Abstract

Title of Thesis: Dietary uptake and toxicity of coal ash and selenium to larval *Hyla versicolor*

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Thesis Directed by: Dr. Christopher Rowe, Associate Professor, Toxicology

In 2009, approximately 24 % of the estimated 92 million tons of coal ash (CA) produced in the U.S. was disposed of in aquatic settling basins. Amphibians are especially at risk of exposure to CA disposed in this manner, as they often breed in ash basins or habitat contaminated by basin effluent. Coal ash is a complex and variable mixture. Although trace elements make up a small percentage of the total mineral content, these CA constituents are of primary concern in the environment. Elevated selenium (Se) inputs to aquatic habitats from CA were associated with increased larval amphibian mortality and malformations. Selenium is an essential micronutrient with a narrow therapeutic concentration range and a propensity to biotransform and bioaccumulate in aquatic food chains. This research contrasts the toxicological effects of dietary exposures of CA, an organic Se-containing compound, selenomethionine (SeMet) and an inorganic Se-containing compound, selenium dioxide (SeO_2) to a larval amphibian (*Hyla versicolor*). Exposure to 50 µg Se g^{-1} wet mass (ww) nominal concentration of SeMet reduced larval metabolic rates, larval growth rates and was lethal to larvae by the conclusion of the 78 d study. The SeMet Low (5 µg Se g^{-1} ww nominal concentration) dose reduced the number of individuals to initiate metamorphosis, reduced size at metamorphosis, reduced survival to initiate and complete metamorphosis, and increased

the frequency of malformations during metamorphosis. In contrast, there was little evidence of CA toxicity relative to control treatments. The SeO_2 High dose (50 µg Se g^{-1} ww nominal concentration) had comparable effects on survival, metamorphosis, and masses of recent metamorphs as the SeMet Low dose. Neither SeO_2 dose (50 µg Se g^{-1} ww or 5 µg Se g^{-1} ww nominal concentration) induced malformations or caused mortality during metamorphic climax. This research provides evidence of Se species-specific toxicity to a larval anuran. Results of this study indicate SeMet is more toxic and bioavailable to *H. versicolor* than SeO_2 and directly link 5 µg Se g^{-1} ww nominal SeMet exposures to rear limb malformations developed during metamorphosis.

Dietary uptake and toxicity of coal ash and selenium to larval *Hyla versicolor*

by
Laura Anne Lockard

Thesis submitted to the faculty of the Graduate School
of the University of Maryland Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
2011

Acknowledgements

Foremost, I would like to thank my advisors, Dr. Christopher Rowe and Dr. Andrew Heyes, for their unwavering guidance throughout the course of this project. Thank you for taking a chance on a "seasoned" Masters student and for providing the financial and intellectual support that enabled me to pursue research in environmental toxicology. I appreciate your involvement and interest in my study. Thank you also for your accessibility, for keeping your office doors open and for always making my academic success such a priority. Chris, I want to thank you in particular for your patient mentorship over the past few years. I have learned so much from you. Andrew, thank you for being such a positive leader. The optimism with which you tackle unanswered scientific questions is contagious. I admire your passion for what you do. I would also like to thank Dr. Katherine Squibb for her contributions as a member of my committee, director of the University of Maryland, Baltimore's Toxicology Department, and professor. Thank you for always having my back. I would not have reached this point in my graduate career without your caring, and at times protective, support.

I also want to recognize the CBL faculty and staff members who contributed their time and unique skills to ensure the success of this research project. Specifically, I'd like to thank Dr. Rodger Harvey and Rachel Pleuthner for their assistance with lipid analyses; Dr. Tom Miller for providing the supplies and equipment to conduct calorimetric analyses; Bud Millsaps for his diligent oversight of the Fisheries Research Center and for his assistance with culturing algae; Kenny Rager, Christy Rager, Mark Griffin, Coach Dale Garner, and the rest of the maintenance staff for their assistance with laboratory setup and upkeep; and, of course, Dr. Carys Mitchelmore for providing the frog eggs that made this work possible. Thanks to the CBL GEC committee for providing funding that allowed me to present my research at national conferences, and thanks also to the UMB GSA for awarding the Graduate Student Research Grant that helped cover the cost of lab supplies.

I would like to thank my labmates in both the Rowe and Heyes labs for their unwavering encouragement and friendship. Special thanks to my best mate, Sarah Downey Ghorpade, for listening to and commiserating with me over the years. I can't convey how important your friendship continues to be to me. Thanks also to my friend and fellow adventurer, Lizzy Jenny, for sharing her creativity, imagination, and excitement for life! Thank you, Emily Stefansson, for your encouraging "pep talks" and for providing the PBR to get me through the bad days. Best of luck with your research! I also want to thank the now Dr. Karen Eisenreich for providing comforting guidance as a senior student and friend from the moment I arrived at CBL in 2007. We finally made it!

Thanks to my Solomons friends: Mariana, Kathleen, Alison, Emily, Todd, the surly Travis Burrell, Kyle, Eli, Owen, Brian, Paine, Becca and the rest of the happyhour-ers. You made life in Southern Maryland so much fun! Thank you to my organic chemistry labmates Kristlyn Araujo, Eileen Beard, Brian Morris and Jay Richards for getting me started. Special thanks to my former roommates and dear friends Melissa

McClendon and Jason Edwards. And thanks especially to Elaine Proctor for watching out for me and the rest of her “kids”. We appreciate it!

I want to thank my parents for their respect, love and support during my lengthy graduate career. Thank you for instilling an appreciation for nature in your children. Thank you to my best friend and sister, Christina, for her steadfast love and acceptance. I’d also like to thank my extended family, my aunt and grandmother in particular, for their unending love and encouragement.

Above all, I want to thank Erin for her help in the lab, for sacrificing many hours to proofread my work, and most of all, for standing beside me through the years. Thank you for waiting for me. I am excited to finally begin our next great adventure together!

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List of Abbreviations

ad lib – ad libitum or “without limit”
ANCOVA – analysis of covariance
ANOVA - analysis of variance
CA – coal ash
dw – dry mass
FFA – free fatty acids
FID – flame ionization detector
GLM – general linear model
IC-ICP-DRC-MS - ion chromatography inductively coupled plasma dynamic reaction cell mass spectrometry
ICP-MS – Inductively Coupled Plasma Mass Spectrometry
LOEC – lowest observed effect concentration
MANOVA – multivariate analysis of variance
Met – methionine
µXANES – micro-x-ray absorption near-edge spectroscopy
Na_2SeO_3 – sodium selenite
ND – non-detect
NR – not reported
PL – phsopholipids
RfD – reference dose
RMR – routine metabolic rate
S – sulfur
S.E. – standard error
SeCys – selenocysteine
SeMet – selenomethionine
SeO_2 – selenium dioxide
SeO_3^{2-} - selenite
SeO_4^{2-} - selenate
SMR – standard metabolic rate
SRM – standard reference material
ST – sterols
SVL – snout to vent length
T_3 – triodothyronine
T_4 – thyroxine
TAG – triacylclycerides
TL – total lipid
TLC – thin layer chromatography
USEPA – United States Environmental Protection Agency
WE – wax esters
ww – wet mass

Chapter 1: Introduction

In 2009, approximately 1.8 trillion kilowatt hrs of electricity was produced from coal combustion in the U.S. That is equivalent to roughly 45 % of the electricity generated in the U.S. that year (EIA 2009). The solid waste, or coal ash (CA), that remains after coal combustion is a complex mixture comprised of mostly inorganic compounds. (See review by Rowe, et al. 2002 for a detailed summary of the chemical constituents of CA.) While the specific components are highly dependent on the mineral composition of the parent coal, CA is generally dominated by nonvolatile aluminum, silicon, calcium and iron oxides (EPRI 2009a). Smokestack residues, collected by scrubber or precipitator units within the power plant, combined with these nonvolatile combustion residues can return some volatile compounds (Hg, Se) to the final CA waste pool (Rowe, et al. 2002). Although trace elements, such as As, Cd, Hg, and Se only make up about 1 % of the total mineral content in the final CA mixture (EPRI 2009a), these constituents are of primary concern in the environment (Carlson and Adriano 1993).

An estimated 92 million tons of CA is produced in the U.S. each year. Of that, 40 % is reused in other applications (concrete and cement manufacturing, various fill applications, and mine reclamation) and 60 % is disposed of in dry landfills or aquatic settling basins. An estimated 40 % (approximately 22.1 million tons per year) is sequestered in aquatic settling basins, where solid ash particles are combined with water and, over time, allowed to settle out of the water column. Most of these basins are housed on-site at coal-fired power plants (EPRI 2009a). Coal ash storage in aquatic settling basins is cheap and, apart from perfunctory measures to prevent direct human

health risks, not well regulated (Carlson and Adriano 1993, Rowe, et al. 2002). In 1993 (USEPA 1993) and again in 2000 (USEPA 2000), the U.S. Environmental Protection Agency maintained that CA does not warrant federal regulation as a hazardous solid waste. Results of a 2009 evaluation are pending (EPRI 2009b). Therefore, CA disposal guidelines fall under state-specific non-hazardous waste regulations that vary in rigor and scope. Most disposal sites employ liner systems to prevent leachate release into groundwater (EPRI 2009a). These measures, combined with the restriction of most disposal sites to power plant property, are thought to reduce risks to human health (EPRI 2009a, b). However, CA exposure risks to wildlife remain (Lemly 2002, Rowe, et al. 2002). The impacts of CA released into the aquatic environment are variable and depend mainly on the specific constituents of the ash and the characteristics of receiving waters. Coal ash inputs have been shown to impact water conductivity, turbidity, temperature, and sedimentation, but the most obvious effects of coal ash releases into aquatic systems are on pH and dissolved element concentrations (Carlson and Adriano 1993).

Both the proximity of CA retention basins to natural water bodies and their use as habitat by wildlife present exposure risks to aquatic and semi-aquatic organisms. Aquatic CA disposal requires the management of large volumes of water; therefore, these disposal sites are often constructed near natural water bodies. Coal ash disposal basins may become hydrologically connected to those water bodies either by design or by seasonal fluctuations in the local water table (Rowe, et al. 2002). Belews Lake, NC is a particularly well studied example of wildlife exposure to CA brought about as a direct consequence of the CA waste handling system design. At that site, runoff water from aquatic CA settling basins at a neighboring coal-fired power plant was pumped directly

into Belews Lake from the mid 1970s to the mid 1980s. This water was enriched in trace elements associated with CA, in particular, high concentrations of dissolved Se (150 – 200 $\mu g\ l^{-1}$) (Lemly 2002, Young 2010a; see Table 1.1 for trace element concentrations observed in different media from Belews Lake.) Alterations in fish community structure in the lake were observed shortly after ash runoff inputs began (Olmsted 1986). Although no large-scale fish kills were recorded, 19 of the 20 fish species residing in the lake were eliminated by the time CA runoff inputs were ceased in 1986 (Lemly 2002). The fishery collapse at Belews Lake was attributed to high Se inputs from CA runoff and subsequent Se bioaccumulation observed in the local aquatic food chain (Olmsted 1986, Lemly 2002, Young 2010a). Selenium exposure reduced reproductive fitness of adult fish by causing sublethal impairments such as gill hemorrhaging and edema. Teratogenic effects of Se exposure to developing embryos and larvae were primarily responsible for causing the gradual collapse of the fishery (Lemly 2002).

In addition to CA runoff, disposal basins may attract wildlife such as amphibians or migrating waterfowl. Amphibians are especially at risk of CA exposure, as they often breed in CA basins or marshes formed from basin runoff (Rowe, et al. 2002, Hopkins, et al. 2006). Amphibians have a highly permeable integument, which facilitates dermal respiration but also leaves them highly susceptible to dissolved contaminants compared to other taxa (Sparling 2003). Typically, aquatic larvae will have greater contaminant exposures because they are often confined to a small habitat, with limited food sources (Roe, et al. 2005). Metamorphosis from fully aquatic larvae to semi-aquatic or fully terrestrial adults is an energetically costly process that occurs in most anuran and many urodelian species. Maintaining homeostasis during toxicant exposures can increase

energetic demands in these animals to non-sustainable levels. A negative energy budget makes survival through metamorphic climax less likely. Reductions in survival through metamorphosis can translate into reductions in reproductive adult populations, which will further reduce survival of future generations, should contamination persist (Rowe, et al. 1998b). The complex life histories of many amphibians also provide a contaminant transport link from aquatic to terrestrial communities (Roe, et al. 2005, Unrine, et al. 2007a).

Many ecological studies conducted at the U.S. Department of Energy's Savannah River Site in Aiken, SC focused on the impacts direct exposure to CA and indirect exposure to CA runoff can have on amphibian species. The coal fired power plant has been in operation at the Savannah River Site for over 50 years and employs aquatic settling basins to dispose of CA (Young 2010a). These settling basins hold combined sluiced fly and bottom ash. Effluent from these settling basins pass into a discharge swamp and finally enter a tributary of the Savannah River (Rowe, et al. 1996; see Table 1.1 for trace element concentrations observed in different media from the Savannah River Site.) Unrine et al (2007a) determined that bullfrog (*Rana catesbeiana*) larvae collected from the CA discharge swamp had accumulated higher trace element concentrations than many macroinvertebrates and fish at the same site. Rowe et al (1998b) observed significantly increased metabolic rates and trace element (As, Ba, Cd, Cr, and Se) concentrations in bullfrog larvae collected from or reared in ash basins on the Savannah River Site. High incidences of craniofacial and axial deformities in larval bullfrogs reared in CA settling basins at the site were also recorded. These malformations severely reduced feeding efficiency and swimming performance (Rowe, et al. 1996, Hopkins, et

al. 2000). The prevalence of axial malformations, in particular, corresponded with larval habitat proximity to CA inputs. Of bullfrog larvae collected from the ash basin, 36 % displayed axial malformations, while 17 % collected from the swamp receiving CA effluent exhibited these same malformations, and only 4 % collected from a reference area were malformed (Hopkins, et al. 2000). Reductions of larval survival in bullfrogs (Rowe, et al. 1998b) and southern toads (*Bufo terrestris*; Rowe, et al. 2001) were also observed in the ash basins at this site. Larvae were not the only amphibian life stage to show CA exposure effects at this site. High trace element (As, Se and V) concentrations were also observed in adult southern toads inhabiting ash basins. Accumulation of these elements was observed in adult toads transplanted to those basins from a reference site within seven weeks of relocation (Hopkins, et al. 1998).

Fish mortality at Belews Lake and the increased mortality and malformations observed in larval amphibians at the Savannah River Site, were strongly associated with increased Se concentrations from CA inputs into those environments (Lemly 1993, Rowe, et al. 1996, Hopkins, et al. 1998, Hopkins, et al. 2000, Rowe, et al. 2001, Lemly 2002). As with all trace elements in CA, Se content is highly variable and dependent on concentrations in the parent coal and the volatile element sequestration technologies in place at the facility at which it was burned. For example, mean Se concentrations in CA samples taken from 42 power plants ranged between <2 - 47 $\mu g\ g^{-1}$ dry mass (Rowe, et al. 2002). Selenium is an essential micronutrient, necessary only at very low concentrations, that becomes toxic when intake surpasses an organism's homeostatic ability to store or excrete excess (Goyer 2001). Typical surface water Se concentrations range from 0.1 – 0.3 $\mu g\ l^{-1}$ and aqueous concentrations of 1 – 5 $\mu g\ l^{-1}$ have been shown to be toxic to

wildlife (Lemly 1993). However, it is now a widely accepted principle that chronic exposure to Se bioaccumulated in sediment and biota is more toxic than acute exposure to dissolved Se in aquatic systems (Fan, et al. 2002, Hamilton 2004). Dissolved Se concentrations, reported by Rowe et al (2002), in sites impacted by CA discharge range from approximately 3 – 110 $\mu g\ l^{-1}$, depending on site characteristics and type of discharge. Selenium concentrations in sediments from these sites ranged from 5.6 – 8.93 $\mu g\ g^{-1}$ dry mass. Selenium can persist in sediment, leading to chronic exposure in benthic organisms. Lemly (1997) reported sediment Se concentrations of 1 - 4 $\mu g\ g^{-1}$ dry mass in Belews Lake more than two decades after Se-laden ash effluent discharge had ceased. Findings by Hopkins et al (2000) confirmed Se bioaccumulation from CA discharge and contaminated sediment to larval bullfrogs at the Savannah River Site. Sediment from the ash basin and discharge swamp contained 6.11 ± 0.3 and 7.11 ± 1.11 $\mu g\ g^{-1}$ dry mass of Se, respectively, while bullfrog larvae collected at each had tissue Se concentrations of 20.25 ± 2.11 and 27.93 ± 1.8 $\mu g\ g^{-1}$ dry mass, respectively. Furthermore, Rowe et al (2001) report elevated Se concentrations (11.85 $\mu g\ g^{-1}$ dry mass) and other CA-associated trace elements in periphyton grown in an ash basin on the Savannah River Site, indicating uptake and Se accumulation by primary producers. In fact, there was strong evidence of Se bioaccumulation in aquatic food webs of both Belews Lake and the Savannah River Site (Hopkins, et al. 2000, Lemly 2002, Unrine, et al. 2007a).

Selenium toxicity is not adequately predicted by total Se concentrations however, but is highly dependent on speciation (Lenz and Lens 2009). Selenium is chemically analogous to sulfur (S) and likely follows a biogeochemical cycle similar to that of S in the natural environment (Figure 1.1; Fan, et al. 2002). The portion of the Se

biogeochemical cycle that is believed to take place in the aquatic environment is driven by biotic transformation of Se-containing compounds (Young 2010b). Much of what is currently understood of Se biotransformation, however, has been derived from agricultural, nutritional and medical research (Fan, et al. 2002). It is thought that dissolved Se in the aquatic environment exists as inorganic Se oxyanions, predominantly selenite (IV) or selenate (VI), and to a lesser extent selenide (-II). These inorganic Se-compounds can be biotransformed by some primary producers to organic Se-containing compounds, such as the amino acids selenocysteine (SeCys) and selenomethionine (SeMet; Schrauzer 2000, Fan, et al. 2002, Young 2010b). Selenomethionine is considered the principal organic Se-containing compound produced by autotrophs in the aquatic food chain (Young 2010b). The biosynthesis of SeMet from selenite and selenate precursors that occurs in some algae and terrestrial plants is shown in Figure 1.2 (reprinted from Schrauzer 2000). Higher organisms are not capable of synthesizing the essential S-based amino acid, methionine (Met), thus it must be provided through dietary sources. The same is true of SeMet. Selenomethionine and Met are incorporated into tissue protein stores of consumers and can be released as those stores are depleted. The presence of SeMet has been shown to increase activity of some proteins, while decreasing the activity of others, compared to their S-containing moieties (Schrauzer 2000). Organic Se-containing compounds, such as SeMet, are more bioactive and bioavailable than inorganic Se-containing compounds and are therefore considered to be more toxic (Fan, et al. 2002). Because of the complicated analytical extraction procedures and instrumentation required to investigate Se speciation in biological matrices, few studies have quantified Se biotranformation in aquatic food webs. Most only compare total Se

concentrations at different trophic levels (Hopkins, et al. 2000, Lemly 2002, Rowe, et al. 2002, Unrine, et al. 2007a). A study by Fan et al (2002) examining Se speciation in organisms from Kesterson Wildlife Refuge (San Joaquin Valley, CA), an aquatic ecosystem contaminated by seleniferous agricultural drainage water, is an exception. Selenium species, specifically SeMet, were quantified in tissue samples from microphytes, macroinvertebrates, fish and aquatic birds collected at Kesterson. Results confirmed Se biotransformation and accumulation as SeMet through the sampled food web.

Nutritional and agricultural studies using mammals form the historical basis of our understanding of Se activity and toxicity (Goyer 2001, Lenz and Lens 2009). Our current understanding of Se ecotoxicology stems from field studies conducted following large-scale ecological disasters, Belews Lake and the contamination of Kesterson Wildlife Refuge being two of the most well studied (Young 2010b). These studies and the laboratory-based investigations that followed focused largely on fish (Lemly 1993, Young 2010a). While amphibians have been examined with respect to effects of the mixture of contaminants in whole ash effluents, there is a lack of ecotoxicological studies addressing the specific effects of Se in the aquatic environment on amphibians (Ohlendorf, et al. 1988, Fan, et al. 2002). The aim of this thesis was to conduct controlled laboratory studies comparing toxicological effects of dietary exposures of CA, an organic Se-containing compound (SeMet) and an inorganic Se-containing acidic oxide (selenium dioxide or SeO_2) to a larval amphibian (*Hyla versicolor*). *Hyla versicolor* is a common arboreal anuran native to the eastern United States. The abbreviated larval

period of *H.versicolor*, roughly 65 d in the wild (Dickerson 1906), make it ideal for laboratory-based toxicity studies.

Assessing toxicity at the larval stage is important, as high mortality at this stage could negatively impact overall community structure by reducing future breeding populations. Previous dosing studies indicated that typical laboratory feeding conditions may enable test organisms to compensate for dosage effects by physiological means not expressed under natural feeding regimens (Lemly 1993, Hopkins, et al. 2002, Rowe, et al. 2009). For instance, wild *H. versicolor* larvae, like the larvae of many anuran species, generally subsist on algae and detritus. Consequently, it is difficult to extrapolate results of laboratory studies utilizing more nutrient-rich, commercial tadpole feed to occurrences in natural systems. Therefore, filamentous green algae was used in studies presented in this thesis to better mimic the natural diet of *H. versicolor* larvae. These studies were conducted in order to satisfy three major research objectives:

1) Determine CA and SeMet dietary exposure toxicity to *H. versicolor* larvae.
2) Determine the impact resource limitation has on CA and SeMet dietary exposure toxicity to *H. versicolor* larvae.
3) Compare the SeMet and SeO_2 dietary exposure toxicity to *H. versicolor* larvae under resource-limited conditions.

The first study, discussed in Chapter 2, addressed the first two objectives. This study assessed chronic toxicity of dietary exposures to CA or SeMet alone and in combination with resource limitation as a possible co-stressor. In the natural environment, resource

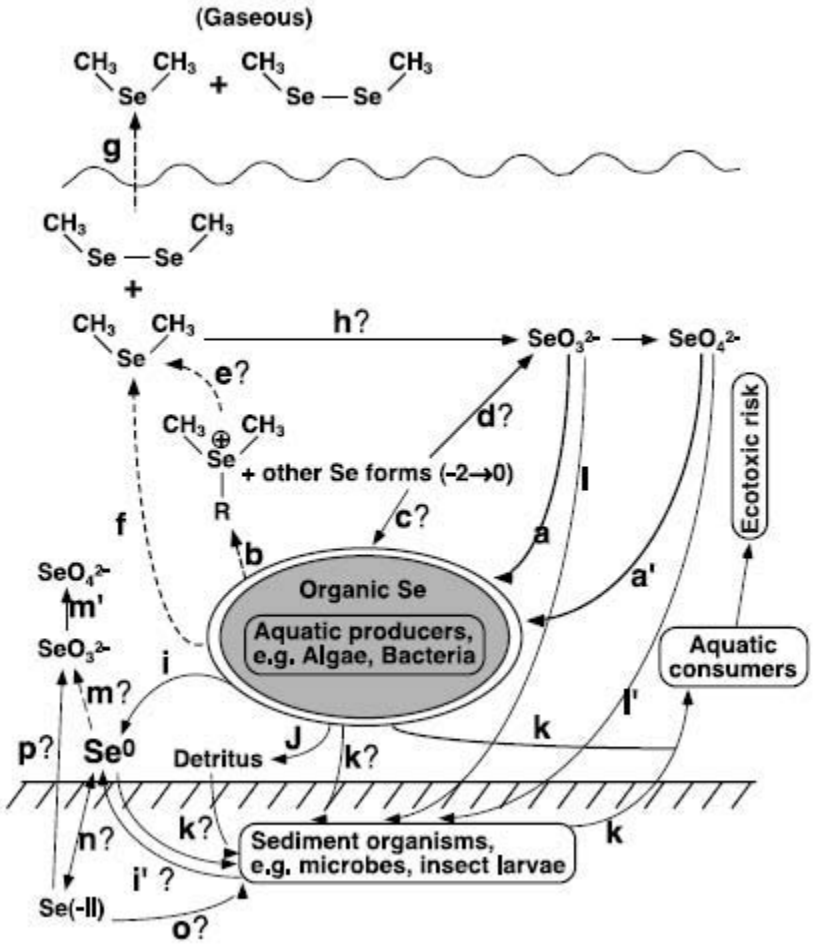
limitation might occur independent of, or as a consequence of, chemical contamination (Hopkins, et al. 2002). Previous CA and Se dosing studies revealed that resource limitation, a natural co-stressor not typically incorporated into laboratory toxicity experiments, can augment the toxicities of these compounds to fish and larval anurans (Hopkins, et al. 2002, Rowe, et al. 2009). Therefore, in this study, each chemical treatment was dosed to larvae via a food-limited (rationed) and an unlimited (*ad libitum* or "*ad lib"*) diet. Lethal and sublethal endpoints were monitored in order to characterize the toxicities of each chemical and food provision level combination to developing *H. versicolor* larvae. Sublethal endpoints, such as respiration rates, growth rates, timing of and mass at metamorphosis, and lipid concentrations at metamorphosis, were chosen due to their influence on adult anuran survival (Scott 1994, Rowe 2003, Scott, et al. 2007). The second feeding study, conducted in parallel to the first and discussed in Chapter 3, addressed the third objective. This study assessed chronic toxicity of dietary exposures to an organic Se-containing compound, SeMet, and an inorganic Se-containing compound, selenium dioxide (SeO_2) under a food-limited (rationed) feeding regimen. Selenium dioxide is an uncharged acidic oxide similar to the oxyanion, selenite. As in selenite, the Se atom in SeO_2 is in the (IV) oxidation state. However, SeO_2 was considered more appropriate for use with a freshwater organism as it does not require application as a Se salt, unlike selenite (Na_2SeO_3). The comparative toxicities of SeO_2 and SeMet were of particular interest, as previous work with other taxa had demonstrated that Se toxicity is heavily dependent on chemical speciation (Maier, et al. 1993, Hamilton 2004, Lenz and Lens 2009). The same larval amphibian species (*H. versicolor*) and toxicity endpoints employed in Chapter 2 were also used in this study.

Table 1.1. Trace element concentrations in environmental media from CA-contaminated areas

Site Description	Environmental Medium	As	Cd	Cr	Cu	Pb	Se
Belews Lake, NC	Ash effluent (µg l^{-1})	190 - 253	NR	NR	NR	NR	157 - 218
	Lake water (µg l^{-1})	6.6	NR	NR	NR	NR	12.6
	Lake Sediment (µg g^{-1} dw)	31.2 - 59.8	NR	NR	NR	NR	6.1 - 8.9
Savannah River Site, SC	Ash settling basin (µg l^{-1})	17.2 - 46.0	0.11-0.3	0.44	2.5	0.1	7
	Ash basin sediment (µg g^{-1} dw)	49.4 - 70.8	0.57 - 0.72	23.9	71.8 - 84.7	45.2	6.11 - 6.21
	Drainage swamp sediment (µg g^{-1} dw)	28.9 - 116.6	1.38-2.32	22.04	43.5 - 147.5	66.2	7.11 - 7.78

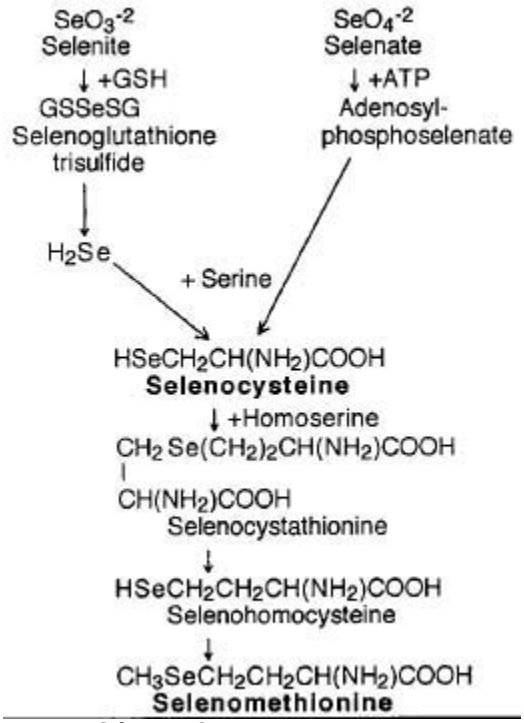
Ranges of concentrations presented in Rowe, et al. 2002 - "NR" indicates element concentrations that were not reported.
Units of measurement for reported values are in parentheses.

Figure 1.1. Selenium biogeochemical cycle



Reprinted from Fan, et al. 2002

Figure 1.2. Conversion of selenite and selenate to selenomethionine



Reprinted from Schrauzer 2000

Chapter 2: Effects of dietary exposure to coal ash and selenomethionine in larval gray tree frogs (*Hyla versicolor*)

Introduction

Approximately 1.8 trillion kilowatt hrs of electricity was produced from coal combustion in the U.S. in 2009, equivalent to 45 % of the electricity generated in the U.S. that year (EIA 2009). An estimated 92 million tons of solid waste, hereafter referred to as coal ash (CA), was produced that year as a byproduct of coal combustion. Of the approximately 60 % of CA produced that was not reused in other applications (concrete and cement manufacturing, various fill applications, and mine reclamation), an estimated 40 % was disposed of in aquatic settling basins (EPRI 2009a). Aquatic CA disposal requires the management of large volumes of water; these disposal sites are often constructed near natural water bodies. The basins are designed to allow solid ash particles to slowly settle out of the water column, while overlying water is diverted to prevent overflow. Coal ash storage in aquatic settling basins is inexpensive and, apart from perfunctory measures to prevent direct human health risks, is not well regulated (Carlson and Adriano 1993, Rowe, et al. 2002). Most are constructed on-site at coal-fired power plants (EPRI 2009a), further limiting exposure to the general population. In 2000 (USEPA 2000), the U.S. Environmental Protection Agency maintained that CA did not warrant federal regulation as a hazardous solid waste. Therefore, CA disposal guidelines fall under state-specific non-hazardous waste regulations that vary in rigor and scope.

Although direct human health risks are low (EPRI 2009a, b), CA exposure risks to wildlife remain (Lemly 2002, Rowe, et al. 2002). Both the proximity of these sites to natural water bodies and their use as habitat by wildlife present exposure risks to aquatic

and semi-aquatic organisms. Amphibians are especially at risk, as they will often breed in ash basins or marshes formed from ash basin runoff (Rowe, et al. 2002). Their highly permeable integument leaves them susceptible to dissolved trace elements released from CA deposits (Carlson and Adriano 1993, Rowe, et al. 2002, Sparling 2003). Their complex life cycles also increase their exposure and susceptibility to CA. Fully aquatic larvae confined to a small contaminated habitat have greater CA exposures compared to the more mobile terrestrial life stage (Roe, et al. 2005). These larvae may retain contaminants in tissues throughout metamorphosis and introduce those contaminants into the terrestrial food chain when they emerge as semi-aquatic adults (Roe, et al. 2005, Unrine, et al. 2007a). Metamorphosis is also an energetically costly process. Maintaining homeostasis during toxicant exposures can increase energetic demands in metamorphosing individuals to non-sustainable levels, making survival through metamorphic climax less likely (Rowe, et al. 1998b).

The U.S. Department of Energy's Savannah River Site in Aiken, SC is one of the most rigorously studied CA-contaminated aquatic sites in the country. The coal-fired power plant at the Savannah River Site has operated for more than 50 y and employs aquatic settling basins to manage CA generated at the facility (Young 2010a). Much of the research conducted at the Savannah River Site has focused on the impacts direct CA exposure and exposure to CA basin effluent has on amphibian species. Bullfrog (*Rana catesbeiana*) larvae, collected from a swamp on the Savannah River Site receiving CA discharge, accumulated higher trace element concentrations (As, Ba, Cd, Cr, and Se) than many macroinvertebrates and fish at the same site (Rowe, et al. 1998b, Unrine, et al. 2007a). Rowe et al. (1998b) also observed significantly increased metabolic rates in

these larvae compared to larvae from a nearby reference site. Researchers also recorded high incidences of craniofacial and axial deformities in larval bullfrogs reared in CA settling basins at the Savannah River Site. These malformations severely reduced feeding efficiency and swimming performance (Rowe, et al. 1996, Rowe, et al. 1998a, Hopkins, et al. 2000). In particular, the prevalence of axial malformations corresponded with larval habitat proximity to CA inputs. Of bullfrog larvae collected from an ash basin, 36 % displayed axial malformations, 17 % collected from the swamp receiving CA effluent exhibited these same malformations, while only 4 % collected from a reference area were malformed (Hopkins, et al. 2000). Larval anuran survival was also reduced within the ash basins at the Savannah River Site (Rowe, et al. 1998b, Rowe, et al. 2001). High trace element (As, Se and V) concentrations were observed in adult southern toads inhabiting these ash basins and trace element accumulation occurred in adult toads relocated to those basins from a reference site (Hopkins, et al. 1998).

The mortality and malformations observed in larval anurans at the Savannah River Site were associated with increased Se as well as other trace element concentrations from CA inputs (Rowe, et al. 1996, Hopkins, et al. 1998, Hopkins, et al. 2002, Rowe, et al. 2002). Selenium is an essential micronutrient involved in cellular regulation of oxidative stress and metabolism. Selenium toxicity occurs when an organism's intake surpasses its ability to store or excrete excess (Goyer 2001). As with all trace elements in CA, Se content is highly variable (ranging from $< 2 - 50$ µg g^{-1} dry mass) and is dependent on the composition of the parent coal and the volatile element sequestration technologies used at each combustion facility. Dissolved Se concentrations in aquatic sites impacted by CA discharge range from approximately $3 – 110$ µg l^{-1},

depending on site characteristics and the type of discharge received (Rowe, et al. 2002). However, it is now widely accepted that dissolved Se concentrations alone do not adequately predict toxicity to aquatic organisms (Fan, et al. 2002, Lemly 2002, Hamilton 2004). Chronic exposure to Se accumulated in sediment and biota present greater toxicity risks within aquatic systems (Fan, et al. 2002, Hamilton 2004).

Selenium is chemically analogous to sulfur (S) and likely follows a similar biogeochemical cycle in the environment (Fan, et al. 2002, Young 2010b). Fan et al (2002) provided a comprehensive review of Se cycling in the aquatic environment, as it is currently understood (Figure 1.1). Dissolved Se exists in either the (IV) or (VI) oxidation state as selenite (SeO_3^{2-}) or selenate (SeO_4^{2-}), respectively (Fan, et al. 2002), once released into aquatic ecosystems. Laboratory studies investigating the effects of these dissolved Se species to algae, invertebrates, fish (Lemly 1993, Maier, et al. 1993, Rosetta and Knight 1995), and waterfowl (Heinz, et al. 1988) revealed low toxicity risks. These same studies indicated Se-contamination toxicity risks increased substantially when aquatic producers biotransformed these inorganic Se-containing compounds into organic, proteinaceous Se-containing compounds. Organic Se-containing molecules are typically formed when Se substitutes for S atoms during amino acid synthesis in high-Se environments (Schrauzer 2000). Selenomethionine (SeMet) is the primary organic Se-containing compound formed at the base of aquatic food chains (Young 2010b) and is also the most toxic to aquatic life (Heinz, et al. 1988, Lemly 1993, Maier, et al. 1993, Rosetta and Knight 1995). Figure 1.2 describes the reaction by which SeO_3^{2-} and SeO_4^{2-} can be converted to selenocystine (SeCys) and finally SeMet within some aquatic bacteria, algae, and macrophyte species (Schrauzer 2000, Fan, et al. 2002).

Selenomenthionine is thought to cause a majority of the toxic effects of Se contamination in aquatic environments because of its structure and bioaccumulative properties (Fan, et al. 2002).

While studies at the Savannah River Site provided a breadth of information concerning CA toxicity to amphibians, there is a lack of laboratory-based ecotoxicological studies specifically addressing the effects of Se contamination to these organisms (Ohlendorf, et al. 1988, Fan, et al. 2002). Selenium in CA from the Savannah River Site exists predominately in the (IV) oxidation state (Jackson and Miller 1998), while selenium in SeMet is in a reduced form (-II) (Fan, et al. 2002). Comparative toxicity studies of Daphnia and bluegill sunfish suggest that SeMet is more acutely toxic than inorganic Se-containing compounds (SeO_3^{2-} and SeO_4^{2-}) (Besser, et al. 1993, Maier, et al. 1993). The current study was designed to contrast the effects of dietary-dosed CA and SeMet to larval *H. versicolor*. Previous CA and Se dosing studies revealed resource limitation, a natural co-stressor not typically addressed in toxicity experiments, can augment the toxicities of these compounds to fish and larval anurans (Hopkins, et al. 2002, Rowe, et al. 2009). To evaluate resource limitation effects on CA and SeMet toxicity to developing *H. versicolor*, both were dosed via a food-limited (rationed) and an unlimited (*ad libitum* or *"ad lib"*) diet.

Hyla versicolor is a common arboreal anuran native to the eastern United States. The abbreviated larval period of *H.versicolor*, roughly 65 d post-hatch in the wild (Dickerson 1906), make it ideal for laboratory-based toxicity studies. We considered numerous lethal and sublethal endpoints to characterize the toxicities of each chemical and food provision level combination to *H. versicolor* larvae and metamorphs. Sublethal

endpoints, such as respiration rates, growth rates, timing of and mass at metamorphosis, and lipid concentrations at metamorphosis, were chosen due to their influence on adult anuran survival (Scott 1994, Scott, et al. 2007).

Methods

Algae Collection and Food Preparation

Filamentous green algae was collected from man-made ponds in Calvert County, Maryland USA. Algae was dehydrated, homogenized, and stored at -4 °C prior to incorporation into tadpole food. Tadpole food consisted of dehydrated algae suspended in a gelatin/agar matrix. These components were combined in a 0.7:1:5:100 g mass-ratio of agar, gelatin, dehydrated algae, and deionized water, respectively. Agar and gelatin were stirred into boiling deionized water for one minute and poured over the dehydrated algae in a shallow glass baking dish. Aqueous solutions of seleno-DL-methionine (SeMet) (Sigma-Aldrich Co.) equivalent to 116.5 µg SeMet g^{-1} wet mass (ww) of food (SeMet high dose) or 11.65 µg SeMet g^{-1} ww of food (SeMet low dose) were added as each gel mixture was cooling to produce nominal concentrations of 50 µg Se g^{-1} ww and 5 µg Se g^{-1} ww , respectively. Coal ash (collected from the margin of an aqueous ash settling basin at the D-Area Power Facility, U.S. Department of Energy's Savannah River Site, Aiken, SC) was added to the cooling food mixture to yield a nominal concentration of approximately 5 µg Se g^{-1} ww, based on previous chemical analyses of the coal ash. Solidified food mixtures were partitioned into 4 g spherical pellets and stored at -4 °C. Dosed food was stored in separate containers to avoid cross-contamination. Subsamples from successive batches of dosed food were lyophilized and analyzed via micro-bomb calorimetry (Parr Instrument Co). Control, SeMet low, and SeMet high food had similar

average caloric contents (4.201 ± 0.049 Cal g^{-1}, 4.23 ± 0.039 Cal g^{-1}, 4.17 ± 0.092 Cal g^{-1}, respectively). Coal ash food had slightly higher caloric content, on average (4.72 ± 0.255 Cal g^{-1}), but the caloric content was highly variable among batches (Table 2.1).

In addition to chemical treatments, this experiment involved a resource-limitation component. Each food-type was provided to larvae via a food-limited (rationed) and an unlimited (*ad libitum* or *"ad lib"*) diet. Feeding regimens were developed based on estimates of energetic requirements calculated from standard metabolic rate (SMR) measurements of 45 larvae not included in this experiment. The median oxygen consumption rate of these fasted individuals, measured by microrespirometry (Micro-Oxymax; Columbus Instruments), was 1.58 µl g^{-1} min^{-1}. This value was converted to units of energy (Joules) according to the relationship: 1 µl min^{-1} oxygen consumed is equivalent to 28.9152 J. A 20 % correction factor was added to compensate for growth and individual variability. The final value was converted to a daily energy requirement expressed as Cal d^{-1}. Tadpole food rations were adjusted based on caloric measurements of control food (micro-bomb calorimetry, Parr Instrument Co). This ensured that organisms receiving rationed diets were still provided adequate calories to maintain survival. A*d lib* replicate tanks received one food pellet every 24 h., while rationed replicate tanks received one food pellet every 48 h. This study was continued until Exposure Day 78, when larvae in all replicate tanks of a single treatment had either died or initiated metamorphosis. This prevented remaining treatments from receiving disproportionately lengthy exposures.

Egg Collection

Three clutches of freshly laid *H. versicolor* eggs were collected on two consecutive evenings in late August 2009 in Saint Mary's County, MD. Eggs were transported to the Chesapeake Biological Laboratory (Solomons, MD) and held in 10 gallon tanks of aerated well water until hatch. Larvae were fed control algae/gel food for one month prior to study initiation.

Experimental Protocol

This study assessed effects of dietary exposures to SeMet or CA alone and in combination with resource limitation as a possible co-stressor over a 78-day time period. Upon reaching Gosner Stage 27 (Gosner 1960), 480 larvae were randomly allocated to 32 polypropylene tanks (Sterilite Corp.) (15 larvae per tank) containing 4 l of aged, UV sterilized well water. Four replicate tanks were assigned per treatment, with a total of eight treatments: 1) control *ad lib*, 2) control rationed, 3) CA *ad lib*, 4) CA rationed, 5) SeMet Low *ad lib*, 6) SeMet Low rationed, 7) SeMet High *ad lib*, and 8) SeMet High rationed. Tank water was constantly aerated and temperature controlled at 23.0 °C (± 1.3 °C) and a 12 h light cycle was maintained throughout the study. Water and air temperatures were monitored regularly and electronically recorded (Onset Computer Corp) through Day 78. Tanks were cleaned, tank water replaced, and water quality parameters (pH, dissolved oxygen, and conductivity) monitored every fourth day. Water samples were collected from each replicate tank, composited, and acidified with concentrated nitric acid (0.5 % of sample volume) prior to each tank cleaning to quantify Se dissolution from food to water throughout the experiment.

Larval Survival, Mass, and Metabolic Rates- Larval health was inspected daily through Day 78. Intact carcasses were collected when observed, placed into individually

labeled plastic (Whirl-Pak, Nasco) bags, and stored at -80 °C until analysis. Total mass and number of individuals in each tank were recorded on Day 0 and on every fourth day until the experiment was complete or until all larvae in that tank had either reached mortality or initiated metamorphosis. Standard metabolic rates (as mean oxygen consumption by fasted, resting animals) were measured via microrespirometry (Micro-Oxymax; Columbus Instruments) at 21° C on one representative individual from each tank on Day 16 and Day 32 of dosing, following methods described by Rowe et al. (1998b). Oxygen consumption ($\mu l\ g^{-1}\ min^{-1}$) by each individual was measured at 3 h intervals for 24 h.

Metamorphosis- Changes to craniofacial and gastrointestinal structures prevent feeding by metamorphosing *H. versicolor* larvae**.** Therefore, individuals were removed and held unfed in individually labeled containers upon attaining Gosner Stage 42 (indicated by forelimb emergence). As individuals completed metamorphosis (Gosner Stage 46 - after complete resorption of the tail), they were euthanized by ventral application of an over-the-counter oral anesthetic (Orajel, Church and Dwight Co., Inc.), containing 20 % benzocaine (Brown and Cai 2007, Torreilles, et al. 2009). Carcasses were stored at -80 °C prior to lipid and Se content analyses. Wet masses of each metamorphosing individual were recorded at Gosner Stages 42 and 46. Snout to vent length (SVL) of each juvenile frog was also measured prior to sacrifice, using digital calipers. Carcasses of any individuals that died prior to completing metamorphosis were weighed, placed into individually labeled plastic (Whirl-Pak, Nasco) bags, and stored at -80 °C until analysis.

Sample Preparation and Analyses

Selenium Analysis- Selenium concentrations in food and larval and juvenile frog carcasses were determined via Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Agilent 4500). Frozen whole-body carcasses were microwave digested (Milestone Ethos EZ) in 2 – 4 ml of high purity concentrated nitric acid as described by Rowe, et al. 2009. Each batch of 10 digests included 8 carcass samples, a blank, and a matrix spike. Every fifth batch included a standard reference material (SRM) (NIST 1566b Se = 2.06 ± 0.15 $\mu g\ g^{-1}$) in place of one of the carcass samples. Matrix spike Se recoveries averaged 92.55 ± 13.84 % and replicate SRM Se recoveries averaged 100.91 ± 13.05 %. The Se content of pre- and post-storage food samples was also analyzed by ICP-MS. Duplicate samples (approx. 1 g ww each) from each treatment batch made throughout the study were microwave digested following the same method used to prepare carcass samples. Digests were diluted with >18 MΩ deionized water to 10x their original volume, prior to analysis. Acidified treatment tank water samples collected throughout the experiment were also analyzed by ICP-MS.

Lipid Class Analysis- Total lipids were extracted and analyzed by modifying a method employed by Ju and Harvey (2004) to analyze the lipid content of Antarctic krill. Based upon the number of animals completing metamorphosis, three to five juvenile frog carcasses were randomly selected from each treatment, with representatives from each replicate. No animals in the SeMet High treatment survived to metamorphosis. Lyophilized carcasses were microwave digested (MARS-5, CEM) in 35 ml CH_2Cl_2:MeOH (2:1) at 200 psi and 80 °C for 30 min. Post-extraction carcasses were removed from microwave digestion vials, rinsed with solvent, and returned to storage at -80 °C for future Se analysis. Post-extraction solvent remaining in digestion vials was

evaporated (BUCHI Labortechnik AG). Evaporated lipid extracts were dissolved in a few milliliters of CH_2Cl_2:MeOH (2:1), gravity filtered through glass wool, and transferred to 8 ml amber vials. Transfer solvent was evaporated from lipid extracts under N_2 gas. Dried extracts remaining in amber vials were diluted to 1 ml with CH_2CL_2:MeOH (2:1) and stored at -80 °C until analysis.

Separation and identification of major lipid classes were performed by thin layer chromatography (TLC) using an Iatroscan MK-V analyzer equipped with a flame ionization detector (FID), as described by Ju and Harvey (2004). Diluted extracts (1-3 µl aliquots) were applied to the bottom of silica-coated glass rods (S-III Chromarods, Mitsubishi Chemical Medicine Co.). Rods were placed in a 55°C oven for 5 min to evaporate any remaining solvent. When cool, extracts were "focused" into tight bands by wicking CH_2Cl_2:MeOH (2:1) onto the end of rods until solvent fronts reached the extract application points. Solvent was again evaporated from rods for 5 min in the 55°C oven. Lipids in each extract were separated into major classes using a hexane:diethyl ether:formic acid (85:15:0.2) development solution. Rods with "focused" extracts were placed in a closed, glass chamber containing sufficient developing solution to wick onto the bottom of the rods. Rods remained in the development chamber for 20 min, before a final 5 min in the 55°C oven. Development separated extracts into five major lipid classes along the length of the rod. Phospholipids (PL) were the most polar and remained near the origin of extract application. The remaining neutral lipids followed: sterols (ST), free fatty acids (FFA), triacylglycerides (TAG), and wax esters (WE), from bottom to top of the rod, respectively. After development, the rods were placed in the Iatroscan for analysis. The FID flame was applied directly to each rod, ionizing lipids from top to

bottom. Rods were held in racks of 10, therefore three extracts could be processed on a rack (each in triplicate) at one time. A 3 µl aliquot of a lipid standards mixture of known class concentrations was applied to the remaining rod on each rack.

Sample chromatograms were integrated using HP ChemStation software. Lipid classes in each sample were identified and quantified based on a calibration curve generated from serial dilutions of a concentrated lipid standards mixture. The concentrated standard mixture (7.077 µg µl^{-1} total lipid) contained a representative compound from each lipid class: 4.168 µg µl^{-1} of 1,2–dipalmitoyl–sn–glycera–3-phosphocholine (PL), 0.396 µg µl^{-1} of cholesterol (ST), 0.172 µg µl^{-1} of nonadecanoic acid (FFA), 2.150 µg µl^{-1} glycerol trioleate (TAG), and 0.191 µg µl^{-1} palmityl state (WE) (Sigma-Aldrich Co.) in CH_2Cl_2:MeOH (2:1). Total lipid (TL) concentrations for each sample were derived through summation of the lipid class concentrations quantified. Samples exceeding calibration limits for any lipid class were diluted with known volumes of CH_2Cl_2:MeOH (2:1) and reanalyzed. Mean intra-sample precision was ± 16.4 % or better for each class and the average percent recovery of lipid classes in the standards mixtures was 109.14 % ± 12.69 %.

Statistical Analyses

Mean responses from each replicate tank were used in statistical analyses. Data were tested to ensure satisfaction of model assumptions and transformed, if necessary. All statistical differences were evaluated based on $\alpha = 0.05$. Time to forelimb emergence, mass at forelimb emergence, time to complete metamorphosis, mass at complete metamorphosis, and SVL at complete metamorphosis were analyzed for treatment specific differences using the general linear model analysis of variance (GLM

ANOVA) and did not require data transformation. Survival to the end of the study, initiation and completion of metamorphosis proportions were arcsine transformed prior to analysis by GLM ANOVA. Proportions of individuals displaying rear leg deformities and post-forelimb emergence mortality proportions were also arcsine transformed and required non-parametric analysis using the Kruskal-Wallis One-Way ANOVA. Larval SMR were analyzed using the GLM ANOVA for each trial measurements were taken (Day 16 and Day 32 of exposure). Larval and metamorph Se body burdens were $\log_{10}$ transformed and the mean duration of larval exposure was used as a covariate in the GLM analysis of covariance (ANCOVA) of larval and metamorph body burdens. A repeated measures GLM ANOVA was conducted to determine treatment effect on larval growth. To calculate larval growth, the mean larval mass of each treatment was recorded until 10 % of the study population initiated metamorphosis. This prevented the mass losses associated with metamorphosis from artificially deflating mean larval mass values as the study progressed. It was determined that greater than 10 % of individuals had initiated metamorphosis by Exposure Day 34. Larval growth was, therefore, determined until the previous day mass measurements were taken, Day 30. The relationship between size at forelimb emergence and survival to complete metamorphosis, independent of treatment, was analyzed by binary logistic regression. The within-treatment relationship between size at forelimb emergence and mortality prior to complete metamorphosis was also analyzed by binary logistic regression, but only for SeMet Low *ad lib* and ration treatments. In all other treatments, 100 % of individuals that initiated metamorphosis survived to complete it. A general multivariate analysis of variance (MANOVA) was used to determine treatment effects on total lipid and lipid class content of metamorphs

and specific treatment effects on individual classes were evaluated using univariate ANOVA.

Results

Selenium in Food

Average Se concentrations in food prior to storage were 0.52 ± 0.09, 3.58 ± 0.3, and 35.55 ± 3.4 µg g^{-1} ww in CA, SeMet Low, and SeMet High treatments, respectively (Table 2.1). Mean Se concentration in control food was below the instrumental detection limit (0.001 µg g^{-1} ww). Mean calculated dw Se concentrations are also presented in Table 2.1. Between 71.6 % and 71.1 % of the target ww Se concentrations were achieved in SeMet Low and High food, respectively. However, only 13.1 % of the target ww Se concentration was achieved in CA food. Food was stored at -4 °C for a maximum of two months. Selenium concentrations (dw) in food were not considerably altered by storage (Table 2.2). The observed increases in Se concentrations are likely due to water loss during storage, as the dw concentrations presented in Table 2.2 were calculated based on the results of wet sample analyses.

Selenium dissolution from food was minimal. Table 2.3 includes the mean and the maximum dissolved Se concentrations (µg ml^{-1}) measured in treatment tank water throughout the experiment prior to any of the 4 d water changes. Tank water was replaced with fresh well water every four days. Selenium concentrations in control and CA treatments were similar to background concentrations in well water.

Selenium Body Burdens

Mean larval tissue Se concentrations per treatment (µg g^{-1} ww) and corresponding mean experimental dates of larval carcass collection are presented in Table 2.4. Experimental date of carcass collection ("Collection date" in Table 2.4) was used as an estimate of exposure duration and was employed as a covariate in *post-hoc* tests. Only one carcass from the CA *ad lib* treatment was available for Se analysis. Therefore, this treatment was excluded from statistical analyses. Larval carcass Se concentrations differed significantly between treatments ($F = 37.38$, $p < 0.0001$) and collection date significantly influenced these differences ($F = 23.99$, $p < 0.0001$), based on *post-hoc* comparisons. Selenium concentrations in larvae from both control *ad lib* and ration treatments and the CA ration treatment did not differ. The Se concentrations did not differ significantly between *ad lib* and ration treatments of the SeMet High dose ($p = 1.000$) or the two SeMet Low dose feeding regimens ($p = 0.9982$). Larvae from the SeMet High *ad lib* treatment did, however, have greater Se body burdens than larvae from the SeMet Low ration ($p = 0.0067$), CA ration, control *ad lib*, and control ration treatments ($p < 0.0001$). Mean larval Se body burdens were not significantly different in the SeMet High *ad lib* and the SeMet Low *ad lib* treatments ($p = 0.0533$). Selenium concentrations in larvae receiving the SeMet High dose under a rationed feeding regimen were higher than in larvae from the SeMet Low *ad lib* ($p = 0.0418$) and ration ($p = 0.0047$) treatments and the CA ration, control *ad lib*, and control ration treatments ($p < 0.0001$ in these cases). Larvae from the SeMet Low *ad lib* treatment had greater mean Se body burdens than larvae in CA ration ($p < 0.0001$), control *ad lib* ($p = 0.0004$), and control ration ($p < 0.0001$). The SeMet Low ration treatment had greater larval Se body

burdens than the CA ration ($p = 0.0019$), control *ad lib* ($p = 0.0026$) and control ration ($p = 0.0001$) treatments.

Mean Se body burdens of metamorphs from each treatment are presented in Table 2.4 in both $\mu g\ g^{-1}$ ww and $\mu g\ g^{-1}$ dw, to facilitate comparisons with ww larval Se body burdens in this study and dw Se body burdens reported in other studies. The mean duration of larval exposure (d) is also included in Table 2.4 and was used as a covariate in *post-hoc* statistical comparisons. Only one individual from the SeMet Low *ad lib* treatment survived to complete metamorphosis, but was not available for Se analysis. There was a significant difference in mean metamorph Se body burdens (dw) between treatments ($F = 330.89$, $p < 0.0001$) and exposure duration showed a significant influence on these values based on *post-hoc* tests ($F = 7.08$, $p = 0.022$). Individuals from the SeMet Low ration treatment had the highest Se body burdens (dw) after metamorphosis ($p < 0.0001$ for all treatments). Metamorphs in the CA *ad lib* treatments had significantly greater Se body burdens (dw) than metamorphs in the control *ad lib* ($p = 0.005$) and control ration ($p = 0.0148$) treatments. Metamorphosed individuals from both the control *ad lib* and ration treatments had greater Se body burdens (dw) than metamorphosed individuals from the CA ration treatment ($p = 0.0028$ and $p = 0.008$, respectively). A similar treatment effect on wet mass-based metamorph Se body burdens was observed ($F = 221.6$, $p < 0.0001$). Metamorph Se body burdens (ww) were also significantly influenced by larval exposure duration ($F = 6.79$, $p = 0.024$). Metamorphs from the SeMet Low ration treatment had significantly greater tissue Se concentrations (ww) than metamorphs from any other treatment ($p < 0.0001$ for all comparisons). Selenium body burdens (ww) in control *ad lib* and ration metamorphs were not significantly different (p

= 1.0000) from each other, but were significantly less than CA *ad lib* metamorphs (p = 0.0181 and p = 0.0325, respectively). Control *ad lib* metamorphs had lower Se body burdens (ww) than CA ration metamorphs (p = 0.0404) as well, but the Se body burdens (ww) of control ration and CA ration metamorphs were not significantly different (p = 0.0678). Selenium body burdens (ww) of CA *ad lib* and ration metamorphs were not significantly different (p = 1.0000).

Toxicity to Larvae

Larvae receiving the SeMet High dose, independent of feeding regimen, displayed abnormal red coloration within 24 h of study initiation. Abnormal coloration began to fade in surviving individuals by Day 7 of dosing. Many larvae from this treatment also displayed severe edema prior to death. These abnormalities were not observed in larvae from any other treatment in this study, including the SeMet Low dose. Fewer individuals in the SeMet Low *ad lib* and ration treatments developed severe edema upon forelimb emergence. These instances of edema coincided with rear leg deformities and mortality prior to metamorphic completion, but were independent of resource provision.

Mean larval SMR per treatment, as measured on Day 16 and 32 of dosing, are presented in Table 2.5 and Figure 2.1. Early larval mortality prevented metabolic rate measurement in the SeMet High *ad lib* treatment on Day 16 and the SeMet High *ad lib* and ration treatments on Day 32. Mortality during SMR measurement also reduced the number of individuals used to generate mean values for the control *ad lib* treatment on both days (Table 2.5). There were significant differences in mean SMR between treatments on Day 16 (p = 0.012) and 32 (p = 0.001). On Day 16, mean SMR of individuals in the SeMet High ration treatment was significantly lower than the mean

SMR of individuals in the CA *ad lib* ($p = 0.0122$) and both control *ad lib* ($p = 0.0079$) and ration ($p = 0.0365$) treatments. By Day 32, mean SMR of individuals from the SeMet Low *ad lib* and ration treatments were significantly lower than the mean SMR of individuals from the control *ad lib* treatment ($p = 0.0007$ and $p = 0.0049$, respectively). Mean SMR in the SeMet Low *ad lib* treatment was also significantly less than mean SMR in control ration ($p = 0.0058$) and CA *ad lib* ($p = 0.0364$) treatments on Day 32.

Mean larval masses per replicate tank were recorded during tank cleanings conducted every four days. Ten percent of larvae initiated metamorphosis prior to tank cleaning on Day 34 (94 d post-hatch). Therefore, mean larval growth rates per treatment were calculated using mass measurements made on Day 30 (90 d post-hatch), the tank cleaning immediately before Day 34 (Figure 2.2). Larval mass did increase over time ($F= 16.65$, $p < 0.0001$) and mean larval growth rates were significantly different among treatments ($F = 78.4$, $p < 0.0001$). Larvae in the SeMet High *ad lib* and ration treatments were of consistently lower mean masses than larvae in all other treatments prior to reaching 100 % mortality.

Figure 2.3 shows mean survival (%) in each treatment for the duration of the experiment. The SeMet High dose was acutely toxic to *H. versicolor* larvae, independent of food provision levels; the SeMet High *ad lib* and ration treatments reached 100 % mortality by Day 10 and 22, respectively. Although not statistically different, a resource limitation effect was seen when the survival trajectories of the *ad lib* and ration feeding regimens were compared in the CA and control doses (Figure 2.3). In both, survival was higher in *ad lib* than rationed treatments. The opposite pattern was observed in the SeMet doses. Overall survival, which included all metamorphosed individuals and

remaining larvae at the end of the experiment (Day 78 of exposure), was significantly influenced by treatment ($p < 0.0001$; Table 2.6). Survival to Day 78 was significantly reduced in both SeMet High treatments compared to all others ($p < 0.0001$ for all CA and control treatments, $p = 0.0053$ for SeMet Low ration), except for SeMet Low *ad lib* ($p = 1.0000$). There was a significant reduction in survival at Day 78 in the SeMet Low *ad lib* treatment compared to the rationed feeding regimen of the same SeMet dose ($p = 0.0095$) and all other treatments ($p < 0.0001$). Survival to Day 78 was higher in CA and control *ad lib* treatments than in control ration ($p = 0.0092$ and $p = 0.0040$, respectively) and all SeMet treatments ($p < 0.0001$). Mean survival at Day 78 was significantly greater in the CA ration treatment than the SeMet Low ration treatment ($p = 0.0076$).

Toxicity During Metamorphosis

Treatment significantly influenced the average number of individuals to initiate metamorphosis by Day 78 ($p < 0.001$; Table 2.6). Specifically, the likelihood to initiate metamorphosis was significantly reduced by SeMet exposure in comparison to other treatments. No individual survived to initiate metamorphosis in either the *ad lib* or ration SeMet High treatments. This was significantly less than the average number to initiate metamorphosis in the control and CA *ad lib* treatments ($p < 0.0001$ for each), the control ration treatment ($p = 0.0002$), and the CA ration treatment ($p = 0.0001$). The failure of individuals to initiate metamorphosis in the SeMet High *ad lib* and ration treatments was not significantly different from the small percentage of individuals to do so in the SeMet Low *ad lib* treatment ($p = 0.9446$), but was significantly less than the 26.67 ± 7.2 % that initiated metamorphosis in the SeMet Low ration treatment ($p = 0.0258$). More individuals survived to initiate metamorphosis in the CA *ad lib* treatment than both the

control and CA rationed treatments ($p = 0.006$ and $p = 0.0252$, respectively). The same was true in the control *ad lib* treatment ($p = 0.0028$ and $p = 0.0123$ compared to control and CA ration, respectively). On average, more individuals initiated metamorphosis in the control and CA ration treatments than in the SeMet Low *ad lib* treatment ($p = 0.0025$ and $p = 0.0006$, respectively).

The toxicity endpoints evaluated specifically for individuals that achieved Gosner stage 42 are presented in Table 2.7. No individuals survived through the larval life stage in either SeMet High treatment, so these treatments were not included in the statistical analyses. There was a slight treatment effect ($p = 0.025$) on mean days post-hatch required for forelimb emergence. *Post-hoc* analysis revealed individuals from the CA ration treatment took significantly longer to reach Gosner stage 42 than individuals from the SeMet Low *ad lib* treatment ($p = 0.01$).

Treatment significantly influenced the mass of individuals at the premetamorph stage ($p < 0.0001$) (Table 2.7). Although the mean masses of premetamorphs in the SeMet Low *ad lib* and ration treatments did not differ ($p = 0.1330$) from each other, mean premetamorph mass in the SeMet Low ration treatment was significantly less than the mean masses of the control and CA *ad lib* treatments ($p = 0.0004$ and $p = 0.0007$, respectively). Resource provision influenced premetamorph masses in the CA treatments, as individuals in the *ad lib* treatment had a greater mean mass than individuals in the ration treatment ($p = 0.0111$). Mean premetamorph mass in the control *ad lib* treatment was significantly greater than the mean mass in the CA ration treatment ($p = 0.0063$), but did not differ from mean premetamorph mass in the control ration treatment ($p = 0.2633$).

Of individuals to enter the premetamorph life stage, rear leg deformities were observed only in the SeMet Low *ad lib* and ration treatments (Table 2.7). These individuals developed weak and sometimes nonfunctional rear legs. The incidences of rear leg deformities significantly coincided with treatment (p = 006). Deformities were more frequent when a rationed diet was provided relative to *ad lib*. There was also a significant treatment effect on mortality of premetamorphs with rear leg deformities (p = 0.004; Table 2.7). All premetamorphs exhibiting rear leg deformities in the SeMet Low ration treatment died prior to completing metamorphosis, whereas one third of deformed individuals in SeMet Low *ad lib* died prior to completion of metamorphosis.

Mean percent survival through metamorphic climax (Gosner stage 46) is also included in Table 2.6. These values are based on the number of replicate tanks in which indviduals had reached Gosner Stage 42 by the end of the experiment. There was a strong treatment effect on mean percentages survived to complete metamorphosis (p < 0.0001). Although there was not a significant resource limitation effect on premetamorph mortality in the SeMet Low treatments (p = 0.6272), significantly more premetamorphs died in the SeMet Low *ad lib* and ration treatments compared to all others. The differences were especially strong between the SeMet Low treatments and the control and CA *ad lib* treatments (p < 0.0001 for each comparison). Survival to metamorphic climax was higher in the CA ration treatment than in the SeMet Low *ad lib* and ration treatments (p = 0.0002 and p = 0.0028, respectively). The same was true when comparing survival through metamorphosis in the control ration treatment with survival in the SeMet Low *ad lib* and ration treatments (p = 0.0006 and p = 0.01, respectively). There was a resource limitation effect on survival through metamorphosis in control and CA treatments. The

CA *ad lib* treatment had greater survival than both the CA ration and control ration treatments ($p = 0.0218$ and $p = 0.0062$, respectively). The same effect was observed in the control *ad lib* treatment ($p = 0.0116$ and $p = 0.0033$ when compared with CA ration and control ration treatments, respectively).

Regardless of treatment, smaller premetamorphs were less likely to survive to complete metamorphosis ($p < 0.0001$ from logistic regression). Even so, it was unclear if the small premetamorph masses were directly related to the treatments applied during the larval stage. To statistically determine the likely cause of premetamorph mortality, the same binary logistic regression analysis was applied to each treatment. Analyses of premetamorph mass and survival data were only possible in the SeMet Low *ad lib* and ration treatments, as all premetamorphs survived to complete metamorphosis in the CA and control treatments (Table 2.6). These analyses indicate that premetamorphs of small mass were more likely to experience mortality prior to completing metamorphosis than those of larger masses in SeMet Low *ad lib* and ration treatments ($p = 0.034$ and $p = 0.026$, respectively).

The sublethal toxicity endpoints evaluated for individuals that survived to complete metamorphosis (Gosner stage 46) are presented in Table 2.8 and Table 2.9. Only one individual from the SeMet Low *ad lib* treatment survived to complete metamorphosis. This treatment, in addition to both SeMet High treatments, was excluded from statistical comparisons (Table 2.8) and lipid analyses (Table 2.9). There were no significant differences in mean time (d post-hatch) required for individuals to complete metamorphosis between treatments ($p = 0.184$). Treatment did have a significant effect on the mass of individuals at metamorphic climax, however ($p = 0.001$). Individual

metamorphs from the SeMet Low ration treatment were, on average, of smaller mass than metamorphs from control *ad lib* ($p = 0.01$) and CA *ad lib* ($p = 0.0294$) treatments. Resource limitation also affected metamorph masses in the CA treatments, as CA *ad lib* animals were of greater mean mass than animals in the CA ration treatment ($p = 0.0085$). Mean mass of metamorphs in the CA ration treatment was also smaller than the mean mass of those from the control *ad lib* treatment ($p = 0.003$). There was no evidence of a significant difference in the mean metamorph masses of the control *ad lib* and ration treatments ($p = 0.2745$). As with mean metamorph mass, SVL was also significantly affected by treatment in this study ($p < 0.0001$). Mean metamorph SVL in the SeMet Low ration treatment was significantly less than in all other treatments ($p = 0.0001$ and $p = 0.001$ when compared with control *ad lib* and ration treatments, respectively, and when compared with CA *ad lib* and ration treatments, $p = 0.0003$ and $p = 0.0263$, respectively). Treatment did not significantly affect the total lipid (TL) content of metamorphs sampled ($p = 0.470$). Triacylglyceride (TAG) and free-fatty acid (FFA) concentrations were significantly influenced by treatment when lipid classes were considered individually ($p = 0.008$ and $p = 0.018$, respectively). Metamorphs from the CA ration and SeMet Low ration treatments had significantly lower TAG levels than metamorphs in the CA *ad lib* treatment ($p = 0.0216$ and $p = 0.01$, respectively). In addition to TAG, resource limitation may have also influenced FFA levels in the CA treatments. Metamorphs in the CA *ad lib* treatment had significantly greater FFA concentrations than metamorps from the CA ration treatment ($p = 0.030$). The mean date of metamorphic climax did not significantly influence total lipid or lipid class levels.

Discussion

Selenium Uptake

Although Se dissolution in tank water was minimal compared to SeMet concentrations in food, the contribution of this exposure route on Se uptake and the overall toxicity observed in these treatments cannot be ruled out. Typical Se concentrations of natural freshwater range from 0.1 to 0.3 $\mu g\ l^{-1}$ (Lemly 1993). The maximum aqueous Se concentrations measured in aged well water and control and CA treatment tank water fall within, or just above this range (Table 2.3). It has been demonstrated, however, that aqueous Se concentrations only an order of magnitude higher (1 – 5 $\mu g\ l^{-1}$) can be toxic to aquatic wildlife (Lemly 1993) and maximum dissolved Se concentrations in SeMet treatment tank water were well above this range (up to 54 $\mu g\ l^{-1}$ in the SeMet *ad lib* treatment). The maximum aqueous Se concentrations measured in all SeMet treatment tanks fall within the range of concentrations reported by Rowe et al (2002) for aquatic CA-contaminated sites, however. Additional laboratory studies investigating the toxicity of the aqueous SeMet concentrations measured in this study, independent of dietary dosing, are needed.

There was strong evidence of Se uptake and accumulation in larvae from the SeMet Low *ad lib* and ration treatments. Greater larval Se body burdens were slightly, though not significantly, associated with increased resource provision at the SeMet Low dosage. There was also evidence of Se uptake from food in SeMet High *ad lib* and ration treatments, though no Se accumulation in larval tissue was observed. It is possible that an accumulation threshold, like that suggested by Lemly (1993) for fish, may have been reached at this high dosage (Schrauzer 2000). However, since SeMet High *ad lib* larvae

died earlier and took up less Se than SeMet High ration larvae, it is more likely larvae succumbed to acute toxicity prior to accumulating Se from food in these treatments. Larvae fed CA food did not accumulate Se relative to control treatments, regardless of feeding regimen. Although CA from the same source was used, mean larval Se body burdens in CA treatments in our study were substantially lower than the mean Se concentrations measured in other species of anuran larvae from the Savannah River Site (Rowe, et al. 2002). This could be because CA exposures in our study were limited to a single, approximately one gram CA application every 24 to 48 h, depending on feeding regimen. While effectively emulating SeMet dosing, the CA dosing method utilized in our experiment does not mimic CA-exposure of larval amphibians developing in a CA-contaminated habitat. In the natural environment, amphibian larvae would most likely be incidentally exposed to CA while grazing periphyton or ingesting sediment and detritus (Hopkins, et al. 2002, Snodgrass, et al. 2004, Unrine, et al. 2007a). High feeding rates are essential to ensure larvae form ample energy stores to survive through the non-feeding period of metamorphosis and successfully transition into terrestrial carnivores, as adults (Unrine, et al. 2007a), but can lead to increased incidental CA exposure in the wild.

Metamorphs in the CA *ad lib* and ration treatments did not retain more Se than control metamorphs. The dry mass Se body burdens of metamorphs from the CA treatments in this study were much lower than those measured in anuran metamorphs at the Savannah River Site (Rowe, et al. 2002, Snodgrass, et al. 2003) or in anuran metamorphs exposed to CA contaminated sediment as larvae within the laboratory (Snodgrass, et al. 2004). The mean Se body burden (dw) of metamorphs from the SeMet

Low ration treatment (80.76 ± 14.4 µg g^{-1} dw) was of the same order of magnitude as the mean Se concentration (dw) provided in the food (50.95 ± 2.96 µg g^{-1} dw). This indicates the Se provided did not become biomagnified in metamorph tissue during this study.

Abnormalities and Malformations

Larvae in the SeMet High treatments displayed abnormal red coloration within 24 h of study initiation. This was attributed to capillary hemorrhaging beneath the integument, as similar symptoms were observed in the gill lamellae of Se-exposed fish (Lemly 2002). Color change was not observed in larvae from the SeMet Low treatments, indicating this dose was not sufficient to elicit this particular response. Additional laboratory studies are required to determine the lowest SeMet dose to produce this toxic response. Many larvae from the SeMet High treatments also displayed severe edema prior to death. Edema is considered a non-specific response to Se exposure (Janz 2010) and has been observed in Se-exposed larval and adult fish and embryonic birds (Lemly 1993, 2002, Ohlendorf 2002, Hamilton 2004). Tadpoles and freshwater fish are hyperosmotic relative to their dilute freshwater habitats. Both taxa maintain osmotic balance by excreting large volumes of dilute urine and using active transport mechanisms to remove excess ions across the integument. Edema signals a loss of control of that osmotic gradient (Ultsch 1999) and is a symptom of oxidative stress (Janz 2010). The role of Se in oxidative stress further illustrates the micronutrient's narrow therapeutic concentration range. At therapeutic concentrations, Se-containing glutathione peroxidase enzymes catalyze the reduction of reactive oxygen species within the cell. However, excess Se concentrations can disrupt this antioxidant reaction and actually increase

oxidative stress (Janz 2010). If Se-induced edema does signal oxidative stress, it is interesting that this symptom was only observed in *H. versicolor* dosed with SeMet. Studies indicate SeMet does not disrupt the glutathione antioxidant system as much as other Se forms. Cellular toxicity studies are necessary to determine the extent SeMet and its metabolites disrupt this and other antioxidant systems before a relationship between SeMet-induced edema and oxidative stress is established.

Premetamorphs with rear leg deformities were only found in SeMet Low treatments and the deformities were most frequent in the SeMet Low ration treatment. Premetamorph mortality was strongly associated with rear limb deformities and was highest in the SeMet Low ration treatment. In this study, we did not observe any of the spinal or cranial malformations in *H. versicolor* larvae that were observed in other anuran species developing in CA-contaminated sites (Rowe, et al. 1996, Raimondo, et al. 1998, Hopkins, et al. 2000). Snodgrass et al (2004) observed rear leg "subluxations," or partial dislocations, of the femur at the pelvic girdle in two *Rana clamitans* larvae from a laboratory study in which larvae were exposed to CA-contaminated sediment. The femoral subluxations observed by Snodgrass et al. (2004) appear similar to those observed in SeMet-exposed premetamorphs from this study.

Biological Responses of Larvae

Selenium toxicity at each life-stage in this study was highly dose-dependent and resource limitation had little effect on the toxicity of each dose. Overall, larvae in CA treatments received low Se dosages, statistically equivalent to Se concentrations in control exposures. Coal ash exposure did not elicit toxic responses in *H. versicolor* during the larval stage. Although tissue concentrations of other trace elements were not

determined, the lack of toxic responses relative to controls and high survival rates and metamorphic success in CA treatments indicate trace element body burdens did not reach toxic levels. Coal ash exposure did not alter larval metabolic rates compared to control animals in our study. This is in contrast to observations made of larval *R. catesbeiana* in CA-contaminated basins at the Savannah River Site (Rowe, et al. 1998b). Selenomethionine exposure did not increase larval SMR, contrary to observations made during SeMet dosing studies of fish (Lemly 1993). In fact, SeMet exposure reduced larval SMR compared to controls as the study progressed. This can be attributed to the acute toxicity of the high doses of this compound to this particular species. The SeMet High doses were particularly toxic to *H. versicolor* larvae and induced 100 % mortality in both *ad lib* and ration treatments within the first 22 d of exposure. Resource limitation did not augment mortality in the SeMet treatments, but reduced survival in the rationed CA and control treatments compared to corresponding *ad lib* treatments. Survival rates were higher in the SeMet High and Low ration treatments relative to corresponding *ad lib* treatments, indicating the toxicity of the slightly greater SeMet exposures in *ad lib* treatments exceeded resource limitation stress in corresponding rationed treatments. Direct SeMet toxicity also had a stronger influence on larval growth rates than resource provision. Mean larval growth rate in the SeMet Low *ad lib* treatment was less than in the SeMet Low ration treatment (Figure 2.2).

Biological Responses During Metamorphic Climax

All surviving individuals in control and CA *ad lib* treatments completed metamorphosis by the end of the study. Few individuals (two in the control ration treatment, three in the CA ration treatment, and nine in the SeMet Low ration treatment)

experienced arrested development and did not progress from the larval stage by Day 78. *Hyla versicolor* do not over-winter as larvae in the wild but metamorphose in late summer (Dickerson 1906). Although considered "survivors" for the purposes of this study, individuals remaining in the larval stage 138 days post-hatch would not likely have survived in the wild. The SeMet Low *ad lib* treatment significantly reduced the number of individuals to reach the premetamorph life stage (Gosner stage 42). Our results indicate resource limitation significantly reduced the likelihood of individuals to become premetamorphs in CA and control treatments. Mean masses at Gosner stage 42 in the CA and SeMet Low ration treatments were significantly reduced compared to corresponding *ad lib* treatments. Since there was no statistical difference in mean masses between the control *ad lib* and ration treatments, we can assume that chemical toxicity coupled with resource limitation reduced individual mean mass at Gosner stage 42.

SeMet Low *ad lib* individuals reached Gosner stage 42 faster than individuals from the CA ration treatment. Larval period plasticity is a life history strategy evolved by many anuran species that may allow individuals to increase survival by rapidly achieving the terrestrial life stage. By decreasing the time spent in the fully aquatic life stage, individuals may be able to reduce the period of exposure to aquatic predators, reduce desiccation risks in ephemeral pools, and avoid high competition for resources (Wilbur and Collins 1973). Therefore, it is also possible that rapid metamorphosis might be employed to reduce exposure to toxicants in larval habitat if toxicants invoke a physiological stress response similar to that imposed by natural stressors. Since the mean larval periods in control treatments were not statistically different from the SeMet *ad lib* mean larval period, it is possible the differences in larval period could merely be an

artifact of the disparity in numbers of individuals to reach this life stage in the SeMet Low *ad lib* and CA ration treatments. The mean time to metamorphosis for individuals, independent of treatment, in this study was 101.5 days post-hatch. The larval period of wild *H. versicolor* typically lasts up to 65 days post-hatch. Timing of the *H. versicolor* larval period is also negatively correlated with temperature in the wild (Dickerson 1906) and high larval densities have been demonstrated to lengthen the larval period of many amphibian species (Wilbur and Collins 1973, Scott 1994). The lengthy larval periods of organisms in this study likely resulted from a combination of low temperatures and high larval densities (Newman 1998).

Survival through metamorphic climax (Gosner stage 42 through 46) was not altered by resource provision. Our results suggest reduced resource provision prevented larvae from surviving to initiate metamorphosis, but did not alter the likelihood of metamorphic completion once an individual reached Gosner stage 42. Selenomethionine Low exposures significantly increased mortality during metamorphic climax, independent of resource provision level. In fact, slightly, though not significantly, more individuals survived to complete metamorphosis in the SeMet Low ration treatment than in the SeMet Low *ad lib* treatment. This is likely due to the increased SeMet dosage that individuals in the *ad lib* treatment received over time. Although there was a treatment effect on larval period length, total time to complete metamorphosis was not significantly altered by toxicant or resource provision level. As expected, individuals lost mass during the non-feeding portion of metamorphosis (Gosner stage 42 through 46) in all treatments (Table 2.7 and Table 2.8). The interactions between toxicant dose, resource provision, and mean wet masses observed at Gosner stage 42, remained at Gosner stage 46.

Therefore, the same conclusion can be drawn: combined toxicant and resource limitation stresses significantly reduced metamorph mean wet mass. Resource limitation affected mean metamorph wet mass but not SVL at this life stage. However, Selenomethionine exposure significantly reduced SVL, further indicating SeMet exposure negatively influenced overall metamorph health. Field studies with salamanders have indicated a strong relationship between size at metamorphosis and adult survival to reproductive age in amphibians (Scott 1994). Thus, SeMet and CA exposure combined with the pressure of resource limitation could reduce the reproductive population of *H. versicolor* in the natural environment, assuming metamorph size correlates with future reproductive success in this species.

Based on metamorph lipid content, however, a treatment effect on adult reproductive success seems less likely. Lipids are the primary energy stores utilized during non-feeding periods of metamorphic climax and early terrestrial life (Fitzpatrick 1976, Scott, et al. 2007) and are most likely a better determinant of adult success than metamorph body size. Neither toxicant exposure nor resource limitation altered metamorph total lipid concentrations or the concentrations of most lipid classes. There was a slight resource limitation effect on TAG and FFA concentrations in the CA exposures, however. Triacylglyceride concentrations in metamorphs from the SeMet Low ration treatment were also reduced compared to those of CA *ad lib* individuals. Lipid class concentrations determined for *H. versicolor* metamorphs in our study agree with those published by Sawant and Varute (1973) for *R. tigrina*, which confirms the validity of the lipid extraction and analysis methods we employed.

Conclusions

Contrary to design, the Se content of CA food was nearly an order of magnitude lower than SeMet Low food (Table 2.1). This inequality could have been avoided had we adjusted Se dosages based on a dry mass (dw) of food instead of wet mass (ww). Nominal SeMet doses were chosen to represent the range of Se typically found in CA (<2 – 50 $\mu g\ g^{-1}$ dw), presented in the review by Rowe et al 2002. However, the CA Se concentrations in this review are presented on a dw basis, as CA contains little moisture. By neglecting to also base our SeMet doses on dw instead of ww of food, we failed to replicate the range of Se concentrations in CA and instead exposed larvae in these treatments to Se concentrations that were not environmentally relevant. Therefore, exposure effects of CA and SeMet treatments in this study cannot be directly compared. Also, toxicant dose was not adjusted based on resource provision in this study. In addition to receiving greater energetic resources, *ad lib* treatments received proportionally more toxicant over time than corresponding ration treatments. The survival rates, metamorphic successes, larval growth rates and Se uptake observed in SeMet treatments reflect this disparity.

Assessing toxicity at the larval stage is important, as high mortality at this stage could negatively impact future breeding populations. Typical field surveys of reproductively mature adults might effectively identify current reductions in breeding populations, but controlled, laboratory-based larval exposure studies can provide a conclusive link between toxicants and adverse responses causing those reductions (Lemly 1993, Snodgrass, et al. 2004). In our study, SeMet exposure reduced larval metabolic rates, growth rates and survival; reduced the number of individuals to initiate

metamorphosis, reduced wet mass and SVL at Gosner stage 46, reduced survival to initiate and complete metamorphosis; and increased the frequency of malformations at Gosner stage 42. In contrast, there was little evidence of CA toxicity relative to control treatments. Resource limitation did not augment the toxicity of SeMet doses applied. Instead, reduced toxicity was observed in rationed SeMet treatments, a likely result of slightly decreased toxicant exposures relative to corresponding *ad lib* SeMet treatments. An interaction between toxicant exposure and resource limitation was only observed in mean wet masses at Gosner stages 42 and 46. Resource limitation may have contributed to reduced survival to the end of the study and reduced the number of individuals initiating metamorphosis in CA and control treatments. Additionally, resource limitation may have reduced TAG and FFA lipid class concentrations in metamorphs exposed to CA. Our study established that exposure to high SeMet concentrations induce rear limb malformations, reduce larval survival and reduce the likelihood of and survival through metamorphosis in *H. versicolor*. Results of this study help fill in the data-gap concerning the possible developmental effects of SeMet exposure to larval amphibians.

Table 2.1. Selenium concentrations and caloric content of tadpole food

Treatment	Se Conc. (µg g^{-1} ww)	Se Conc. (µg g^{-1} dw*)	% of Target ww Conc.	Se dose per pellet (µg)	Cal g^{-1}
Control	ND	ND			4.21 ± 0.05
CA	0.52 ± 0.09	3.88 ± 0.54	13.1	2.09 ± 0.35	4.72 ± 0.26
SeMet Low	3.58 ± 0.3	50.95 ± 2.96	71.6	14.32 ± 1.20	4.23 ± 0.04
SeMet High	35.55 ± 3.4	482.03 ± 33.02	71.1	142.19 ± 19.3	4.17 ± 0.09

Values are means ± 1 S.E. * Dry mass (dw) Se concentrations were calculated based on wet mass (ww) concentrations and the average moisture content of subsamples from each food type. "ND" indicates Se concentrations were below the method detection limit (0.001 µg g^{-1} ww or 0.0126 µg g^{-1} dw).

Table 2.2. Selenium concentrations in food prior to and after storage at -4 °C

Treatment	Pre-Storage Se Conc. (µg g^{-1} dw*)	Post-Storage Se Conc. (µg g^{-1} dw*)
Control	ND	2.30 ± 0.20
CA	3.88 ± 0.54	5.05 ± 0.76
SeMet Low	50.95 ± 2.96	48.42 ± 1.79
SeMet High	482.03 ± 33.02	529.03 ± 46.9

Values are means ± 1 S.E. * Dry mass (dw) Se concentrations were calculated based on wet mass (ww) concentrations and the average moisture content of subsamples from each food type. "ND" indicates mean Se concentration was below the method detection limit (0.0126 µg g^{-1} dw).

Table 2.3. Dissolved Se concentrations in tank water

Treatment	Mean Se Conc. ($\mu g\ ml^{-1}$)	Maximum Se Conc. ($\mu g\ ml^{-1}$)
well water	0.002 ± 0.0002	0.005
Control *ad lib*	0.002 ± 0.0002	0.003
Control ration	0.002 ± 0.0002	0.004
CA *ad lib*	0.003 ± 0.0002	0.004
CA ration	0.002 ± 0.0002	0.004
SeMet Low *ad lib*	0.010 ± 0.0004	0.015
SeMet Low ration	0.006 ± 0.0003	0.009
SeMet High *ad lib*	0.047 ± 0.003	0.054
SeMet High ration	0.025 ± 0.002	0.029

Values are means ± 1 S.E.

Table 2.4. Larval and metamorph Se body burdens of *H. versicolor*

Treatment	Larvae			Metamorphs			
	n	Se (µg g^{-1} ww)	Collection Date	n	Se (µg g^{-1} ww)	Se (µg g^{-1} dw)	Larval Exposure (d)
Control ad lib	4	0.113 ± 0.07[a]	31 ± 7.5	4	0.269 ± 0.01[a]	2.04 ± 0.06[a]	41.8 ± 1.8
Control ration	3	0.096 ± 0.07[a]	56 ± 10.9	3	0.27 ± 0.23[ab]	2.11 ± 0.23[a]	40.7 ± 2.5
CA ad lib	1	0.223	71	4	0.413 ± 0.15[c]	3.19 ± 0.15[b]	40.8 ± 3.5
CA ration	3	0.155 ± 0.09[a]	48 ± 18.6	3	0.469 ± 0.05[bc]	3.95 ± 0.05[b]	17.1 ± 1.7
SeMet Low ad lib	4	28.36 ± 3.5[b,d]	47 ± 3.3	0			
SeMet Low ration	4	14.89 ± 1.5[b]	63 ± 5.5	4	9.26 ± 14.4[d]	80.76 ± 14.4[c]	34.1 ± 6.3
SeMet High ad lib	4	26.25 ± 1.3[c,d]	7 ± 0.4	0			
SeMet High ration	4	37.21 ± 3.5[c]	11 ± 1.7	0			

Values presented are mean larval carcass Se concentrations (µg g^{-1} ww) ± 1 S.E. and mean Se concentrations in metamorphs (expressed in both µg g^{-1} ww and µg g^{-1} dw). Mean experimental date of carcass collection ± 1 S.E. (thru Day 78) for larvae and mean larval exposure ± 1 S.E. (thru Day 78) are included as estimates of exposure duration. Dissimilar superscripts indicate statistically significant differences.

Table 2.5. Larval standard metabolic rates

Treatment	Day 16		Day 32	
	n	SMR (µl O_2 min^{-1} g^{-1})	*n*	SMR (µl O_2 min^{-1} g^{-1})
Control *ad lib*	3	1.72 ± 0.11^{a}	2	1.95 ± 0.37^{a}
Control ration	3	1.51 ± 0.31^{a}	3	1.63 ± 0.07^{ac}
CA *ad lib*	4	1.61 ± 0.16^{a}	4	1.43 ± 0.06^{ac}
CA ration	3	1.21 ± 0.12	3	1.40 ± 0.11
SeMet Low *ad lib*	4	1.34 ± 0.07	3	0.899 ± 0.14^{bc}
SeMet Low ration	4	1.26 ± 0.14	4	1.10 ± 0.06^{c}
SeMet High *ad lib*	0		0	
SeMet High ration	2	0.567 ± 0.18^{b}	0	

Values are mean larval standard metabolic rates (SMR) expressed as µl O_2 consumed per min per g wet mass ± 1 S.E. on Day 16 and 32 of Se exposure. Numbers of individuals (n) measured per treatment each day are also included. High mortality prevented SMR measurements in the SeMet High *ad lib* treatment on Day 16 and both SeMet High treatments on Day 32. Dissimilar superscripts indicate statistically significant differences.

Table 2.6. Survival at each life stage

Treatment	Replicate tanks *n*	Survival to Day 78 (%), metamorphs + remaining larvae	Survivors initiating metamorphosis by Day 78 (%)	Survival through metamorphic climax by Day 78 (%)
Control *ad lib*	4	73.33 ± 4.7^{a}	73.33 ± 4.7^{a}	73.33 ± 4.7^{a}
Control ration	3	48.89 ± 4.4^{bd}	44.44 ± 2.2^{b}	44.44 ± 2.2^{b}
CA *ad lib*	4	71.67 ± 5.0^{a}	71.67 ± 5.0^{a}	71.67 ± 5.0^{a}
CA ration	3	55.56 ± 2.2^{ab}	48.89 ± 4.4^{b}	48.89 ± 4.4^{b}
SeMet Low *ad lib*	4	1.67 ± 1.7^{c}	8.33 ± 5.0^{cd}	1.67 ± 2.4^{c}
SeMet Low ration	4	28.33 ± 5.7^{d}	26.67 ± 7.2^{bc}	13.33 ± 4.7^{c}
SeMet High *ad lib*	0	0^{c}	0^{d}	0
SeMet High ration	0	0^{c}	0^{d}	0

Values are means ± 1 S.E. Dissimilar superscripts indicate statistically significant differences.

Table 2.7. Toxicity endpoints evaluated at Gosner stage 42

Treatment	Replicate tanks with premetamorphs *n*	Time to forelimb emergence (d post-hatch)	Mass at forelimb emergence (g ww)	Incidences of rear leg deformities (% of those to initiate metamorphosis)*	Mortality with rear leg deformities (% of those with rear leg deformaties)*	Survival through metamorphic climax (% of those to initiate metamorphosis)
Control *ad lib*	4	101.7 ± 1.3	0.484 ± 0.01[a]	0	0	100 ± 0.0
Control ration	3	100.7 ± 2.5	0.402 ± 0.01	0	0	100 ± 0.0
CA *ad lib*	4	100.9 ± 3.5	0.472 ± 0.02[a]	0	0	100 ± 0.0
CA ration	3	108.6 ± 3.7[a]	0.325 ± 0.03[b]	0	0	100 ± 0.0
SeMet Low *ad lib*	2	87.5 ± 5.5[b]	0.396 ± 0.08	33.33 ± 33.3	33.33 ± 33.3	25 ± 25.0
SeMet Low ration	4	98.0 ± 2.7	0.286 ± 0.02[b]	70.42 ± 12.4	100 ± 0.0	40 ± 13.5

Values are means ± 1 S.E. Dissimilar superscripts indicate statistically significant differences. * Indicates statistically significant differences among treatments according to non-parametric analyses (p = 0.006 for incidences of rear leg deformities and p = 0.004 for mortality with rear leg deformities).

Table 2.8. Toxicity endpoints evaluated at Gosner stage 46

Treatment	Replicate tanks with metamorphs *n*	Time to complete metamorphosis (d post-hatch)	Mass at complete metamorphosis (g ww)	SVL at complete metamorphosis (mm)
Control *ad lib*	4	105.1 ± 1.4	0.376 ± 0.01[a]	13.81 ± 0.03[a]
Control ration	3	104.2 ± 2.3	0.323 ± 0.01	13.37 ± 0.29[a]
CA *ad lib*	4	104.3 ± 7.0	0.361 ± 0.02[a]	13.53 ± 0.26[a]
CA ration	3	111.4 ± 2.6	0.253 ± 0.03[b]	12.58 ± 0.52[a]
SeMet Low *ad lib*	1*	86	0.36	
SeMet Low ration	3	97.8 ± 5.9	0.272 ± 0.01[b]	11.12 ± 0.11[b]

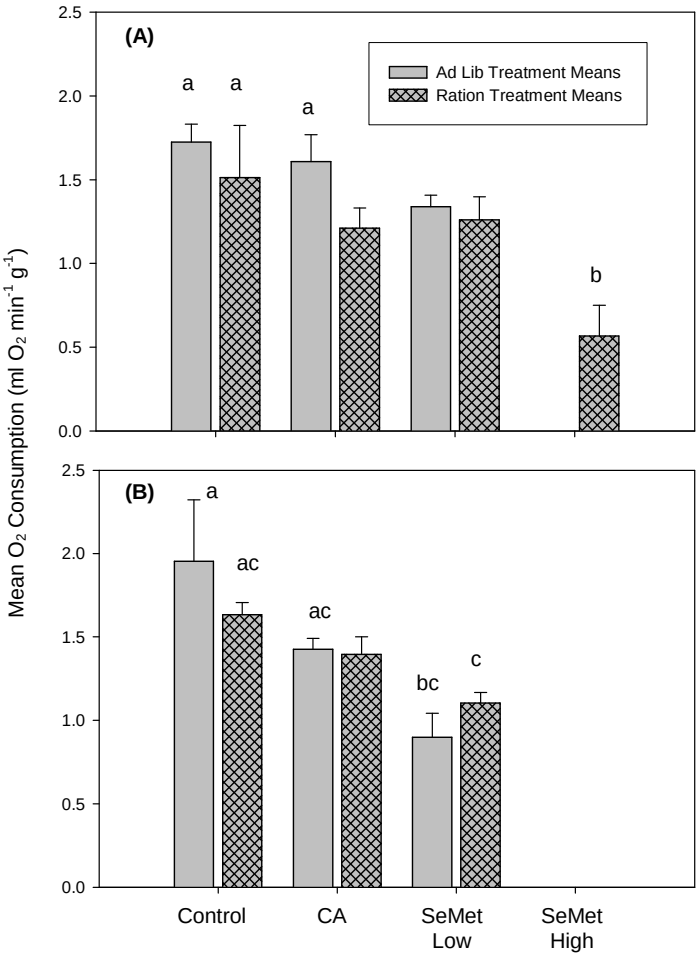
Values are means ± 1 S.E. *Only one individual from the SeMet Low *ad lib* treatment survived to complete metamorphosis. Therefore, this treatment was excluded from statistical analyses. Dissimilar superscripts indicate statistically significant differences.

Table 2.9. Total lipid and lipid class content of metamorphs (Gosner stage 46)

Treatment	Replicate tanks with metamorphs *n*	Total Lipids (mg/g dw)	WE (mg/g dw)	TAG (mg/g dw)	FFA (mg/g dw)	ST (mg/g dw)	PL (mg/g dw)
Control *ad lib*	4	247.3 ± 29.1	2.51 ± 0.2	40.8 ± 8.9	10.1 ± 0.9	11.3 ± 1 .2	182.6 ± 29.6
Control ration	3	238.6 ± 22.6	3.15 ± 0.6	38.2 ± 2.4	10.9 ± 0.6	12.3 ± 0.1	174.1 ± 21.5
CA *ad lib*	4	238.2 ± 22.1	3.05 ± 0.4	52.7 ± 3.2[a]	10.9 ± 0.5[a]	10.7 ± 0.7	160.9 ± 20.6
CA ration	3	297.7 ± 33.4	3.02 ± 0.4	22.5 ± 6.4[b]	7.3 ± 1.0[b]	13.7 ± 1.2	251.1 ± 39.4
SeMet Low ration	3	283.5 ± 44.1	3.35 ± 0.6	18.9 ± 2.2[b]	8.3 ± 0.3	12.4 ± 1.3	240.5 ± 45.1

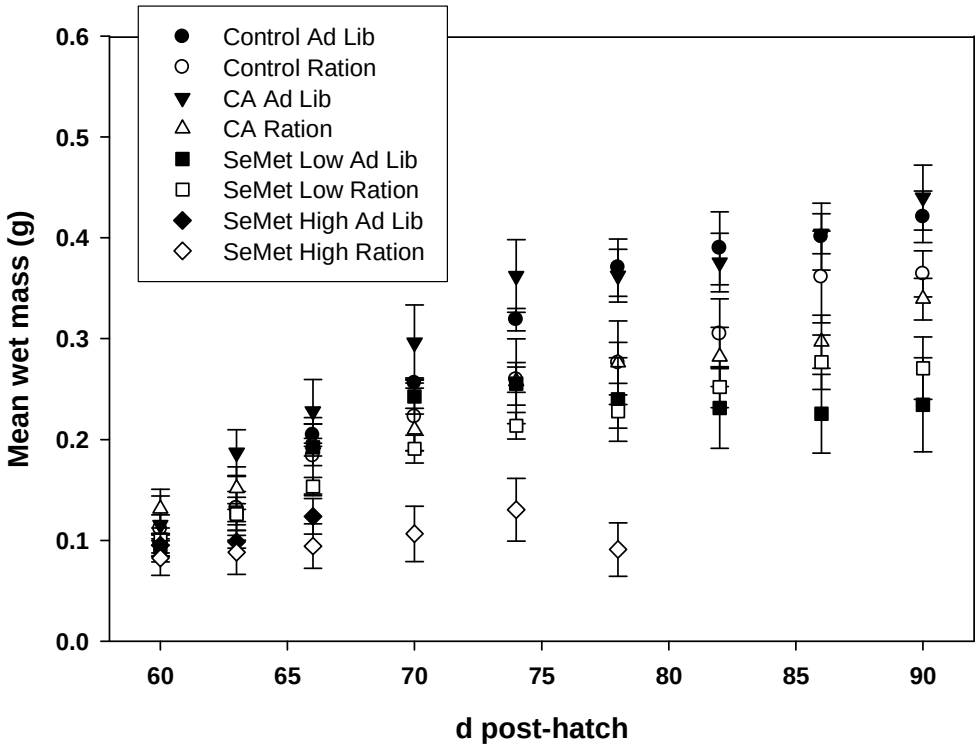
Values are means ± 1 S.E. Dissimilar superscripts indicate statistically significant differences.

Figure 2.1. Larval standard metabolic rates on Day 16 (A) and Day 32 (B)



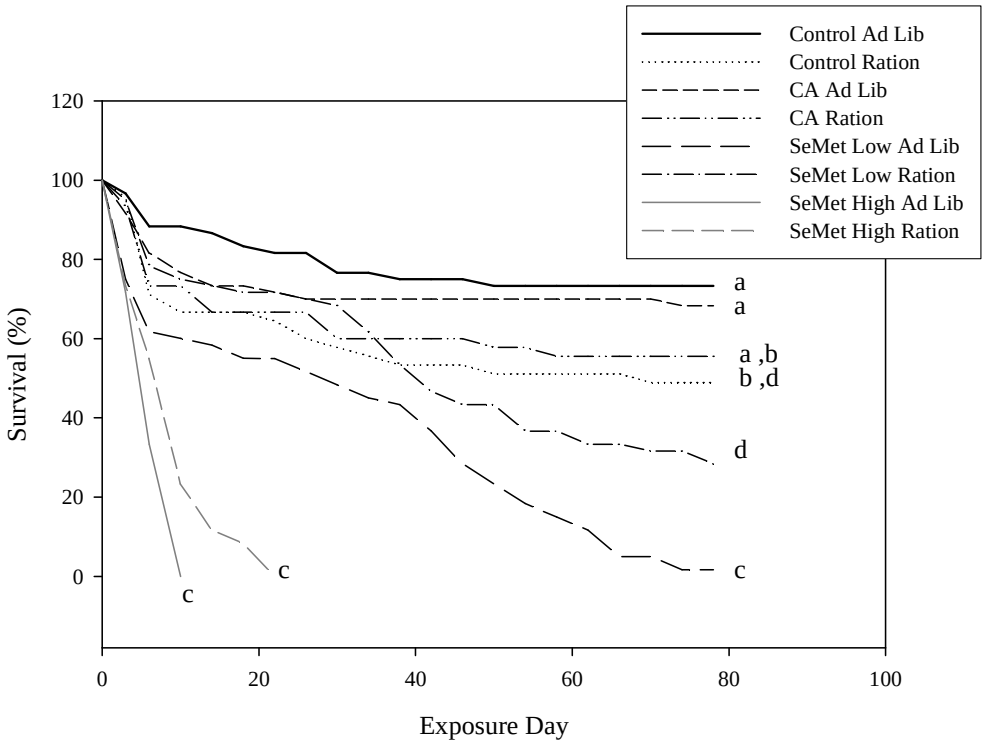
Values are mean larval standard metabolic rates (SMR) expressed as µl O_2 consumed per min per g wet mass ± 1 S.E. on Day 16 (A) and 32 (B) of Se exposure. High mortality prevented SMR measurements in the SeMet High *ad lib* treatment on Day 16 and both SeMet High treatments on Day 32. Dissimilar letters indicate statistically significant differences.

Figure 2.2. Larval growth rates over the first 30 d of exposure



Larval growth rates are presented from 60 to 90 d post-hatch (equivalent to the first 30 d of exposure). Ten percent of larvae initiated metamorphosis by 94 d post-hatch, therefore larval growth rates are only presented until the 90 d post-hatch measurements. Error bars represent ± 1 S.E.

Figure 2.3. Survival through 78 d exposure



Mean percent survival of individuals, regardless of life stage, in each treatment through exposure Day 78. Dissimilar letters indicate statistically significant differences in percent survival on Day 78.

Chapter 3: Toxicity of dietary-dosed selenomethionine and selenium dioxide to larval gray tree frogs (*Hyla versicolor*)

Introduction

Selenium Essentiality

Selenium is a micronutrient essential to cellular antioxidant and metabolic processes. Naturally occurring selenoproteins have been identified in mammalian systems; two of the most well known and well understood are glutathione peroxidase and selenoprotein P. The former is vital in mitigating cellular oxidative stress, while the latter has chelative properties involved in metal detoxification (eg. Hg and Cd; Young 2010b). Selenocystine (SeCys) and selenomethionine (SeMet) are Se-containing analogs of the sulfur (S)-based proteins cysteine and methionine, which higher organisms must acquire through dietary sources (Schrauzer 2000). Selenocystine and SeMet are not considered selenoproteins, but instead are considered "selenium-containing proteins", since Se integration is nonspecific (Young 2010b). Selenocystine is a precursor to both glutathione peroxidase and type I iodothyronine deiodinase, the catalyst for the converstion of thyroxine (T_4) to triodothyronine (T_3) in the thyroid gland. Selenium deficiencies can lead to hypothyroidism and myocardial lesions in humans (Goyer 2001). However, Se has a narrow therapeutic concentration range. In fact, the Se reference dose (RfD) for humans is only 5 $\mu g\ kg^{-1}$ body mass day^{-1} (Schrauzer 2000). Toxicity to humans and other organisms occurs when Se intake surpasses the homeostatic ability to store or excrete excess (Goyer 2001).

Selenium Contamination in Aquatic Environments

Toxic effects of Se in the environment have been observed in aquatic habitats impacted by coal ash (CA) disposal and agricultural waters used to irrigate Se-laden soils (Lemly 1993, Fan, et al. 2002, Ohlendorf 2002, Rowe, et al. 2002). Belews Lake, NC and the Savannah River Site in Aiken, SC are two of the most rigorously studied aquatic sites contaminated by CA and CA basin effluent (Lemly 1993, Rowe, et al. 2002, Young 2010a). Belews Lake, NC received spill-water from a nearby CA retention basin from the mid-1970s to the mid-1980s. The spill-water introduced high dissolved Se concentrations (150 – 200 $\mu g\ l^{-1}$) into the lake (Lemly 2002), but dissolved Se concentrations in the lake water never exceeded 35 $\mu g\ l^{-1}$, the USEPA designated chronic exposure limit at the time (Young 2010a). Selenium bioaccumulated into the aquatic food chain and eventually caused the collapse of the lake fishery (Lemly 2002). Evidence of teratogenesis was still observed in larval fish even 10 y after CA drainage inputs to the lake had ceased and aqueous Se concentrations had returned to near background levels. Selenium cycling through the food chain is slow and uptake from sediment was likely the cause of the observed latent toxic effects (Lemly 2002). In 1987, the USEPA adopted 5 $\mu g\ l^{-1}$ as the chronic dissolved Se exposure limit for aquatic life as a measure to better protect aquatic ecosystems from the bioaccumulative properties of Se in light of the Belews Lake disaster (Young 2010a). Selenium toxicity investigations at Belews Lake focused mainly on fish and benthic prey species. Selenium-contamination effects on populations of other aquatic and semi-aquatic taxa within the lake habitat, such as amphibians or reptiles, were not assessed. It is unclear if the USEPA chronic dissolved Se exposure limit is equally protective to other taxa.

The coal-fired power plant located at the Savannah River Site has been active for over 50 years and employs aquatic settling basins to manage ash wastes generated by the facility (Young 2010a). Effluent from these settling basins passes into a discharge swamp and eventually enters a tributary of the Savannah River (Rowe, et al. 1996). Mean aqueous Se concentrations are low at this site (approximately 2 $\mu g\ l^{-1}$) and fall below the USEPA chronic dissolved Se exposure limit (Young 2010a). However, potentially toxic Se concentrations were accumulated throughout the aquatic food chain. Selenium concentrations measured in periphyton, algae, and macrophytes, which form the base of the aquatic food chain, range from 6 – 12 $\mu g\ g^{-1}$ dw. Tissue Se concentration increases coincided with increasing trophic levels. Fish and amphibian larvae had tissue Se concentrations between 15 – 25 $\mu g\ g^{-1}$ dw (Rowe, et al. 2002, Unrine, et al. 2007a) and the highest Se concentrations were measured in top predators (100 $\mu g\ g^{-1}$ dw in snake livers; Rowe, et al. 2002, Hopkins, et al. 2006, Young 2010a). Selenium toxicity was implicated in larval amphibian deformities and high mortalities observed at this site (Rowe, et al. 1996, Hopkins, et al. 2000, Rowe, et al. 2001). However, since CA effluents contain numerous potentially toxic metals, additional laboratory studies are needed before these toxic responses can be specifically linked to Se contamination.

In addition to sites of CA-contamination, our current understanding of Se ecotoxicology results from field studies conducted following contamination by Se-laden agricultural drainage waters at the Kesterson Reservoir, San Joaquin Valley, CA. Selenium contamination at Kesterson Reservoir was identified following widespread mortality and reproductive failures in migrating waterfowl and aquatic bird species. Selenium concentrations in avian food sources ranged from 20 – 332 $\mu g\ g^{-1}$ dry wt. and

included algae, plankton, aquatic plants, and macroinvertebrates (Ohlendorf 2002). Elevated Se concentrations were also identified in adult snakes and frogs at the site. Studies addressing reproductive and developmental effects of Se exposure in amphibians and reptiles were recommended based on the toxic effects of Se identified in birds and fish (Ohlendorf, et al. 1988).

Selenium Biogeochemistry, Biotransformation and Associated Toxicities

Selenium is a S analog and likely follows a similar biogeochemical cycle in the environment (Fan, et al. 2002, Young 2010b). Fan et al (2002) provided a comprehensive review of Se cycling in the aquatic environment, as it is currently understood. The complexities of the Se biogeochemical cycle are illustrated in Figure 1.1, (reprinted from Fan, et al. 2002). Dissolved Se in aquatic ecosystems exists in either the (IV) or (VI) oxidation state, as the oxyanions selenite (SeO_3^{2-}) or selenate (SeO_4^{2-}), respectively (Fan, et al. 2002). Laboratory studies investigating the effects of these dissolved Se species to algae, invertebrates, fish (Lemly 1993, Maier, et al. 1993, Rosetta and Knight 1995), and waterfowl (Heinz, et al. 1988) revealed low toxicity risks. These same studies indicated Se toxicity risks increase substantially when these inorganic Se-containing compounds are biotransformed into organic, proteinaceous Se-containing compounds by aquatic producers. Organic Se-containing molecules are typically formed when Se substitutes for S atoms during protein synthesis in high-Se environments (Schrauzer 2000). Figure 1.2 (Schrauzer 2000) describes the reaction by which SeO_3^{2-} and SeO_4^{2-} can be converted to SeCys and finally to SeMet by some aquatic bacteria, algae, and macrophyte species (Schrauzer 2000, Fan, et al. 2002). Selenomethionine is the primary organic Se-containing compound formed at the base of aquatic food chains

(Young 2010b) and is also the most toxic to aquatic life (Heinz, et al. 1988, Lemly 1993, Maier, et al. 1993, Rosetta and Knight 1995). Selenomenthionine is thought to cause a majority of the toxic effects of Se contamination in aquatic environments because of its bioaccumulative properties and its amino acid structure (Fan, et al. 2002).

Much of what is currently understood about Se biotransformation to SeMet in aquatic environments is a result of extensive research following Se-contamination at the Kesterson Reservoir in San Joaquin Valley, CA. Selenium speciation analyses of organisms from diverse trophic levels within the reservoir indicated that greater SeMet concentrations were incorporated into protein stores of top predators than organisms at the base of the aquatic food chain. This suggested that SeMet was likely responsible for much of the ecotoxicity following Se contamination. The molecular mechanisms responsible for the severity of SeMet toxicity, relative to other Se-containing species, are still unknown (Fan, et al. 2002). It is possible that the presence of SeMet can alter the activity of certain enzymes compared to those containing S-based methionine moieties (Schrauzer 2000). There is also debate surrounding the effects of SeMet on protein structure. Excessive substitution of Se for S in amino acids may prevent formation of the disulfide bonds necessary for proper protein folding (Lemly 2002). It has also been suggested that, although SeMet does not directly oxidize glutathione, SeMet metabolites might. Increasing glutathione oxidation decreases its antioxidant functionality, causing increased cellular vulnerability to oxidative damage. Selenomethionine toxicity likely results from a combination of improper protein folding and increased cellular oxidative stress, although additional cellular toxicity studies are needed to confirm this theory (Janz 2010).

The mechanisms behind Se toxicity are beyond the scope of this study. This study was instead designed to investigate Se species toxicity to a representative of an understudied taxonomic group: amphibians. Nutritional and agricultural mammalian studies form the historical basis for our understanding of Se activity and toxicity. The current ecotoxicological understanding of Se comes as a result of field studies conducted following large-scale ecological disasters, the contamination of Kesterson Reservoir being one of the most famous and well-studied. These studies, and the laboratory-based investigations that followed, focused largely on fish species (Lemly 1993, 2002, Young 2010a). Amphibians, as a group, receive disproportionately little attention in ecotoxicology (Sparling 2003). As a result of their complex lifecycles, many amphibian species connect aquatic and terrestrial food chains and, in turn, may act as vectors for contaminant transport between these habitats (Snodgrass, et al. 2003, Roe, et al. 2005). Their unique life histories and physiology also make amphibians some of the most sensitive vertebrates to environmental perturbations, and are often viewed as sensitive sentinels of habitat disturbance (Sparling 2003).

Specifically, there is a lack of ecotoxicological studies addressing the effects of Se contamination to amphibians in the aquatic environment (Ohlendorf, et al. 1988, Fan, et al. 2002). The primary objective of this study was to contrast the developmental toxicities of an inorganic Se-containing compound, selenium dioxide (SeO_2), and an organic Se-containing compound, SeMet, dosed through the diet to larval *Hyla versicolor*. Selenium dioxide is an uncharged acidic oxide similar to the oxyanion, selenite. As in selenite, the Se atom in SeO_2 is in the (IV) oxidation state, but unlike selenite, SeO_2 is not applied as a Se salt (Na_2SeO_3). Selenium dioxide was deemed more

appropriate for use with a freshwater organism. Results of previous comparative toxicity studies implied SeMet was more acutely toxic to Daphnia and bluegill sunfish than inorganic forms (SeO_3^{2-} and SeO_4^{2-}) (Besser, et al. 1993, Maier, et al. 1993). This study was designed to determine if there is a similar Se species-dependent toxicity to larval *H. versicolor*.

Hyla versicolor is a common arboreal anuran native to the eastern United States. The abbreviated larval period of *H.versicolor*, roughly 65 d post-hatch in the wild (Dickerson 1906), make it ideal for laboratory-based toxicity studies. We quantified numerous lethal and sublethal endpoints to characterize the toxicities of each chemical to *H. versicolor* larvae. Sublethal endpoints, such as respiration rates, growth rates, timing of and mass at metamorphosis, and lipid concentrations at metamorphosis, were chosen because of their influence on adult anuran survival (Scott 1994, Scott, et al. 2007).

Methods

Algae Collection and Food Preparation

Larvae were provided a filamentous green algae-based diet to mimic the natural diet of *H. versicolor*. Algae was collected from man-made ponds in Calvert County, MD, dehydrated, homogenized, and stored at -4 °C prior to suspension in a gelatin/agar matrix. These components were combined in a 0.7:1:5:100 g mass-ratio of agar, gelatin, dehydrated algae, and deionized water, respectively. Agar and gelatin were stirred into boiling deionized water for one minute and poured over the dehydrated algae in a shallow glass baking dish. Aqueous solutions of seleno-DL-methionine (SeMet) (Sigma-Aldrich Co.) equivalent to 116.5 µg SeMet g^{-1} wet mass (ww) of food (SeMet High dose) or

11.65 µg SeMet g^{-1} ww of food (SeMet Low dose) were added as each gel mixture was cooling to produce nominal concentrations of 50 µg Se g^{-1} ww and 5 µg Se g^{-1} ww , respectively. Aqueous selenium dioxide (SeO_2) (Sigma-Aldrich Co.) equivalent to 65.8 µg SeO_2 g^{-1} ww of food (SeO_2 High dose) or 6.58 µg SeO_2 g^{-1} ww of food (SeO_2 Low dose) were added to cooling gel mixtures to produce the same nominal Se concentrations as the SeMet treatments. Solidified food mixtures were partitioned into 4 g spherical pellets and stored at -4 °C. Dosed food was stored in separate containers to avoid cross-contamination. Subsamples from successive batches of dosed food were lyophilized and analyzed via micro-bomb calorimetry (Parr Instrument Co). Actual Se concentrations and the mean calories per pellet of food from each treatment are presented in Table 3.1.

For this experiment, larvae were provided a food-limited (rationed) diet only, as an *ad libitum* diet might not mimic natural conditions. The same calculations as in Chapter 2 of this thesis were used to estimate larval daily energetic requirements and develop a feeding regimen. Briefly, the resting oxygen consumption rates of 45 fasted individuals, not included in either experiment, were measured over a 24 h period (Micro-Oxymax; Columbus Instruments). The median value (1.58 µl g^{-1} min^{-1}) was converted to an individual daily energy requirement (Cal d^{-1}) using the same calculations and 20 % correction factor used in Chapter 2. As in Chapter 2, daily food allocations were developed based on the calorie content of control food (micro-bomb calorimetry, Parr Instrument Co) to make certain that individuals in this experiment, although experiencing resource-limitation, were still provided adequate calories to maintain survival. Each replicate tank received one food pellet every 48 h. The study was continued until Exposure Day 78, when all larvae in a single treatment had either died or initiated

metamorphosis. This prevented remaining treatments from receiving disproportionately lengthy exposures.

Egg Collection

Three clutches of freshly laid *Hyla versicolor* eggs were collected on two consecutive evenings in late August 2009 in Saint Marys County, MD. Eggs were transported to the Chesapeake Biological Laboratory (Solomons, MD) and held in 10 gallon tanks of aerated laboratory water until hatch. Larvae were fed control algae/gel food for a month prior to study initiation.

Experimental Protocol

This study assessed chronic toxicity of long-term (78 d) dietary exposures to SeMet or SeO_2 via a resource-limited feeding regimen. Upon reaching Gosner Stage 27 (Gosner 1960), 300 larvae were randomly allocated to 20 polypropylene tanks (Sterilite Corp.) (15 larvae per tank) containing 4 L of aged, UV sterilized well water. Four replicate tanks were assigned per treatment, with a total of five treatments: 1) control, 2) SeO_2 Low - 5 µg Se g^{-1} ww, 3) SeO_2 High - 50 µg Se g^{-1} ww, 4) SeMet Low - 5 µg Se g^{-1} ww, and 5) SeMet High - 50 µg Se g^{-1} ww. Each tank received one 4g algae/gel food pellet every 48 hrs. Tank water was constantly aerated and temperature controlled at 23.0 °C (±1.3 °C) and a 12 h light cycle was maintained throughout the study. Water and air temperatures were monitored regularly and electronically recorded (Onset Computer Corp) through Day 78. Tanks were cleaned, tank water replaced, and water quality parameters (pH, dissolved oxygen, and conductivity) monitored every fourth day. Water samples were collected from each replicate tank, composited, and acidified with

concentrated nitric acid (0.5 % of sample volume) prior to each tank cleaning, to quantify Se dissolution from food to water throughout the experiment.

Larval Survival, Mass, Growth and Metabolic Rates- Larval health was tracked daily until the end of the study. Intact carcasses were collected when observed, placed into individually labeled plastic (Whirl-Pak, Nasco) bags, and stored at -80 °C until analysis. Total mass and number of individuals in each tank were recorded on Day 0 and on every fourth day until experimentation was complete or until all larvae in that tank had either reached mortality or initiated metamorphosis. Routine metabolic rates ("RMR" as mean oxygen consumption by resting animals) were measured via microrespirometry (Micro-Oxymax; Columbus Instruments) at 21° C on one non-fasted individual from each tank on Day 9 and Day 29 of dosing, following methods described by Rowe et al. (1998b). Oxygen consumption ($\mu L\ g^{-1}\ min^{-1}$) by each individual was measured at 3 h intervals for 24 h.

Metamorphosis- Craniofacial developments prevent feeding by metamorphosing *H. versicolor* larvae; therefore, individuals were removed to individually labeled containers upon attaining Gosner Stage 42 (indicated by forelimb emergence). As individuals completed metamorphosis to juvenile frogs at Gosner Stage 46 (after complete resorption of the tail), they were euthanized by ventral application of an over the counter oral anesthetic (Orajel, Church and Dwight Co., Inc.), containing 20 % benzocaine (Brown 2004, Torreilles, et al. 2009). Carcasses were stored at -80 °C prior to lipid and Se content analyses. Wet masses of each metamorphosing individual were recorded at Gosner Stages 42 and 46. Snout to vent length (SVL) of each juvenile frog was also measured prior to sacrifice, using digital calipers. Carcasses of any individuals

that died prior to completing metamorphosis were weighed, placed into individually labeled plastic (Whirl-Pak, Nasco) bags, and stored at -80 °C until analysis.

Sample Preparation and Analyses

Selenium Analysis- Selenium concentrations in food and larval and juvenile frog carcasses were determined via Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Agilent 4500). Frozen whole-body carcasses were microwave digested (Milestone Ethos EZ) in 2 – 4 ml of high purity concentrated nitric acid as described by Rowe, et al. 2009. Each batch of 10 digests included 8 carcass samples, a blank, and a matrix spike. Every fifth batch included a standard reference material (SRM) (NIST 1566b Se = 2.06 ± 0.15 $\mu g\ g^{-1}$) in place of one of the carcass samples. Matrix spike Se recoveries averaged 92.55 ± 13.84 % and replicate SRM Se recoveries averaged 100.91 ± 13.05 %. The Se content of pre- and post-storage food samples was also analyzed by ICP-MS. Duplicate samples (approx. 1 g ww each) from each treatment batch made throughout the study were microwave digested following the same method used to prepare carcass samples. Digests were diluted with >18 MΩ deionized water to 10x their original volume, prior to analysis. Acidified treatment tank water samples collected throughout the experiment were also analyzed by ICP-MS.

Lipid Class Analysis- Total lipids were extracted and analyzed by modifying a method by Ju and Harvey (2004) to analyze the lipid content of Antarctic krill. Five juvenile frog carcasses were randomly selected from each treatment, with representatives from each replicate. Lyophilized carcasses were microwave digested (MARS-5, CEM) in 35 ml CH_2Cl_2:MeOH (2:1) at 200 psi and 80 °C for 30 min. Post-extraction carcasses were removed from microwave digestion vials, rinsed with solvent, and returned to

storage at -80 °C for future Se analysis. Post-extraction solvent remaining in digestion vials was evaporated (BUCHI Labortechnik AG). Evaporated lipid extracts were dissolved in a few milliliters of CH_2Cl_2:MeOH (2:1), gravity filtered through glass wool, and transferred to 8 ml amber vials. Transfer solvent was evaporated from lipid extracts under N_2 gas. Dried extracts remaining in amber vials were diluted to 1 ml with CH_2CL_2:MeOH (2:1) and stored at -80 °C until analysis.

Separation and identification of major lipid classes were performed by thin layer chromatography (TLC) using an Iatroscan MK-V analyzer equipped with a flame ionization detector (FID), as described by Ju and Harvey (2004). Diluted extracts (1-3 µL aliquots) were applied to the bottom of silica-coated glass rods (S-III Chromarods, Mitsubishi Chemical Medicine Co.). Rods were placed in a 55°C oven for 5 min to evaporate any remaining solvent. When cool, extracts were "focused" into tight bands by wicking CH_2Cl_2:MeOH (2:1) onto the end of rods until solvent fronts reached the extract application points. Solvent was again evaporated from rods for 5 min in the 55°C oven. Lipids in each extract were separated into major classes using a hexane:diethyl ether:formic acid (85:15:0.2) development solution. Rods with "focused" extracts were placed in a closed, glass chamber containing sufficient developing solution to wick onto the bottom of the rods. Rods remained in the development chamber for 20 min, before a final 5 min in the 55°C oven. Development separated extracts into five major lipid classes along the length of the rod. Phospholipids (PL) were the most polar and remained near the origin of extract application. The remaining neutral lipids followed: sterols (ST), free fatty acids (FFA), triacylglycerides (TAG), and wax esters (WE), from bottom to top of the rod, respectively. After development, the rods were placed in the Iatroscan for

analysis. The FID flame was applied directly to each rod, ionizing lipids from top to bottom. Rods were held in racks of 10, therefore three extracts could be processed on a rack (each in triplicate) at one time. A 3 µl aliquot of a lipid standards mixture of known class concentrations was applied to the remaining rod on each rack.

Sample chromatograms were integrated using HP ChemStation software. Lipid classes in each sample were identified and quantified based on a calibration curve generated from serial dilutions of a concentrated lipid standards mixture. The concentrated standard mixture (7.077 µg µl^{-1} total lipid) contained a representative compound from each lipid class: 4.168 µg µl^{-1} of 1,2–dipalmitoyl–sn–glycera–3-phosphocholine (PL), 0.396 µg µl^{-1} of cholesterol (ST), 0.172 µg µl^{-1} of nonadecanoic acid (FFA), 2.150 µg µl^{-1} glycerol trioleate (TAG), and 0.191 µg µl^{-1} palmityl state (WE) (Sigma-Aldrich Co.) in CH_2Cl_2:MeOH (2:1). Total lipid (TL) concentrations for each sample were derived through summation of the lipid class concentrations quantified. Samples exceeding calibration limits for any lipid class were diluted with known volumes of CH_2Cl_2:MeOH (2:1) and reanalyzed. Mean intra-sample precision was ± 23.5 % or better for each class and the average percent recovery of lipid classes in the standards mixtures was 103.1 % ± 7.93 %.

Statistical Analyses

Mean replicate tank responses were used in statistical analyses. Data were tested and transformed, if necessary, to meet model assumptions. Statistical differences were evaluated based on $\alpha = 0.05$. Time to forelimb emergence and time to complete metamorphosis data did not require transformation prior to analysis for treatment specific differences using the general linear model analysis of variance (GLM ANOVA). Larval

exposure duration was used as a covariate in the GLM analysis of covariance (ANCOVA) used to determine treatment effects on metamorph wet mass (ww) and dry mass (dw) Se body burdens, wet mass at forelimb emergence, and wet mass and SVL at complete metamorphosis. These data did not require transformation to meet model assumptions. Larval RMR were $\log_{10}$ transformed and analyzed using the GLM ANOVA for each trial (Day 9 and Day 29 of exposure). Larval ww Se body burdens were rank transformed and date of carcass collection was used as a covariate in the GLM ANCOVA. Proportion data (survival to Day 78 and initiation and completion of metamorphosis) were arcsine transformed prior to analysis with the GLM ANOVA. A repeated measures GLM ANOVA was conducted to determine treatment effect on larval growth. To calculate larval growth, the mean larval mass of each treatment was recorded until 10 % of the study population initiated metamorphosis. This ensured that mass losses associated with metamorphosis did not artificially deflate larval growth values. It was determined that > 10 % of individuals had initiated metamorphosis by Exposure Day 34. Larval growth was, therefore, determined until the previous day mass measurements were taken, Day 30. The relationship between size at forelimb emergence and survival to complete metamorphosis, independent of treatment, was analyzed by binary logistic regression. The within-treatment relationship between size at forelimb emergence and mortality prior to complete metamorphosis was also analyzed by binary logistic regression, but only for the SeMet Low treatment, as 100 % of individuals in all other treatments that initiated metamorphosis survived to complete it. A general multivariate analysis of variance (MANOVA) was used to determine treatment effects on metamorph total and class-specific lipid concentrations.

Results

Selenium in Food

Actual mean Se concentrations in food were 37.33 ± 7.22 and 584.5 ± 103.5 µg g^{-1} dry mass (dw) in SeO_2 Low and High, respectively, and 50.95 ± 2.96 and 482.03 ± 33.02 µg g^{-1} dw in SeMet Low and High, respectively (Table 3.1). The mean Se concentration in control food was below the 0.0126 µg g^{-1} method detection limit. Selenium dosages were originally calculated to achieve 5 and 50 µg g^{-1} ww nominal Se concentrations in Low and High dose food, respectively. The ww-based mean Se concentrations of each food type are also presented in Table 3.1. Approximately 58.8 % and 71.6 % of the nominal 5 µg g^{-1} ww target Se concentration was achieved in SeO_2 Low and SeMet Low foods, respectively. In the SeO_2 High and SeMet High foods, 71.6 % and 71.1 % of the nominal 50 µg g^{-1} ww target Se concentration, respectively, was achieved. Food was stored at -4°C for a maximum of two months prior to use. Values in Table 3.2 indicate storage did not appreciably alter Se concentrations.

Selenium dissolution from food was minimal, as tank water was replaced every four days. The mean and maximum dissolved Se concentrations (µg ml^{-1}) measured in pooled replicate tank water prior to water change are presented in Table 3.3. Selenium dissolution was dose dependent and highest in the SeO_2 High treatment (0.140 µg ml^{-1}, maximum). The mean dissolved Se concentration in control tank water (0.002 ± 0.0002 µg ml^{-1}) was the same as the mean background Se concentration in measured well water (0.002 ± 0.0002 µg ml^{-1}).

Selenium Body Burdens

Larval Se body burdens (ww) were significantly influenced by treatment ($F = 44.01$, $p < 0.0001$) and length of Se exposure (approximated by date of carcass collection in Table 3.4) ($F = 5.03$, $p = 0.042$). Control larvae had significantly lower mean Se body burdens than larvae in SeO_2 High ($p < 0.0001$), SeMet High ($p < 0.0001$), and SeMet Low ($p = 0.0216$) treatments. The mean Se body burdens of SeO_2 Low larvae were not significantly different from controls ($p = 0.7639$). Selenium body burdens in the SeO_2 High and SeMet High treatments did not differ significantly ($p = 0.7639$). Selenium body burdens were dosage-dependent. Larvae from the SeO_2 High treatment had significantly greater Se tissue concentrations than larvae in the SeO_2 Low and SeMet Low treatments ($p = 0.0003$ and $p = 0.0073$, respectively). The SeMet High treatment larvae also had significantly greater Se body burdens than SeO_2 Low and SeMet Low larvae ($p < 0.0001$ and $p = 0.0006$, respectively). The Se body burdens of larvae in the SeO_2 Low and SeMet Low treatments did not differ ($p = 0.3703$).

The mean Se body burdens of metamorphs from each treatment are also presented in Table 3.4 along with the corresponding mean larval period duration (d), as an approximation of Se exposure duration. Metamorph Se body burdens are presented in ww and dw concentrations. The SeO_2 High treatment could not be included in statistical analyses because only one individual from this treatment completed metamorphosis. Individuals in the SeMet High treatment reached 100 % mortality as larvae. Exposure duration was used as a covariate in *post-hoc* comparisons but did not significantly influence metamorph Se body burdens ($p = 0.219$ for ww Se concentrations and $p = 0.619$ for dw Se concentrations). Treatment did have a significant impact on both ww

and dw Se tissue concentrations in metamorphs ($p < 0.0001$). The dw Se body burdens of control metamorphs were significantly less than SeO_2 Low metamorphs ($p = 0.0063$) and SeMet Low metamorphs ($p < 0.0001$). Metamorphs from the SeMet Low treatment had significantly greater dw Se tissue concentrations than metamorphs from the SeO_2 Low treatment ($p = 0.0009$). The same patterns of significance were observed when ww Se body burdens were compared. The ww Se body burdens of control metamorphs were significantly lower than SeO_2 Low metamorphs ($p = 0.0041$) and SeMet Low metamorphs ($p < 0.0001$). Metamorphs in the SeO_2 Low treatment had significantly lower ww Se tissue concentrations than meatmorphs in the SeMet Low treatment ($p = 0.0005$).

Toxicity to Larvae

Larvae receiving the SeMet High dose displayed abnormal red coloration beneath the integument within 24 h of study initiation. Abnormal coloration began to fade in surviving individuals by Day 7 of dosing. Many larvae from this treatment also displayed severe edema prior to death. These abnormalities were not observed in larvae from any other treatment in this study, including the SeMet Low dose. Two individuals from different replicate tanks of the SeMet Low treatment developed edema upon forelimb emergence. These individuals did not display other deformities during metamorphic climax. Both survived to complete metamorphosis, despite exhibiting edema.

Mean routine metabolic rates (RMR) per treatment are displayed in Table 3.5 and Figure 3.1. There was a significant treatment effect on mean RMR on Day 9 ($F = 4.68$, $p < 0.0001$). Mean RMR in the SeMet High treatment was significantly less than that of

control (p = 0.0369), SeO_2 Low (p = 0.0008), SeO_2 High (p = 0.0014), and SeMet Low (p = 0.0007) treatments on Day 9. Larvae from the SeMet High treatment were no longer available for RMR measurements by Day 29. Treatment still had a significant effect on RMR by Day 29, however (F = 4.68, p = 0.022). Mean RMR of larvae from the SeO_2 High treatment was significantly less than control mean larval RMR (p = 0.0188). Mean RMR in other treatments did not differ significantly.

Mean larval masses per replicate tank were recorded during tank cleanings conducted every four days. Ten percent of larvae initiated metamorphosis prior to tank cleaning on Day 34 (94 d post-hatch). Therefore, mean larval growth rates per treatment were calculated using mass measurements made on Day 30 (90 d post-hatch), the tank cleaning immediately before Day 34 (Figure 3.2). Larval mass did increase over time (F= 25.35, $p < 0.0001$) and mean larval growth rates were significantly different among treatments (F = 4.72, p = 0.011). Larvae in the SeMet High treatment were of consistently lower mean mass than larvae in all other treatments until Day 30.

Mean survival in each treatment on Day 78 is included in Table 3.6. Larvae and premetamorphs remaining at the end of the study and individuals that successfully completed metamorphosis prior to Day 78 were considered survivors. Treatment significantly influenced mean survival on Day 78 (F = 31.32, $p < 0.0001$). The SeMet High treatment was acutely toxic to *H. versicolor* larvae. Replicate tanks receiving this treatment reached 100 % mortality by exposure Day 34 (Figure 3.3). No individuals in this treatment survived to initiate metamorphosis. Survival to Day 78 in the SeMet High treatment was significantly less than all other treatments ($p < 0.0001$), except the SeO_2 High treatment (p = 0.9848). The mean percent survival to Day 78 in the SeO_2 High

treatment (3.333 ± 1.9 %) was significantly less than that in control ($p < 0.0001$), SeO_2 Low ($p < 0.0001$), and SeMet Low ($p = 0.0002$) treatments.

Toxicity During Metamorphosis

The percentage of survivors to initiate metamorphosis (Gosner stage 42) prior to Day 78 significantly differed among treatments ($F = 39.80$, $p < 0.0001$) (Table 3.6). High larval mortality rates in the SeMet High treatment prevented any individuals from reaching Gosner stage 42. This was significantly less than the number of individuals to enter Gosner stage 42 in control, SeO_2 Low, and SeMet Low treatments ($p < 0.0001$). Survival to initiate metamorphosis was also low in the SeO_2 High treatment (1.667 ± 1.7 %) and not significantly different from the SeMet High treatment ($p = 0.9981$). Significantly more individuals initiated metamorphosis in the SeO_2 Low and SeMet Low treatments ($p < 0.0001$ and $p = 0.0001$, respectively) than in the SeO_2 High treatment. The proportion of individuals to initiate metamorphosis in the SeO_2 Low and SeMet Low treatments did not differ significantly ($p = 0.2997$) and were not significantly different from the control treatment ($p = 0.9491$ and $p = 0.6892$, respectively).

Toxicity endpoints evaluated at Gosner stage 42 are included in Table 3.7. No individuals survived to initiate metamorphosis in the SeMet High treatment and only one individual initiated metamorphosis in the SeO_2 High treatment. These treatments were, therefore, excluded from statistical comparisons. The mean days post-hatch required for individuals to reach forelimb emergence (Gosner stage 42) in each treatment did not differ significantly ($F = 0.63$, $p = 0.553$). Mean masses of individuals at Gosner stage 42 did vary significantly by treatment ($F = 5.21$, $p = 0.036$), but were not significantly influenced by the mean length of the larval period ($F = 3.29$, $p = 0.107$ see Table 3.4).

Premetamorphs from the SeMet Low treatment were of significantly smaller masses than control premetamorphs ($p = 0.0323$), but did not differ in mass from SeO_2 Low premetamorphs ($p = 0.4244$). Mean premetamorph masses in the control treatment were also not significantly different from the SeO_2 Low treatment ($p = 0.1640$).

Rear limb deformities were observed in 8.3 ± 4.8 % of premetamorphs from the SeMet Low treatment, but were absent in other treatments. Of the SeMet Low premetamorphs that displayed rear limb deformities, 37.5 ± 23.9 % died prior to completing metamorphosis (Gosner stage 46). Survival through metamorphic climax of individuals that initiated metamorphosis was also reduced in the SeMet Low treatment (87.5 ± 8.0 %), but not in any others (Table 3.7).

The proportion of individuals to complete metamorphosis (Gosner stage 46) significantly varied by treatment ($F = 35.64$, $p < 0.0001$; see Table 3.6). No individuals survived to complete metamorphosis in the SeMet High treatment and only one individual completed metamorphosis from the SeO_2 High treatment. The proportion of individuals to reach Gosner stage 46 in the control treatment was significantly greater than in the SeMet High and SeO_2 High treatments ($p < 0.0001$ for each comparison). Metamorphic success in the SeO_2 High treatment was also significantly lower than in the SeO_2 Low and SeMet Low treatments ($p < 0.0001$ and $p = 0.0004$, respectively). Significantly more individuals completed metamorphosis in the SeO_2 Low and SeMet Low treatments than in the SeMet High treatment ($p < 0.0001$ and $p = 0.0002$, respectively). The proportion of individuals to achieve Gosner stage 46 in the SeO_2 Low and SeMet Low treatments did not differ significantly ($p = 0.0728$) and neither differed from the control treatment ($p = 0.9547$ and $p = 0.2358$, respectively). Wet mass at

Gosner stage 42 did not influence the likelihood of survival through metamorphic climax, independent of treatment ($p = 0.208$). Mortality during metamorphic climax (Gosner stage 42 through 46) was only observed in the SeMet Low treatment, but was not dependent on premetamorph mass ($p = 0.629$).

Sublethal toxicity endpoints monitored at Gosner stage 46 are presented in Tables 3.8 and 3.9. As previously mentioned, only one individual completed metamorphosis in the SeO_2 High treatment and no individuals receiving the SeMet High treatment survived to complete metamorphosis. These treatments were excluded from statistical comparisons in Table 3.8 and were not analyzed for total lipid or lipid class concentrations (Table 3.9). As with time to forelimb emergence, time (days post-hatch) to complete metamorphosis (Gosner stage 46) did not significantly differ among treatments ($F = 1.59$, $p = 0.256$). Larval exposure duration (Table 3.4) did not have a significant influence on metamorph mean wet mass ($F = 4.76$, $p = 0.061$) or mean SVL ($F = 4.56$, $p = 0.065$). However, including larval exposure duration as a covariate revealed a significant treatment effect on mass ($F = 6.58$, $p = 0.020$) and SVL ($F = 11.32$, $p = 0.005$) at Gosner stage 46. Control metamorphs were of larger mass and SVL than SeO_2 Low ($p = 0.044$ and $p = 0.0085$, respectively) and SeMet Low ($p = 0.0323$ and $p = 0.0096$, respectively) metamorphs. Metamorphs in the SeO_2 Low and SeMet Low treatments did not differ in mean wet mass ($p = 0.4244$) or mean SVL ($p = 0.9494$). Metamorph total lipid (TL) and specific lipid class concentrations (Table 3.9) did not differ between treatments and were not significantly influenced by the timing (d post-hatch) of metamorphosis.

Discussion

Selenium Uptake

It is a widely accepted principle that aqueous Se concentrations in contaminated aquatic ecosystems do not adequately predict Se toxicity to wildlife due to the propensity of Se to bioaccumulate in the aquatic food chain (Hamilton 2002, Lenz and Lens 2009). The contribution of aqueous Se exposure to tissue Se concentrations and associated toxic responses observed in this study cannot be omitted, however. Aqueous Se concentrations of 1 - 5 $\mu g\ l^{-1}$ can be toxic to aquatic wildlife (Lemly 1993). Background Se concentration of aged well water used in this study fell within that range, as did the mean Se concentration of control tank water. Although minimal compared to Se concentrations in the food, maximum dissolved Se concentrations in tank water from Se-dosed treatments were 2 - 35 times the background concentration (Table 3.3). Maximum dissolved Se concentrations in SeO_2 Low and SeMet Low treatments were similar to aqueous Se concentrations measured at Kesterson Reservoir and Belews Lake at the height of Se contamination (Fan, et al. 2002, Rowe, et al. 2002). Combination dietary and aqueous Se exposure studies determined bluegill (*Lepomis macrochirus*) accumulated more Se from diet than from aqueous exposure, independent of the Se species applied (Besser, et al. 1993). Further study is required to determine the toxicity of these dissolved SeMet and SeO_2 concentrations to developing *H. versicolor* larvae in combination and independent of dietary dosing.

In general, SeMet accumulated in *H. versicolor* tissue to a greater extent than SeO_2, particularly at the High doses. Mean larval tissue Se concentrations in all dosed treatments, except SeO_2 Low, were greater than in the control treatment. Larvae in the

SeO_2 Low treatment did not take up or retain significantly more Se than larvae in the control treatment, although the control mean larval exposure period (approximated by collection date) was longer (Table 3.4). Larval exposure periods prior to carcass collection in the SeMet High treatment were short due to high larval mortality (100 % by Day 34 of the experiment). Larvae in the SeMet High treatment still took up slightly more Se than larvae in the SeO_2 High treatment, though the differences in mean tissue Se concentrations in these treatments were not significant. Mean larval exposure periods in the SeMet High and SeO_2 Low treatments were almost identical, although SeO_2 Low larval exposures prior to carcass collection were short because of high rates of metamorphosis, not mortality (see Table 3.6). Although the mean Se body burden of SeO_2 Low larvae was approximately 50 times less than that of SeMet Low larvae, these mean Se concentrations were not significantly different. This is likely an artifact of the disparity in carcass collection dates between treatments, which was used as a covariate in the statistical model. Mean larval exposure in the SeO_2 Low treatment was brief (15 ± 2.7 d) compared to the mean larval exposure period in the SeMet Low treatment (53 ± 8.8 d). There was no evidence of Se bioconcentration or biomagnification from food, based on comparisons of ww Se concentrations in larvae and corresponding food sources.

High doses of both Se-containing compounds were toxic to larvae. Metamorphosis was severely reduced by larval exposure to the SeO_2 High dose, and acute toxicity of the SeMet High dose prevented any individuals from surviving to initiate metamorphosis (Table 3.6). Metamorph Se body burdens were greater in Se-exposed treatments (SeO_2 Low and SeMet Low treatments) than in the control treatment, indicating Se accumulated during the larval stage in Se-exposed treatments was retained

through the non-feeding period of metamorphosis. Field measurements of *Rana catesbeiana* at the coal ash contaminated Savannah River Site support this idea. Snodgrass et al (2003) observed Se concentrations accumulated in tissues during the larval period were maintained after metamorphosis. Unfortunately, definitive comparisons between the ww-based larval and metamorph Se body burdens could not be conducted in the present study due to high variability in tissue water. Tadpoles can lose more than half of their body water during metamorphosis while dry mass and lipid content remain unchanged (Beck and Congdon 2003, Brown and Cai 2007). Analyzing Se concentrations in lyophilized larvae and metamorphs would have eliminated body water inconsistencies from affecting the inter- and intra-life stage Se body burden comparisons. It should also be noted that larvae were not fasted prior to carcass collection in this study. Therefore, it is likely that measured larval tissue Se concentrations also include Se from undigested gut contents. Metamorph tissue concentrations represent fasted Se body burdens, since physical and chemical changes during metamorphic climax prevent active feeding.

Biological Responses

We observed the same physical indications of toxicity (increased red coloration, edema, and deformed rear limbs) in the SeMet treatments of this study as in SeMet treatments from the CA and SeMet dosing study discussed in Chapter 2. Many larvae in the SeMet High treatment developed abnormal red coloration within the first 7 d of dosing. The red color appeared to result from release or pooling of blood in capillaries beneath the larval integument. Similar capillary leakage was observed in gill lamella of juvenile bluegill (*Lepomis macrochirus*) exposed to a much lower dietary concentration

(5 ug g^{-1} dw) of SeMet over 180 d (Lemly 1993). The fading red color observed after Day 7 indicates surviving larvae may have engaged a mechanism for coping with the vascular osmotic stress induced by SeMet High exposure. Color change was not observed in larvae from the SeMet Low treatments in either study (see Chapter 2), which suggests the SeMet Low dosing concentrations (see Tables 2.1 and 3.1) were below the lowest observed effect concentration (LOEC) to elicit this particular response. Additional laboratory studies are needed to determine 1) if a cellular mechanism for restoring capillary homeostasis in the presence of high SeMet concentrations exists and 2) the LOEC of dietary SeMet to cause this response.

In addition to abnormal coloration, some larvae in the SeMet High treatment displayed edema prior to death. Like freshwater fish, tadpoles are hyperosmotic and hyperionic relative to their dilute freshwater habitats. They maintain osmotic homeostasis by excreting large volumes of dilute urine and by using active transport mechanisms within the integument to remove excess ions. Edema occurs when an organism can no longer maintain control of its osmotic gradient and a sudden influx of water results (Ultsch 1999). Only individuals in the SeMet High treatment developed edema as larvae. Two individuals in the SeMet Low treatment developed edema following forelimb emergence (Gosner stage 42). As in Chapter 2, premetamorph edema only occurred in the SeMet Low treatment. However, it did not coincide with rear limb deformities or mortality prior to metamorphic completion in this study. Selenium exposure elicited edema in larval and adult fish and embryonic birds in previous studies (Lemly 1993, 2002, Ohlendorf 2002, Hamilton 2004). It has also been suggested that edema is a symptom of oxidative stress, a common sublethal response to Se exposure

(Janz 2010). Selenium's role in oxidative stress management provides another illustration of the narrow therapeutic range of this essential micronutrient. At therapeutic concentrations, Se incorporated into selenoproteins, specifically the glutathione peroxidase enzyme, play an integral role in cellular antioxidant systems (Young 2010b). However, excess concentrations of Se can actually increase oxidative stress by disrupting this and other antioxidant systems (Janz 2010). If Se-induced edema does signal oxidative stress, it is interesting that this symptom was only observed in *H. versicolor* receiving SeMet in their diets. Studies indicate SeMet does not disrupt the glutathione antioxidant system as much as other Se forms. Further research is needed to determine if SeMet metabolites might instead be responsible for the observed oxidative stress.

A smaller proportion (Table 3.7) of SeMet Low premetamorphs developed the same weak, malformed rear limbs as in the CA and SeMet dosing study (Chapter 2). Again, these rear limb deformities were only observed in premetamorphs from the SeMet Low treatment and are similar to rear limb deformities described by Snodgrass et al (2004). Less than 40 % of premetamorphs with deformed rear limbs died prior to completing metamorphosis (Table 3.7). It was unclear whether these deformities directly contributed to premetamorph mortality.

Selenium toxicity to larvae in this study was dose and Se species-dependent. As expected, SeMet was more toxic to developing *H.versicolor* larvae than SeO_2, especially at the high concentrations used in this study (Fan, et al. 2002). In addition to the malformations and abnormalities discussed above, exposure to nominal SeMet concentrations of 50 µg Se g^{-1} ww in the diet caused reductions in larval RMR and growth rates, increased larval Se concentrations, and induced 100 % larval mortality by

Day 34 of dosing. Exposure to SeO_2 of the same nominal Se concentration did not induce physical abnormalities in larvae and decreased larval RMR and survival to a lesser extent than the SeMet High dose. Based on the endpoints evaluated at the larval life stage, effects of SeO_2 and SeMet at the lower nominal Se concentration (5 µg Se g^{-1} ww in the diet) were statistically indistinguishable from the control treatment in this study. Toxic responses in the SeMet Low treatment were not observed until metamorphosis was initiated (Gosner stage 42). Neither Low-dose Se exposures altered the proportion of individuals to initiate metamorphosis (Table 3.6), but premetamorphs that emerged from the SeMet Low treatment were significantly smaller than control or SeO_2 Low premetamorphs (Table 3.7). A small percentage of premetamorphs in the SeMet Low treatment developed rear limb deformities and edema. Mortality during metamorphic climax only occurred in this treatment, but was unrelated to reduced premetamorph mass. As expected, individuals lost mass during the non-feeding portion of metamorphosis (Gosner stage 42 through 46) in all treatments. Metamorphs from SeO_2 Low and SeMet Low treatments were significantly smaller than control metamorphs (Table 3.8). Field studies have indicated there is a strong relationship between size at metamorphosis and adult survival to reproductive age in amphibians (Scott 1994). All treatments were under rationed feeding regimens, and excess food was not observed in tanks prior to each new food addition. Therefore, individuals were likely experiencing food-limitation stress in combination with chemical toxicity, although this effect was not evaluated by the study design. We can therefore only infer that sublethal responses to SeMet and SeO_2 exposure combined with the pressure of resource limitation could cause reductions in the reproductive population of *H. versicolor* in the natural environment.

Lipids are the primary energy stores utilized during non-feeding periods of metamorphic climax and early terrestrial life (Fitzpatrick 1976, Scott, et al. 2007) and therefore, are likely a better determinant of adult success than metamorph wet mass. Larval exposures to the Low dose of each compound did not alter metamorph lipid concentrations in comparison to controls, however. This indicates wet mass reductions in these treatments are due to other factors, perhaps increased water or protein losses, during the non-feeding period of metamorphic climax (Lemly 1993, Beck and Congdon 2003, Brown and Cai 2007). The time (d post-hatch) required for individuals to reach Gosner stages 42 and 46 was not affected by Low-dose exposures to either Se-containing compound, nor were the proportions of individuals to complete metamorphosis.

Conclusions

This study provides further evidence of Se species-specific toxicity by contrasting toxic responses of developing *H. versicolor* exposed to high concentrations of an inorganic Se-containing compound, SeO_2, and an organic Se-containing compound, SeMet. The mechanisms of Se toxicity are largely unknown, but the results of our study indicate that SeMet is more toxic and bioavailable to aquatic consumers than inorganic Se-containing compounds, such as SeO_2. Selenomethionine exposure increased oxidative stress in larvae and premetamorphs, decreased survival and metamorphosis, and caused reductions in masses and sizes of recent metamorphs that could negatively affect juvenile survival. The SeO_2 High dose had comparable effects on survival, metamorphosis, and masses of recent metamorphs, but neither SeO_2 dose induced symptoms of oxidative stress or caused mortality during metamorphic climax. In this study, nominal Se doses

were developed on a wet mass instead of a dry mass basis, and were therefore elevated beyond concentrations typically found at even the most contaminated sites (Fan, et al. 2002, Lemly 2002, Rowe, et al. 2002). Additional research evaluating the toxicities of more environmentally relevant SeMet and SeO_2 concentrations is necessary before Se-contamination risks to larval amphibians can be properly assessed. Future studies contrasting the effects of food limitation on toxic responses of *H. versicolor* to SeMet and SeO_2 exposure are also needed.

Table 3.1. Selenium concentrations and caloric content of tadpole food

Treatment	Se Conc. (µg g^{-1} ww)	% of Target ww Conc.	Se Conc. (µg g^{-1} dw)	Se Dose per Pellet (µg ww)	Cal g^{-1}
Control	ND		ND		4.21 ± 0.05
SeO_2 Low	2.94 ± 0.6	58.8	37.33 ± 7.22	11.76 ± 2.4	4.15 ± 0.03
SeO_2 High	36.91 ± 9.7	73.8	584.5 ± 103.5	147.63 ± 38.8	4.20 ± 0.02
SeMet Low	3.58 ± 0.3	71.6	50.95 ± 2.96	14.32 ± 1.20	4.23 ± 0.04
SeMet High	35.55 ± 3.4	71.1	482.03 ± 33.02	142.19 ± 19.3	4.17 ± 0.09

Values are means ± 1 S.E. * Dry mass (dw) Se concentrations were calculated based on wet mass (ww) concentrations and the average moisture content of subsamples from each food type. "ND" indicates Se concentrations were below the method detection limit (0.001 µg g^{-1} ww or 0.0126 µg g^{-1} dw).

Table 3.2. Selenium concentrations in food prior to and after storage at -4 °C

Treatment	Pre-Storage Se Conc. ($\mu g\ g^{-1}$ dw)	Post-Storage Se Conc. ($\mu g\ g^{-1}$ dw)
Control	ND	2.30 ± 0.2
SeO_2 Low	37.33 ± 7.22	51.80 ± 9.02
SeO_2 High	584.5 ± 103.5	570.45 ± 19.4
SeMet Low	50.95 ± 2.96	48.42 ± 1.79
SeMet High	482.03 ± 33.02	529.03 ± 46.9

Values are means ± 1 S.E. * Dry weight (dw) Se concentrations were calculated based on wet mass (ww) concentrations and the average moisture content of subsamples from each food type. Post-storage concentrations were measured after storage for a maximum of two months. "ND" indicates mean Se concentration was below the method detection limit (0.0126 $\mu g\ g^{-1}$ dw).

Table 3.3. Dissolved Se concentrations in tank water

Treatment	Mean Se Conc. ($\mu g\ ml^{-1}$)	Maximum Se Conc. ($\mu g\ ml^{-1}$)
well water	0.002 ± 0.0002	0.005
Control ration	0.002 ± 0.0002	0.004
SeO_2 Low ration	0.009 ± 0.001	0.012
SeO_2 High ration	0.106 ± 0.006	0.140
SeMet Low ration	0.006 ± 0.0003	0.009
SeMet High ration	0.029 ± 0.002	0.043

Values are means ± 1 S.E.

Table 3.4. Larval and metamorph Se body burdens of *H. versicolor*

Treatment	Larvae			Metamorphs			
	n	Se (µg g^{-1} ww)	Collection Date	*n*	Se (µg g^{-1} ww)	Se (µg g^{-1} dw)	Larval Exposure (d)
Control ration	4	0.057 ± 0.06^{a}	36 ± 14.3	4	0.361 ± 0.071^{a}	2.05 ± 0.57^{a}	40.7 ± 4.7
SeO_2 Low ration	4	0.197 ± 0.2^{ac}	15 ± 2.7	4	6.53 ± 1.3^{b}	41.4 ± 6.2^{b}	40.5 ± 3.02
SeO_2 High ration	4	29.36 ± 3.4^{b}	47 ± 2.7	1*	27.3	217.2	42
SeMet Low ration	4	11.18 ± 0.9^{c}	53 ± 8.8	4	15.1 ± 1.3^{c}	100.6 ± 7.7^{c}	34.2 ± 0.41
SeMet High ration	4	41.99 ± 7.6^{b}	14 ± 2.0	0			

Values presented are mean larval carcass Se concentrations (µg g^{-1} ww) ± 1 S.E. and mean Se concentrations in metamorphs (expressed in both µg g^{-1} ww and µg g^{-1} dw). Mean experimental date of carcass collection ± 1 S.E. (thru Day 78) for larvae and mean larval exposure ± 1 S.E. (thru Day 78) for metamorphs are included as estimates of exposure duration. * Excluded from statistical analyses. Dissimilar superscripts indicate statistically significant differences.

Table 3.5. Larval routine metabolic rates

Treatment	Day 9		Day 29	
	n	RMR (µl O_2 min^{-1} g^{-1})	*n*	RMR (µl O_2 min^{-1} g^{-1})
Control ration	4	0.904 ± 0.02^{a}	4	2.02 ± 0.16^{a}
SeO_2 Low ration	4	1.34 ± 0.15^{a}	4	1.69 ± 0.18
SeO_2 High ration	4	1.30 ± 0.21^{a}	4	1.16 ± 0.19^{b}
SeMet Low ration	4	1.34 ± 0.10^{a}	4	1.81 ± 0.21
SeMet High ration	4	0.516 ± 0.08^{b}	0	

Values are mean larval routine metabolic rates (RMR) expressed as µl O_2 consumed per min per g wet mass ± 1 S.E. on Day 9 and 29 of Se exposure. Numbers of individuals (n) measured per treatment each day are also included. High mortality prevented RMR measurements in the SeMet High treatment on Day 29. Dissimilar superscripts indicate statistically significant differences.

Table 3.6. Survival to Day 78 and proportion to initiate and complete metamorphosis by Day 78

Treatment	Replicate tanks *n*	Survival to D78 (%), metamorphs + remaining larvae	Initiate metamorphosis by Day 78 (%)	Complete metamorphosis by Day 78 (%)
Control ration	4	48.33 ± 6.3[a]	45.00 ± 3.2[a]	45.00 ± 3.2[a]
SeO_2 Low ration	4	48.33 ± 5.7[a]	48.34 ± 5.7[a]	48.33 ± 5.7[a]
SeO_2 High ration	4	3.333 ± 1.9[b]	1.667 ± 1.7[b]	1.667 ± 1.7[b]
SeMet Low ration	4	41.67 ± 1.7[a]	38.33 ± 4.2[a]	33.33 ± 4.7[a]
SeMet High ration	0	0.0[b]	0.0[b]	0.0[b]

Values are means ± 1 S.E. Dissimilar superscripts indicate statistically significant differences.

Table 3.7. Toxicity endpoints evaluated at Gosner stage 42

Treatment	Replicate tanks with premetamorphs *n*	Time to forelimb emergence (d post-hatch)	Mass at forelimb emergence (g ww)	Incidences of rear leg deformities (% of those to initiate metamorphosis)	Mortality with rear leg deformities (% of those with rear leg deformaties)	Survival through metamorphic climax (% of those to initiate metamorphosis)
Control ration	4	101.4 ± 5.3	0.417 ± 0.03[a]	0	0	100
SeO_2 Low ration	4	102.4 ± 4.1	0.359 ± 0.01[ab]	0	0	100
SeO_2 High ration	1*	102	0.315	0	0	100
SeMet Low ration	4	96.43 ± 2.0	0.342 ± 0.01[b]	8.333 ± 4.8	37.50 ± 23.9	87.50 ± 8.0
SeMet High ration	0					

Values are means ± 1 S.E. Dissimilar superscripts indicate statistically significant differences. * Excluded from statistical analyses.

Table 3.8. Toxicity endpoints evaluated at Gosner stage 46

Treatment	Replicate tanks with metamorphs *n*	Time to complete metamorphosis (d post-hatch)	Mass at complete metamorphosis (g ww)	SVL at complete metamorphosis (mm)
Control ration	4	104.4 ± 4.9	0.342 ± 0.04[a]	13.93 ± 0.45[a]
SeO_2 Low ration	4	105.8 ± 3.9	0.262 ± 0.01[b]	12.41 ± 0.14[b]
SeO_2 High ration	1*	106	0.245	12.34
SeMet Low ration	4	95.42 ± 1.7	0.272 ± 0.01[b]	12.65 ± 0.25[b]
SeMet High ration	0			

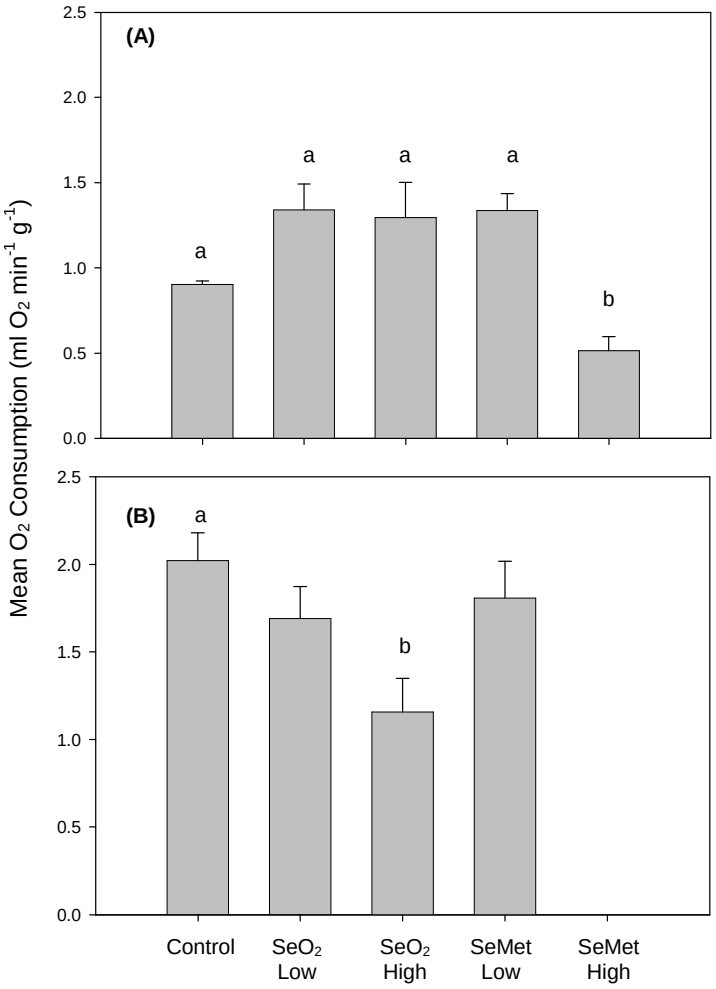
Values are means ± 1 S.E. Dissimilar superscripts indicate statistically significant differences. * Excluded from statistical analyses.

Table 3.9. Total lipid and lipid class content of metamorphs (Gosner stage 46)

Treatment	Replicate tanks with metamorphs *n*	Time to complete metamorphosis (d post-hatch)	Total Lipids (mg/g dw)	WE (mg/g dw)	TAG (mg/g dw)	FFA (mg/g dw)	ST (mg/g dw)	PL (mg/g dw)
Control ration	4	106.8 ± 10.2	353.6 ± 46.1	3.14 ± 0.3	33.03 ± 3.8	8.85 ± 0.5	12.9 ± 0.9	295.7 ± 49.1
SeO_2 Low ration	4	109.5 ± 6.4	374.3 ± 71.0	3.09 ± 0.6	38.4 ± 6.9	9.41 ± 1.0	13.3 ± 0.4	310.1 ± 70.1
SeMet Low ration	3	100.7 ± 1.9	294.7 ± 45.4	3.19 ± 0.3	34.9 ± 2.03	10.2 ± 1.1	12.2 ± 0.05	234.3 ± 48.04

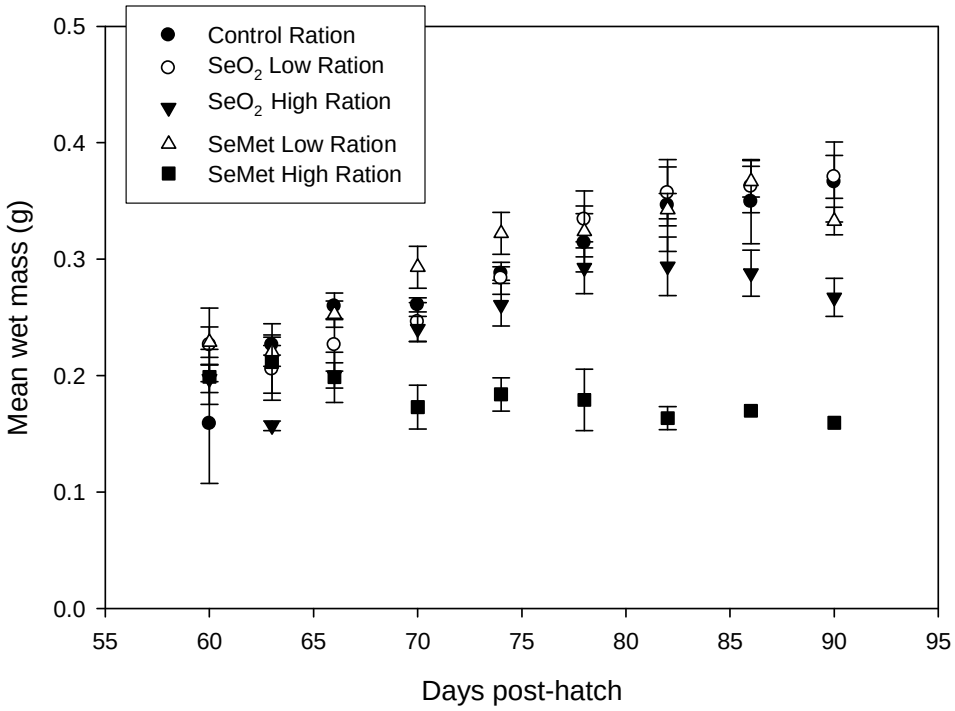
Values are means ± 1 S.E. Dissimilar superscripts indicate statistically significant differences.

Figure 3.1. Larval routine metabolic rates on Day 9 (A) and Day 29 (B)



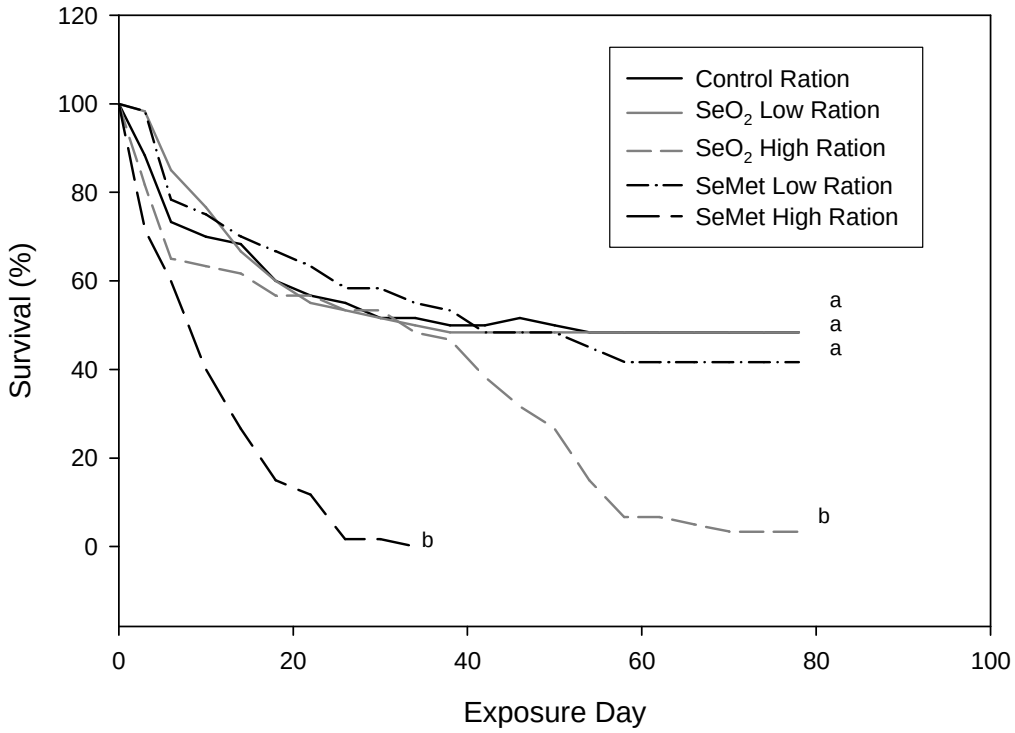
Values are mean larval routine metabolic rates (RMR) expressed as µl O_2 consumed per min per g wet mass ± 1 S.E. on Day 9 (A) and 29 (B) of Se exposure. High mortality prevented RMR measurements in the SeMet High treatment on Day 29. Dissimilar superscripts indicate statistically significant differences.

Figure 3.2. Larval growth rates over the first 30 d of exposure



Larval growth rates are presented from 60 to 90 d post-hatch (equivalent to the first 30 d of exposure). Ten percent of larvae initiated metamorphosis prior to 94 d post-hatch, therefore larval growth rates are only presented until the 90 d post-hatch measurements. Error bars represent ± 1 S.E.

Figure 3.3. Survival through 78 d exposure



Mean percent survival of individuals, regardless of life stage, in each treatment through exposure Day 78. Dissimilar letters indicate statistically significant differences in percent survival on Day 78.

Chapter 4: Conclusion

This research provides evidence of Se species-specific toxicity to a larval anuran, *Hyla versicolor*. Although the mechanisms of Se toxicity remain unknown, the results of this study (Chapter 3) indicate that SeMet is more toxic and bioavailable to aquatic consumers than inorganic Se-containing compounds, such as SeO_2. In contrast, there was little evidence of CA toxicity (Chapter 2) to *H. versicolor*. However, this is likely an artifact of our dosing methods, as larvae were only exposed to CA when fed every 24 or 48 h. In the natural environment, amphibian larvae would be continually exposed to CA while grazing periphyton or ingesting sediment and detritus (Hopkins, et al. 2002, Snodgrass, et al. 2004, Unrine, et al. 2007a). Utilizing a study design where CA is incidentally ingested during feeding (Snodgrass, et al. 2004) would be more appropriate. Hopkins et al. (2002) conducted a similar study addressing the effects of CA exposure and resource limitation on lake chubsuckers and discovered a direct relationship between the amount of food provided and Se accumulated from CA mixed with treatment tank substrate. This study design would better address the toxic effects of combined CA contamination and resource limitation to larval amphibians.

Results of both experiments (Chapters 2 and 3) demonstrated SeMet was more toxic to larval *H. versicolor* than the other Se-containing compounds studied (CA and SeO_2). Each used the same High (50 µg Se g^{-1} ww nominal concentration) and Low (5 µg Se g^{-1} ww nominal concentration) doses of SeMet and similar toxic responses were observed in both studies. However, the structure of the particular SeMet molecule (seleno-DL-methionine; Sigma-Aldrich Co.) used may preclude comparisons of these results to the effects of SeMet in the natural environment. The L isomer is the

predominant amino acid configuration present in nature and the only isomer incorporated into proteins (Lemly 1993, Hamilton 2004). The SeMet applied in this study was a 50:50 mixture of the D and L SeMet isomers. Avian dosing studies indicated SeMet toxicity was isomer-specific, and dietary application of D-SeMet elicited greater toxicity (Heinz and Hoffman 1996, Heinz, et al. 1996. The same avian studies confirmed that L-SeMet was more toxic than inorganic forms (selenite and selenate; Heinz, et al. 1988, Heinz and Hoffman 1996, Heinz, et al. 1996). Therefore, Se species-specific toxicities observed in the studies presented here (Chapter 2 and 3) are valid. However, use of the racemic SeMet mixture makes contrasting the results with observed effects of Se-contamination in the environment more difficult. Future experiments should utilize environmentally relevant dosing concentrations of pure L-SeMet.

Future studies investigating Se biotransformation in freshwater habitats are also necessary. Selenium speciation determination requires advanced analytical instrumentation that was not available for either study (Chapters 2 and 3). Therefore, only total Se concentrations are reported. We were unable to verify that Se species applied in food were not altered when dissolved in tank water or accumulated in anuran tissue. Applying analytical methods such as ion chromatography inductively coupled plasma dynamic reaction cell mass spectrometry (IC-ICP-DRC-MS) or x-ray absorption near-edge spectroscopy (μXANES) to determine Se biotransformation would be valuable in order to fully understand Se species-specific toxicity (Punshon, et al. 2005, Unrine, et al. 2007b).

Despite these shortcomings, the work presented here provides valuable information concerning the developmental effects of SeMet and SeO_2 exposure to larval

amphibians. Although the Se exposure concentrations utilized were elevated compared to those typically encountered in even the most contaminated areas (Fan, et al. 2002, Ohlendorf 2002, Rowe, et al. 2002), these studies were designed primarily to contrast toxic effects of three distinct Se-containing compounds. The results of this work established that larval exposure to high SeMet concentrations (50 µg Se g^{-1} ww and 5 µg Se g^{-1} ww nominal concentrations) may induce rear limb malformations, reduce larval survival and reduce the likelihood of and survival through metamorphosis in *H. versicolor*. The SeO_2 High dose (50 µg Se g^{-1} ww nominal concentration) elicited effects on larval survival and metamorphic success comparable to both SeMet doses, but did not induce malformations. The SeO_2 Low dose (5 µg Se g^{-1} ww nominal concentration) was not toxic to larval *H. versicolor*, based on the measured endpoints. Similar studies contrasting effects of environmentally relevant, dry mass-based doses of these Se-containing compounds should be conducted to determine the lowest effect concentrations (LOEC) of each to larval anurans. The embryotoxicity and maternal transfer of each Se-containing compound should also be addressed in future studies, as anuran exposure in a Se-contaminated environment is likely to occur in advance of the period modeled here (Gosner Stages 27 through 42).

Chapter 5: References

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