific models. CVA was calculated as $(\sqrt{V_A}/\bar{X}) \times 100$, where $\bar{X}$ equals the population x environment-specific trait mean.

size GS25

pop	env	VA	VM	VD	VP	CVA
REF	REF	0.0009 (0.0005-0.0018)	0.0004 (0.0000-0.0019)	0.0008 (0.0004-0.0014)	0.0017 (0.0012-0.0032)	3.22 (2.30-4.48)
	ASH	0.0009 (0.0004-0.0016)	0.0007 (0.0000-0.0026)	0.0007 (0.0004-0.0012)	0.0019 (0.0012-0.0038)	3.08 (2.18-4.32)
ASH	REF	0.0006 (0.0003-0.0012)	0.0006 (0.0000-0.0023)	0.0005 (0.0002-0.0008)	0.0016 (0.0010-0.0032)	2.70 (1.99-3.69)
	ASH	0.0007 (0.0004-0.0012)	0.0006 (0.0000-0.0021)	0.0006 (0.0003-0.0010)	0.0017 (0.0012-0.0032)	2.77 (2.03-3.79)

size 1

pop	env	VA	VM	VD	VP	CVA
REF	REF	0.002 (0.0007-0.0049)	0.0028 (0.0000-0.0163)	0.0014 (0.0006-0.0031)	0.0079 (0.0036-0.023)	4.04 (2.46-6.52)
	ASH	0.0021 (0.0007-0.0051)	0.0022 (0.0000-0.0118)	0.0019 (0.0007-0.0041)	0.0068 (0.0033-0.0175)	3.85 (2.36-6.26)
ASH	REF	0.0012 (0.0005-0.0023)	0.0005 (0.0000-0.0022)	0.0010 (0.0005-0.0019)	0.0042 (0.0030-0.0074)	3.07 (2.13-4.39)
	ASH	0.0011 (0.0005-0.0021)	0.0003 (0.0000-0.0016)	0.0010 (0.0005-0.0018)	0.0040 (0.0027-0.0075)	2.89 (1.99-4.10)

Table A6 (continued)

early growth

pop	env	VA	VM	VD	VP	CVA
REF	REF	0.0073 (0.0013-0.0244)	0.0057 (0.0000-0.0347)	0.0152 (0.0021-0.0464)	0.0596 (0.0397-0.1104)	12.454 (5.50-24.91)
	ASH	0.0089 (0.0014-0.0300)	0.0045 (0.0052-0.0296)	0.0188 (0.0023-0.0548)	0.0399 (0.0269-0.0690)	13.49 (5.73-27.37)
ASH	REF	0.0109 (0.0016-0.0356)	0.0104 (0.0000-0.0420)	0.0192 (0.0022-0.0570)	0.1163 (0.0878-0.1778)	17.35 (6.86-35.21)
	ASH	0.0029 (0.0009-0.0070)	0.0009 (0.0000-0.0051)	0.0033 (0.0010-0.0080)	0.0214 (0.0169-0.0303)	7.10 (4.10-11.49)

ttm

pop	env	VA	VM	VD	VP	CVA
REF	REF	0.0024 (0.0008-0.0060)	0.0021 (0.0000-0.0126)	0.0023 (0.0008-0.0052)	0.0096 (0.0052-0.0229)	3.08 (1.82-5.12)
	ASH	0.0172 (0.0020-0.0586)	0.0088 (0.0000-0.0549)	0.0147 (0.0019-0.0497)	0.0715 (0.0371-0.1637)	7.23 (2.70-14.59)
ASH	REF	0.0028 (0.0010-0.0064)	0.0003 (0.0000-0.0026)	0.0028 (0.0010-0.0059)	0.0096 (0.0070-0.0156)	3.31 (1.99-5.11)
	ASH	0.0054 (0.0012-0.0150)	0.0010 (0.0000-0.0072)	0.0086 (0.0016-0.0231)	0.0524 (0.0352-0.1016)	4.18 (2.11-7.29)

Table A6 (continued)

						svl					
pop	env	VA		VM		VD		VP		CVA	
REF	REF	0.0016	(0.0006-0.0038)	0.0019	(0.0000-0.0102)	0.0012	(0.0005-0.0025)	0.0047	(0.0020-0.0140)	4.21	(2.67-6.69)
	ASH	0.0016	(0.0006-0.0039)	0.0019	(0.0000-0.0116)	0.0014	(0.0006-0.0029)	0.0049	(0.0021-0.0153)	4.28	(2.68-6.85)
ASH	REF	0.0011	(0.0005-0.0021)	0.0004	(0.0000-0.0016)	0.0011	(0.0005-0.0019)	0.0033	(0.0022-0.0064)	3.50	(2.43-5.01)
	ASH	0.0010	(0.0005-0.0020)	0.0003	(0.0000-0.0013)	0.0013	(0.0007-0.0023)	0.0027	(0.0018-0.0051)	3.43	(2.38-4.89)

						growth rate					
pop	site	VA		VM		VD		VP		CVA	
REF	REF	0.0010	(0.0004-0.0020)	0.0018	(0.000-0.0090)	0.0006	(0.0003-0.0012)	0.0032	(0.0010-0.0106)	75.76	(17.25-251.09)
	ASH	0.0014	(0.0005-0.0032)	0.0020	(0.000-0.0104)	0.0011	(0.0005-0.0022)	0.0042	(0.0014-0.0133)	107.41	(8.36-663.49)
ASH	REF	0.0005	(0.0003-0.0009)	0.0003	(0.000-0.0012)	0.0004	(0.0002-0.0006)	0.0014	(0.0007-0.0035)	11.69	(28.35-121.82)
	ASH	0.0006	(0.0003-0.0011)	0.0003	(0.000-0.0012)	0.0005	(0.0003-0.0008)	0.0018	(0.0010-0.0040)	75.36	(33.04-224.94)

Table A6 (continued)

		metamorphosis probability				
pop	env	VA	VM	VD	VP	CVA
REF	REF	0.3602 (0.0004-1.6691)	0.2889 (0.0000-1.9845)	0.3138 (0.0003-1.4389)	1.7325 (1.0523-3.9680)	NA
	ASH	0.2687 (0.0004-1.2915)	0.2937 (0.0000-1.8203)	0.4175 (0.0007-1.8251)	2.0123 (1.1160-5.0534)	NA
ASH	REF	0.1084 (0.0001-0.5154)	0.0434 (0.0000-0.3446)	0.4172 (0.0017-1.3487)	1.3476 (1.0627-2.0872)	NA
	ASH	0.3220 (0.0009-1.1359)	0.0637 (0.0000-0.5311)	0.2287 (0.0003-0.9653)	1.6360 (1.1630-2.9991)	NA

Table A7. Principal component loadings of water quality and trace element levels in environmental water samples taken in field. Specific conductance (spc) was modeled in µS/cm and trace element concentrations as ug/L. The first two PCs sufficiently explained the environmental variation in the data.

parameter	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
spc	-0.322	-0.142	0.069	-0.207	0.111	-0.154	0.279	-0.234	0.032	-0.140	0.247	0.761
pH	-0.318	-0.180	0.073	-0.103	-0.223	0.059	-0.685	-0.084	-0.492	0.248	0.080	0.107
Fe	0.274	0.278	-0.349	-0.697	-0.346	-0.326	-0.075	-0.078	0.043	-0.009	-0.004	-0.005
Al	-0.192	0.578	-0.289	-0.069	0.314	0.568	-0.138	-0.305	0.045	-0.047	0.046	0.035
V	-0.301	0.272	-0.129	0.127	-0.475	0.127	0.418	0.296	-0.473	-0.200	-0.180	0.045
Ni	-0.320	0.092	-0.016	-0.300	0.529	-0.219	-0.138	0.650	-0.045	-0.130	-0.083	-0.065
Cu	-0.214	0.554	0.159	0.401	-0.027	-0.624	-0.138	-0.183	0.089	0.104	0.024	-0.013
Zn	0.160	0.308	0.853	-0.312	-0.076	0.209	0.033	0.042	-0.034	-0.036	-0.004	0.013
As	-0.324	-0.102	0.054	0.000	-0.341	0.069	-0.242	0.024	0.432	-0.623	0.277	-0.224
Se	-0.320	-0.147	0.078	-0.252	0.178	-0.139	0.352	-0.417	-0.275	0.033	0.179	-0.590
Sr	-0.323	-0.135	0.072	-0.138	-0.068	0.009	-0.019	-0.224	0.286	0.051	-0.845	0.015
Ba	-0.328	0.034	-0.017	-0.095	-0.235	0.154	0.180	0.262	0.417	0.676	0.262	-0.055
eigenvalue	9.20	1.70	0.81	0.13	0.10	0.04	0.01	0.01	0.00	0.00	0.00	0.00
variance	76.64	14.16	6.74	1.10	0.83	0.36	0.09	0.05	0.02	0.01	0.00	0.00
cumulative variance	76.64	90.80	97.54	98.64	99.46	99.83	99.92	99.96	99.99	99.99	100.00	100.00

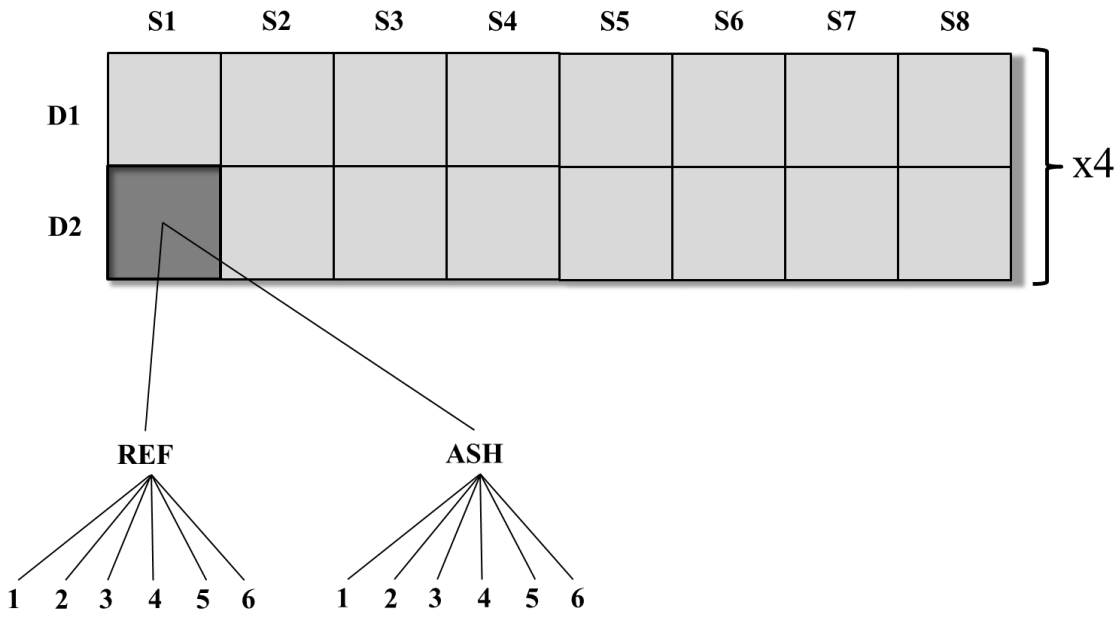

Figure A1. Schematic of NC II breeding design used for each population. In each 2 x 8 breeding block, two dams were crossed with eight unique sires (each square represents a full-sib family). Six larvae from each cross were reared in each environment.

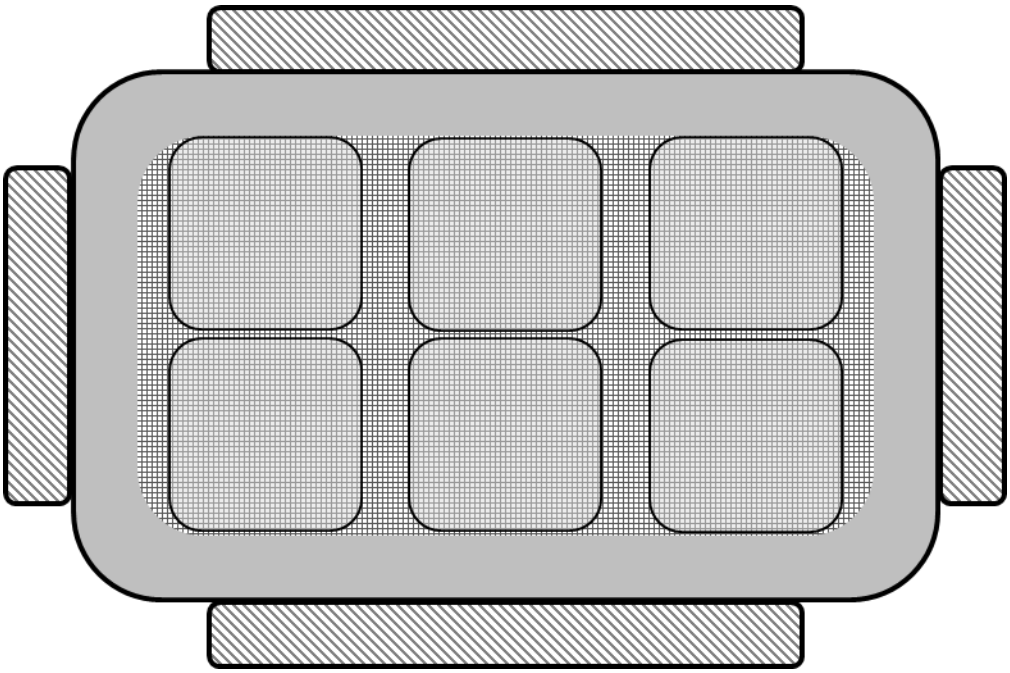

Figure A2. Detailed Field Enclosure Design

Standardized foam floatation (diagonal fill) was attached with UV-resistant plastic zip-ties to all four sides of the larger enclosures to maintain a standardized depth of bin and volume of water in the experimental units. The lids and bottoms of both the floating field enclosures and the individual rearing containers within each enclosure (i.e. the six smaller containers seen at top) were removed and fit with non-metallic window screen (crosshatched fill).

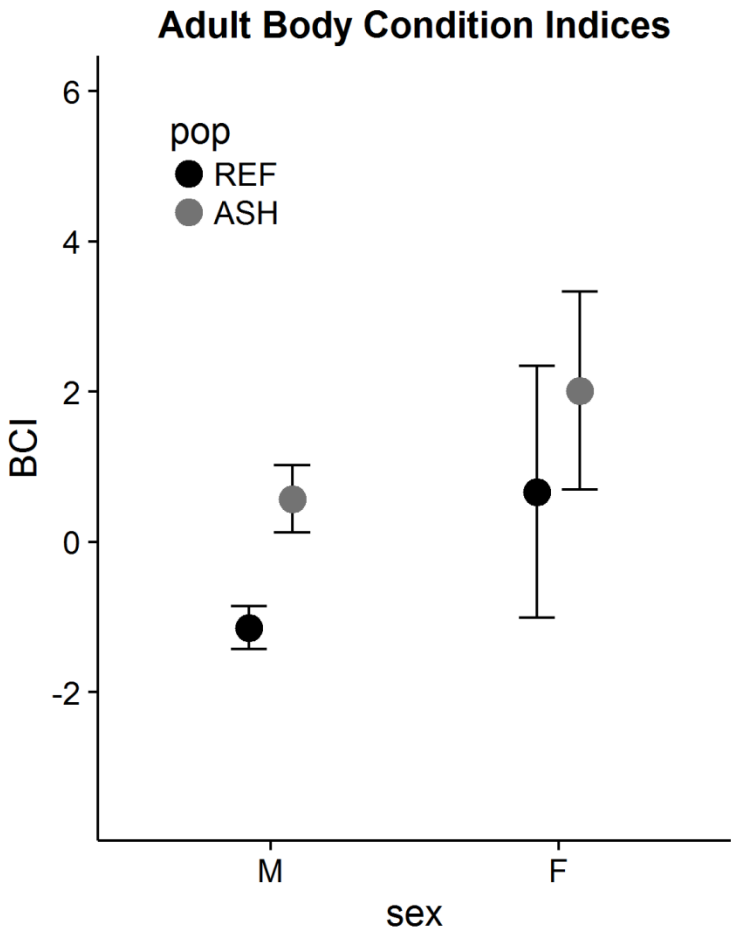

Figure A3. Mean (±SE) body condition indices (BCI) for parental toad from REF and ASH sites. Overall BCI was greater for ASH parents, but only see a significant difference between males when breaking down by sex.

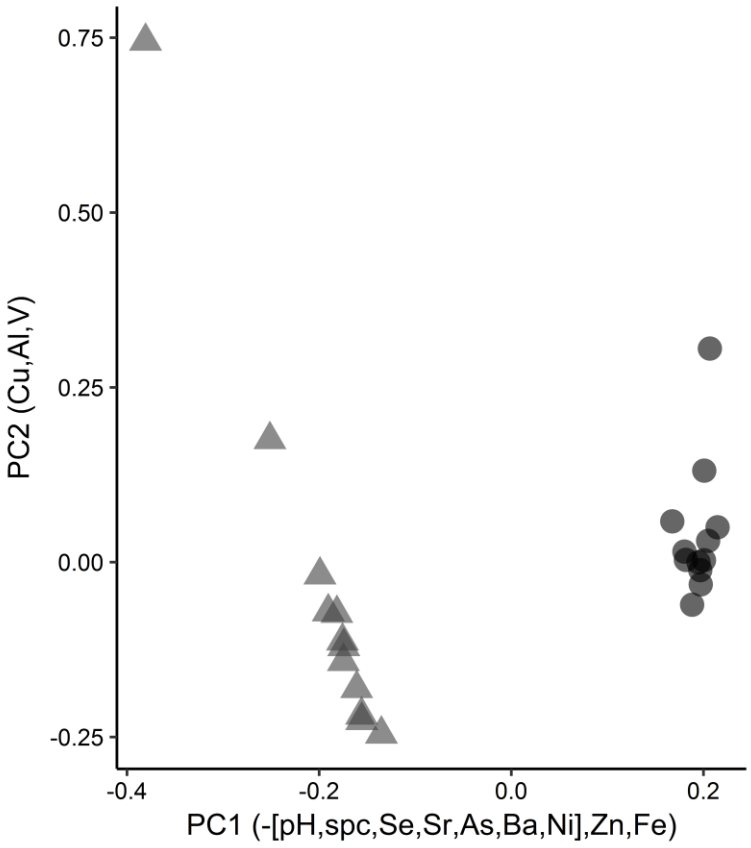

Figure A4. Plot of first two, varimax rotated PC-axes. PC1 explains 59.1% and PC2 31.7% of the variance in trace element and water quality of the aquatic environments (90.8% total). The two rearing environments separated primarily along the PC1, while PC2 mostly explained within site variation. See Table A6 for PC eigenvalues, % variance, and loading scores prior to rotation.

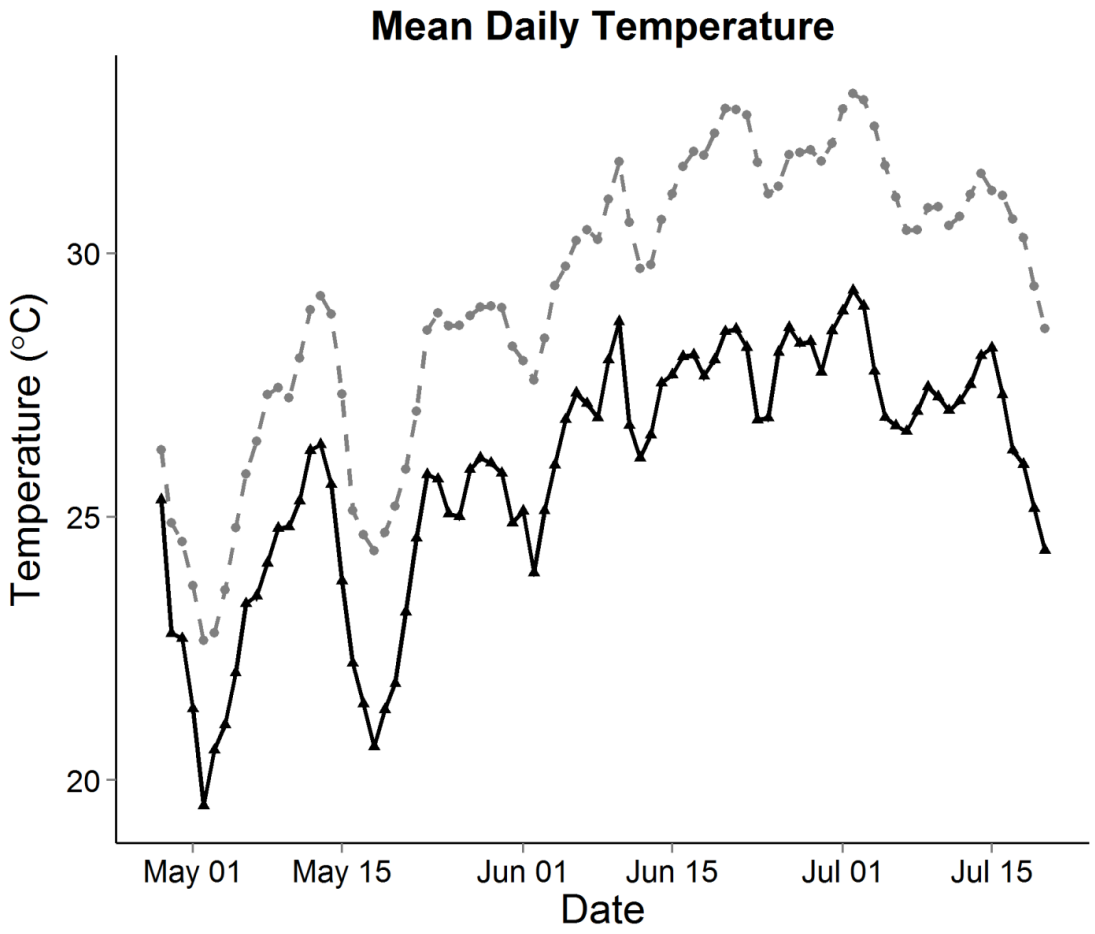

Figure A5. Mean daily water temperature profiles across field sites with the REF site in black and the ASH site in gray. The ASH environment was consistently warmer and had less thermal variability.

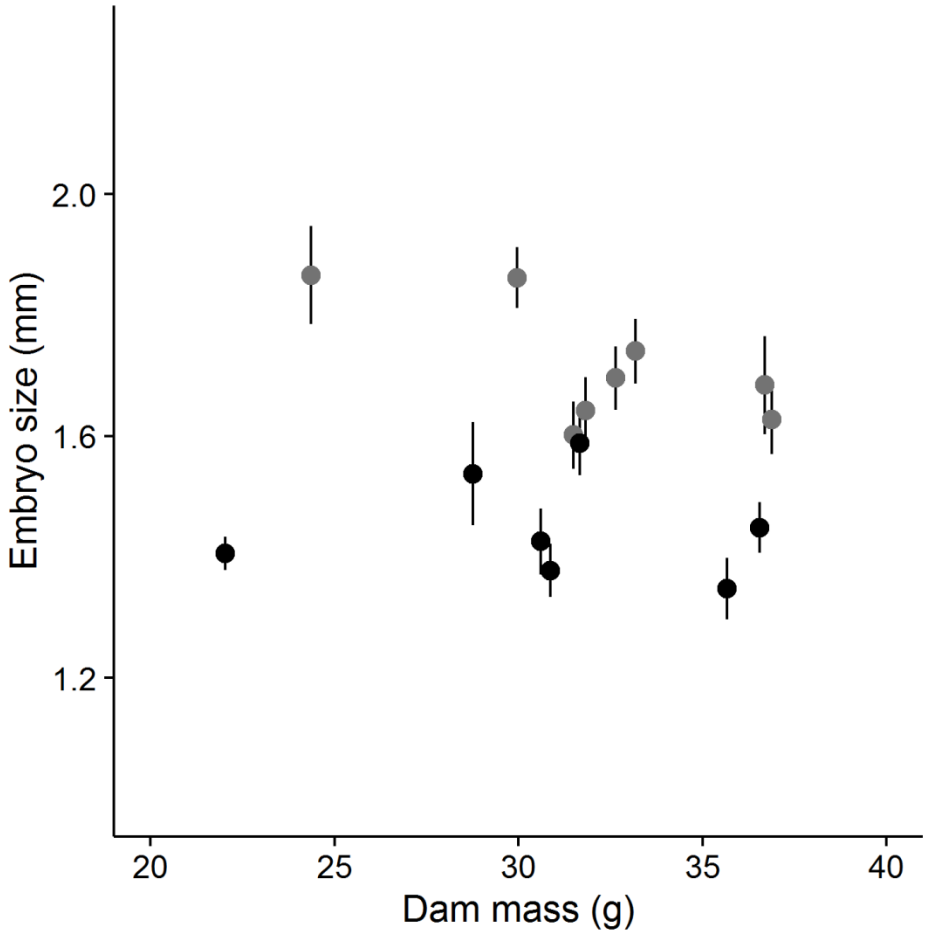

Figure A6. Plot of mean embryo size (95%CI) against dam mass. While there was no relationship between dam size and embryo size in the REF population (black), there was a slight negative relationship between the two in the ASH population counter to other research examining this relationship in amphibians (Laugen et al. 2002). Laugen et al. (2002) found this relationship only in some populations however, in others there was no discernable relationship between the two.

APPENDIX B

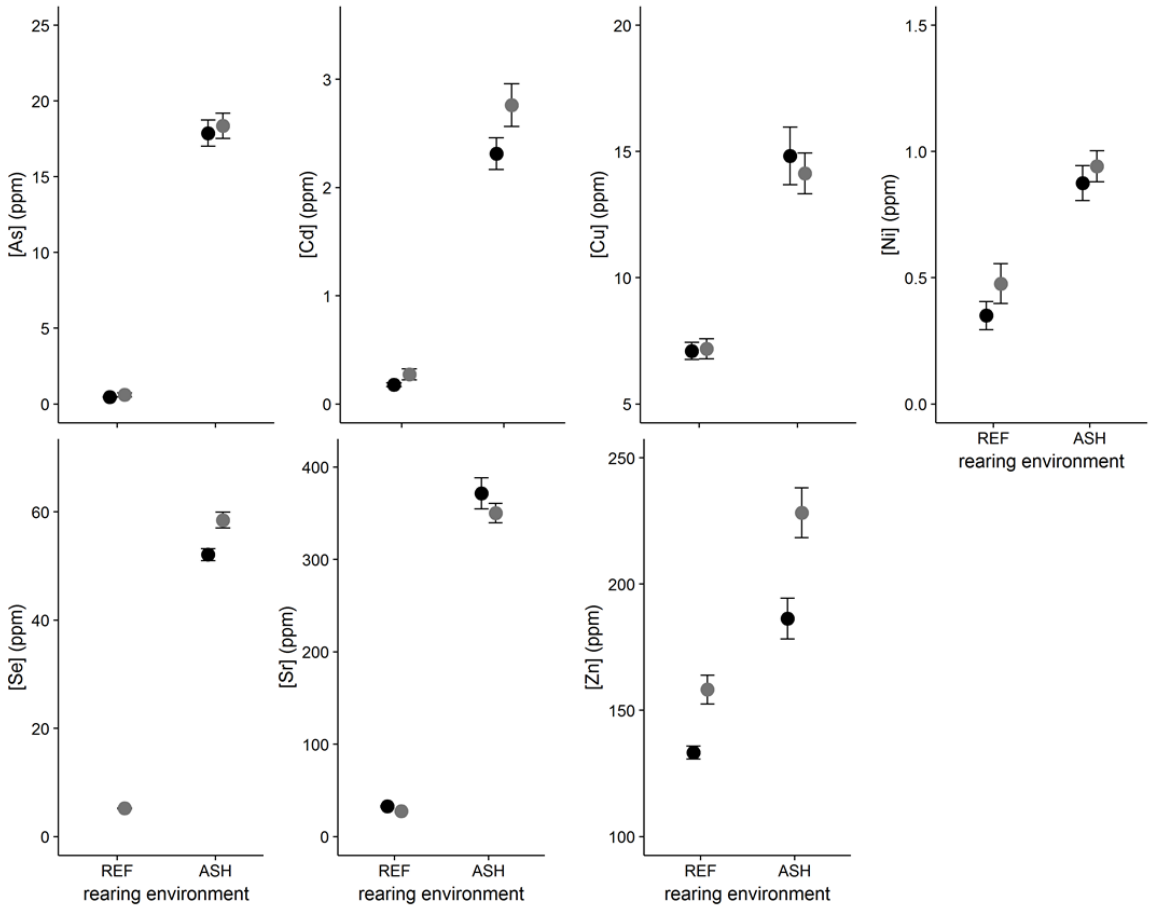

Figure B1. Plots of mean (+/-SE) values of PC1 elements by population and rearing environment. The REF population is in black and the ASH population is in gray.

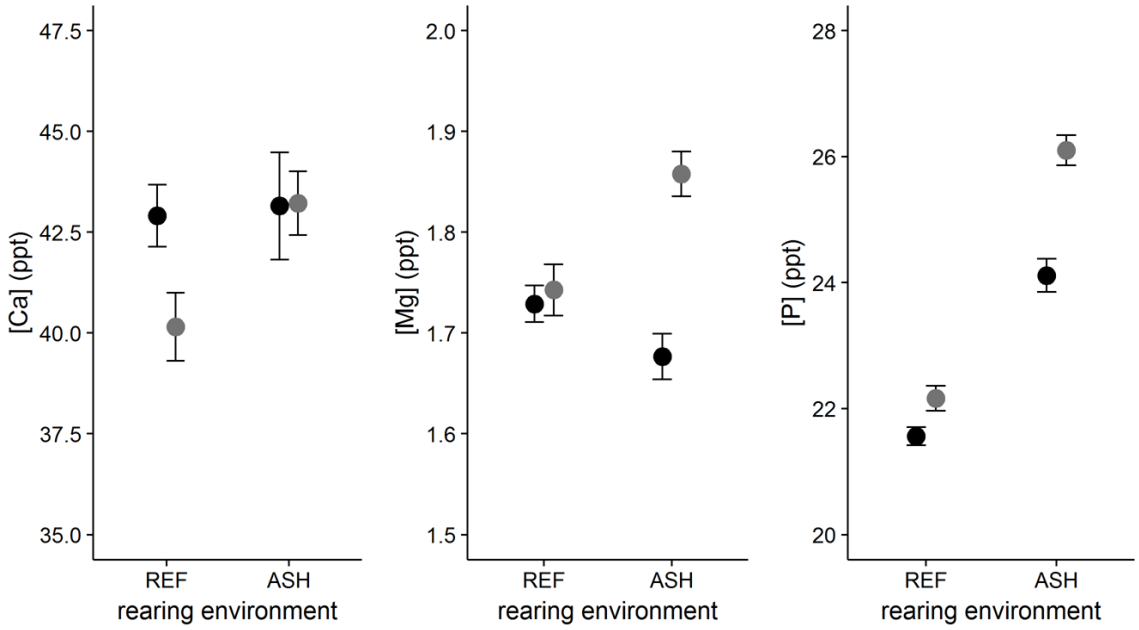

Figure B2. Plot of mean (+/- SE) concentrations of calcium (Ca), magnesium (Mg), and phosphorus (P) by population and environment. Black points are for the REF population and gray are the ASH population.

APPENDIX C

Table C1. Water quality and trace element loadings on first two, rotated principal component axes. For clarity, only showing values with loadings > |0.40| and values bolded for axis on which that variable loaded most heavily

	Rotated PC axes	
	PC1	PC2
pH	**-0.94**	
spc	**-0.93**	
Se	**-0.93**	
Sr	**-0.92**	
As	**-0.90**	0.41
Ba	**-0.82**	0.57
Ni	**-0.76**	0.62
Zn	**0.62**	
Fe	**0.90**	
V	-0.59	**0.79**
Cu		**0.96**
Al		**0.95**
SS loadings	7.09	3.81
% variance	59	32
cumulative % variance	59	91

Table C.2. Summary of linear mixed effect models testing effect of rearing environment on host life history traits. Effect estimates given are for individuals reared in the ASH environment relative to those reared in REF.

trait	estimate	*p*
early growth	**1.33**	**0.01**
ttm	**14.87**	**0.03**
mass	1.36	0.53
svl	-0.01	0.97
BCI	**14.58**	**0.04**
growth rate	-0.16	0.22

Table C3. Summary of linear mixed effect models testing effect of rearing environment on trace element body burdens in metamorphic toads used in this study. Effect estimates given are for individuals reared in the ASH environment relative to those reared in REF.

element	estimate	*p*
As	3.59	<**0.0001**
Cu	0.61	<**0.0001**
Ni	0.75	<**0.0001**
Sr	2.56	<**0.0001**
Zn	0.37	<**0.0001**

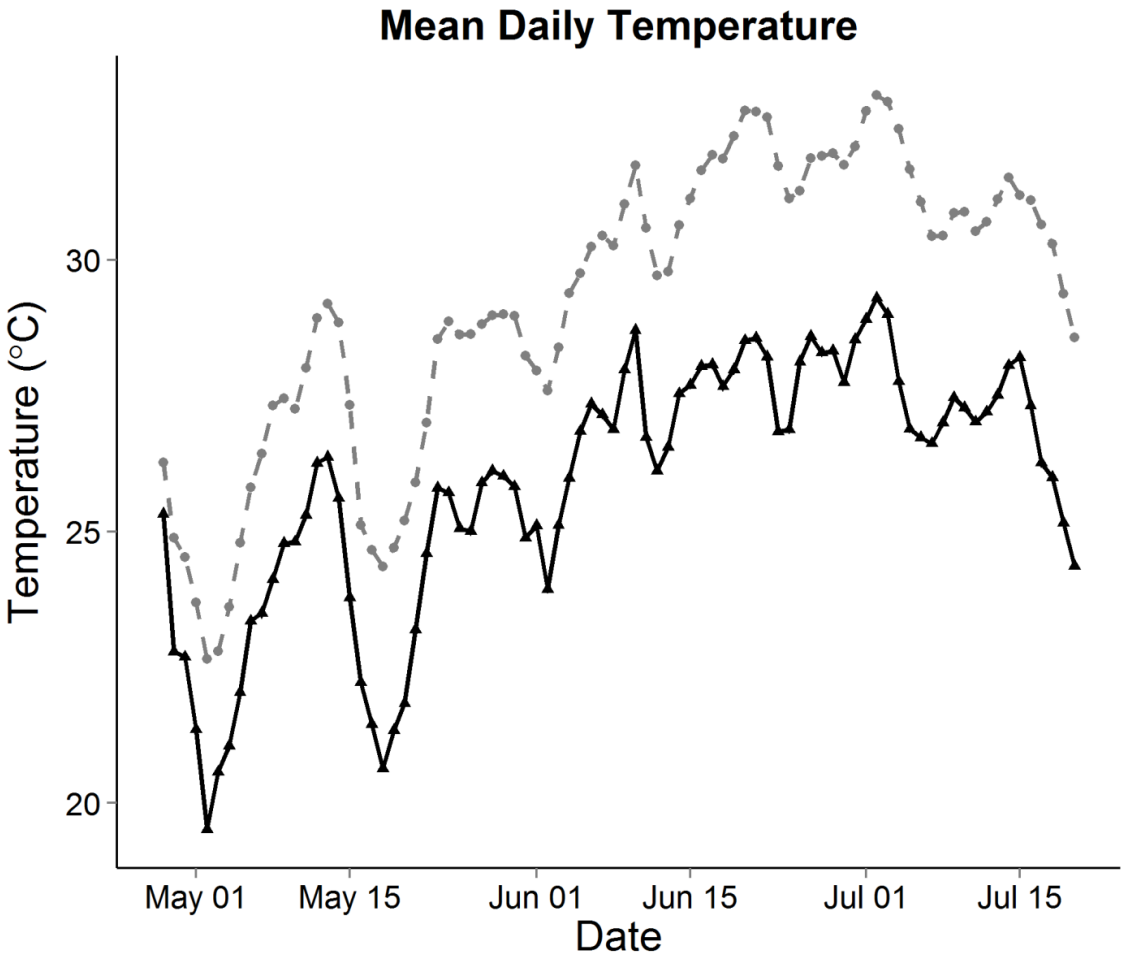

Figure C1. Plots of daily mean water temperatures in REF (black) and ASH (gray) environments. Eight loggers were distributed in every other bin at each location at each site and programed to log a data point every hour.

Text C1. Detailed DNA extraction protocol

Bead Beater DNA Extraction Protocol

Whole gut samples were transferred from RNAlater® to 2 mL Beadbeater tubes containing a single, sterilized 3.5 mm steel bead. We added 350 µL of Working Lysis Solution (see *Recipes* below) to each tube, before capping and running on Beadbeater for 3 minutes at 2,500 oscillations/minute. Tubes were then centrifuged at 14,000 rcf for 3 minutes (note all centrifugation steps were done at 14,000 rcf).

The supernatant from each tube was carefully pipeted, avoiding debris at bottom of tube, onto a spin column (Epoch®, #1920-050) placed in a fresh 2 mL collection tube and centrifuged for 1 minute. After discarding the flow-through and transferring spin columns to fresh collection tubes, we added 700 µL of Working Wash Buffer (see *Recipes* below) and centrifuged for 1 minute. Flow-through was once again discarded and columns placed in a fresh collection tube. This wash was repeated two more times for a total of three washes. After third wash, caps on columns were opened before spinning for 5 minutes to minimize ETOH contamination.

Last, filter columns were transferred to fresh 1.5 mL microcentrifuge tubes for elution. To elute DNA, we added 50 µL of ultra-pure DEPC-treated water to filters and incubated at room temperature for 4 minutes. Tubes were centrifuged for 1 minute to collect DNA.

Recipes

Premix stock solution:

Stock Lysis Buffer: Guanidium thiocyanate (4 M), Tris HCl (pH 7.5, 0.01 M)

Stock Wash Buffer: Potassium acetate (162.8 mM), Tris HCl (pH 7.5, 27.1 mM)

Preparation of working solutions:

Working Lysis solution (1 part BME:49 parts Lysis Buffer)

Beta-mecaptoethanol (BME): 7uL/sample (i.e. 2% of final solution)

RNA Lysis Buffer: 343 uL/sample (98% of final solution)

Working Wash Buffer (6 parts ETOH:4 parts buffer)

ETOH: 18.9 mL (60%)

Wash Buffer: 12.6 mL (40%)

APPENDIX D

Table D1: Summary of number of clutches from each population used in time to death trials grouped by whether the population was sourced from a site with (METAL) or without (REF) a history of trace element contamination

METAL		
	A01	13
	APW	30
	DUN	3
REF		
	FB	5
	LP	3
	RB	22