SPECIES AND STAGE SPECIFIC DEVELOPMENTAL TOXICITY OF ENDOSULFAN EXPOSURE IN *HYLA CINEREA* AND *RANITOMEYA IMITATOR*

by

Matthew K. Edwards

April, 2015

Director of Thesis: Krista McCoy

Major Department: Biology

During ontogeny, organisms pass through critical periods of heightened vulnerability to disruptive exogenous agents. In organisms with complex life cycles, these developmental windows result in variable susceptibility across life stages. Consequently, developmental anomalies that occur during early life may carry-over to affect later stages and ultimately individual fitness. This study investigated stage specific and carry-over effects on anuran growth and development following low dose exposure to the insecticide endosulfan. For this purpose, *Hyla cinerea* and *Ranitomeya imitator* embryos and larvae were continuously exposed, either individually or in combination, to a gradient of environmentally relevant concentrations of endosulfan (0, 0.1, 1, 10, 100, and 1,000 ng/L) until completion of metamorphosis. Though effects on mortality, deformity, and timing to and condition at multiple developmental points were not significant, observed abnormalities implicated endosulfan exposure. Embryonic *R. imitator* exposed to endosulfan experienced greater mortality and deformity than did those exposed as larvae, though *H. cinerea* life stages did not exhibit large differences with either endpoint. These findings indicate that traditional indicators of developmental toxicity may not provide an adequate characterization of pesticide hazards across species and life stages.

SPECIES AND STAGE SPECIFIC DEVELOPMENTAL TOXICITY OF ENDOSULFAN EXPOSURE IN *HYLA CINEREA* AND *RANITOMEYA IMITATOR*

A Thesis

Presented to the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Master of Science in Biology

by

Matthew K. Edwards

April, 2015

SPECIES AND STAGE SPECIFIC DEVELOPMENTAL TOXICITY OF ENDOSULFAN EXPOSURE IN *HYLA CINEREA* AND *RANITOMEYA IMITATOR*

by

Matthew K. Edwards

APPROVED BY:

DIRECTOR OF THESIS: _____
(Krista McCoy, PhD)

COMMITTEE MEMBER: _____
(Michael McCoy, PhD)

COMMITTEE MEMBER: _____
(Kyle Summers, PhD)

COMMITTEE MEMBER: _____
(Jamie Dewitt, PhD)

CHAIR OF THE DEPARTMENT
OF BIOLOGY: _____
(Jeff McKinnon, PhD)

DEAN OF THE
GRADUATE SCHOOL: _____
Paul J. Gemperline, PhD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: GENERAL INTRODUCTION

Amphibians possess numerous characteristics that make them effective biological indicators of environmental health. By filling diverse trophic niches and representing substantial portions of the community biomass and prey base, amphibians play crucial roles in food web dynamics (McCoy *et al.* 2009). Amphibians have complex life histories that often rely on both aquatic and terrestrial habitats, thereby increasing their exposure and vulnerability to physical and chemical stressors (Dunson *et al.* 1992). Amphibians develop in eggs that lack protective shells, and as larvae and adults they have permeable, highly vascularized skin used for both gas exchange and osmoregulation (Hopkins 2007). Moreover, the eggs, larvae, and adults of many amphibian species have distinct interactions with their environments, and so experience different stressors at individual life stages. Amphibians also have small home ranges and high habitat fidelity (Smith and Green 2005), so an individual may also be influenced by the same environmental stressor throughout its lifetime. These life history characteristics make amphibians valuable models for examining the effects of environmental stressors on specific developmental periods and on development as a whole.

Because of their perceived value as indicators of environmental health, recent global declines in amphibian populations have caused widespread public concern. Nearly one-third of all amphibian species are threatened with extinction, and more than 43% are experiencing population declines (Stuart *et al.* 2004). In the last three decades, 122 (~2%) amphibian species are believed to have gone extinct, representing an extinction rate over 200 times the background rate (Stuart *et al.* 2004; McCallum 2007).

While some losses can be traced to well-known processes such as habitat destruction, overexploitation, and invasive species, the causes of population declines in areas without obvious disturbance are not well understood (Collins and Storfer 2003). Factors implicated in these enigmatic declines, which are most commonly seen in the species rich Neotropics, include emergence of infectious diseases, global climate change, and environmental contamination (Collins and Storfer 2003). Numerous studies have hypothesized associations between amphibian declines and global warming induced changes in precipitation patterns that cause reduced water depth of oviposition sites, increased exposure to ultraviolet radiation, and a consequential increase in susceptibility to infectious disease (Pounds and Crump 1994; Kiesecker *et al.* 2001; Daszak *et al.* 2003). However, early life mortality may not always have the assumed deleterious, population level consequences (Vonesh and De la Cruz 2002).

Little is known about mechanisms through which exposures to environmental pollutants drive amphibian population declines. Agricultural pesticides are considered an important source of pollution linked to this phenomenon (Davidson 2004; Hayes *et al.* 2006). Many agricultural chemicals have been shown to induce deformity, affect sexual differentiation, impair growth, alter the timing of metamorphosis, and cause suppression of the immune system (reviewed by Mann *et al.* 2009). Due to their persistence in soils and water and their bioaccumulation in the tissues of organisms, pesticides can remain in agricultural sites long after application, therefore increasing the opportunity for exposure (Boethling *et al.* 2009). Importantly, some pesticides can enter the atmosphere through spray drift or volatilization, where they can be transported for long distances before contaminating new sites via precipitation or dry deposition (Simonich and Hites 1995). The prevalent use, atmospheric mobility, and persistence of certain pesticides have made

their residues pervasive within the environment (Peterle 1969; Freed et al. 1972; Tanabe *et al.* 1982; Weber *et al.* 2010).

Although low, environmental levels of contaminants were once believed to be harmless, exposures during development are increasingly recognized for their negative effects that carry over into later life stages (e.g. Carey and Bryant 1995; Hayes *et al.* 2002; Park and Kidd 2005). In order to evaluate the capacity of pollutants to drive amphibian population declines, we must first understand how ecologically relevant concentrations of common contaminants influence growth and development throughout early life.

Endocrine System Function and Disruption

While ubiquitous, environmental pesticide concentrations are often too low to induce direct mortality to amphibians (Hall and Henry 1992; Hayes *et al.* 2006). However, many pesticides can impact non-target organisms at low concentrations through sub-lethal disruption of normal endocrine system function (Fenner-Crisp 1997; Lutz and Kloas 1999). The endocrine system is a series of ductless glands that mediate an organism's homeostasis, reproduction, development and behavior by secreting chemical messengers called hormones (Zoeller *et al.* 2012). Hormones travel through the blood and associate with plasma membrane or intracellular proteins called hormone receptors, initiating gene transcription, protein synthesis, and physiological change (Zoeller *et al.* 2012). Pesticides may act as endocrine disrupting chemicals (EDCs) by interfering with the synthesis, secretion, transport, binding, action, or elimination of endogenous hormones to promote or antagonize biological responses (Kavlock *et al.* 1996). Thus, investigations into the developmental effects of pesticide exposure must be conducted in the context of the basic principles of endocrinology.

Because of their high receptor affinities, endogenous hormones can exert effects at very low concentrations, frequently within the picomolar range (Vandenberg *et al.* 2012). Although EDCs often have receptor affinities that are much lower than those of endogenous hormones, they can still generate low dose effects (Vandenberg *et al*. 2012). This is possible because changes in hormone concentration produce proportionally larger effects at low doses than at high doses, a trend that follows a sigmoidal dose-response curve resulting from the saturability of finite hormone receptor binding sites (Zoeller *et al*. 2012). The relationship between hormone levels and cellular response can alternatively be non-monotonic, with the slope of the curve changing sign across the dose-response (Vandenberg *et al.* 2012). This relationship can be produced by homeostatic negative feedback mechanisms that reduce endogenous hormone production, increase their metabolism, or promote receptor down regulation with excessive hormone levels (Bergman *et al.* 2012). Through this process, the effects of low hormone concentrations may be greater than those induced by large doses. Because receptors also mediate the effects of exogenous disruptors, EDCs share the characteristic of non-linear dose-responses (Bergman *et al.* 2012). Therefore responses to high doses of EDCs are not indicative of responses to low doses, which can induce large physiological effects (Vandenberg *et al.* 2012).

The specificity of endocrine response to endogenous hormones is further affected by receptor availability. The extent of a hormone's influence is limited by the expression of its receptors, which may be localized in single cell types or may be found throughout the body (Vandenberg *et al.* 2012). Furthermore, some hormones can bind multiple receptor isoforms that vary in quantity and elicited effect across tissue types (Vandenberg *et al*. 2012). Differences in receptor expression between tissues, different life stages, and among organisms can also drive variable effects of EDC exposure at each of these levels. In amphibians, pesticides demonstrate

tissue-specific effects by impairing the actions of retinoids, sex steroids, and thyroid hormones involved in early developmental processes and later adult physiology.

Disruption of retinoic acid homeostasis

The 1995 discovery of deformed frogs in Minnesota agricultural sites brought the teratogenic effects of environmental pollutants to the forefront of discussion on threats to amphibian biodiversity. Although there is debate about the relative influences of agricultural chemicals, UV-radiation, and infestation by larval trematodes in the induction of deformity in wild amphibians (Gardiner *et al.* 2003; Ballengee and Sessions 2009), the severity, abundance, and spread of such abnormalities has increased dramatically and now occurs in more than 60 amphibian species across North America, Europe, and Japan (Blaustein and Johnson 2003). While deformities may be seen in up to 5% of a population due to injury or mutation, field studies that find correlations between agricultural intensity and malformations document severe cases at frequencies as high as 15-90% (Ouellet *et al.* 1997; Stocum 2000). Aberrant phenotypes observed in wild populations are diverse, including absent or supernumerary limbs and digits, incomplete limb development, and misshapen jaws, tail, and eyes (Blaustein and Johnson 2003).

The hypothesis that agricultural chemicals are directly responsible for these abnormalities is supported by the induction of limb deformities following the application of retinoic acid (RA) (Loeffler *et al.* 2001). Retinoids are oxidized vitamin A derivatives that bind a heterodimer complex composed of the nuclear RA and retinoid X receptors (Inoue *et al.* 2010). This binding promotes the transcription of target *Hox* genes through the RA response element (Inoue *et al.* 2010). By controlling the expression of these genes and heterodimerizing with other nonsteroidal nuclear receptors, such as those for thyroid hormone, this system exerts pleiotropic effects on

embryonic morphogenesis (Inoue *et al.* 2010). Pesticides that alter this ligand-receptor interaction can cause deficient or excess signaling, affecting the patterning of the axial skeleton, differentiation and proliferation of cells and tissues, and development of the limbs (reviewed in Novák *et al.* 2008).

Disruption of the gonad axis

Unlike the conspicuous anatomical malformations induced by teratogens, endocrine disruptors alter the expression of genes during early developmental stages to produce less visible functional change. These new phenotypes, however, may be equivalently detrimental.

In larval amphibians, reproductive development is regulated by the hypothalamus-pituitary-gonad (HPG) axis. Hypothalamus induced gonadotropin release from the pituitary stimulates the secretion of estrogen, androgen, and progestogen sex steroid hormones from the gonads (Kloas *et al.* 2009). These hormones control reproductive organ differentiation and growth, promote or inhibit germ cell maturation, and stimulate the development of secondary sex characteristics (Kloas *et al.* 2009).

The genetically predetermined differentiation of the bipotential gonad into ovaries or testes can be altered by imbalances in sex steroids (Haczkiewicz and Ogielska 2013). For this reason, much of the research on endocrine disruption has focused on environmental pollutants with estrogenic and anti-androgenic activities, as well as those that affect the actions of aromatase, the enzyme responsible for the biosynthesis of estrogens from androgens (Propper 2005). As the balance of endogenous sex steroids is critical for the development of the reproductive system, certain EDCs can impact early reproductive events and later reproductive function (Wallace *et al.* 1999; Akingbemi and Hardy 2001).

Many environmental contaminants are known to disrupt sexual differentiation and steroidogenesis in amphibians (Palmer *et al.* 1998; Crews *et al.* 2000; Noriega and Hayes 2000; Hayes *et al.* 2002). For instance, acute exposure of male *Xenopus laevis* tadpoles to the herbicide atrazine reduced the number of spermatogonial cell nests, which serve as the main source of germ cells throughout adult life (Tavera-Mendoza *et al.* 2002). Increasing agricultural land use, and by extension pesticide exposure, is also associated with gonadal abnormalities and altered sex hormone levels in wild amphibians (McCoy *et al*. 2008). These reproductive effects of EDC exposure can contribute to amphibian population declines through the processes of sex ratio bias, reduced recruitment, and infertility.

Disruption of the thyroid axis

Pesticides are also known to disrupt the actions of thyroid hormones (TH) necessary for larval somatic growth, brain development, and the initiation of metamorphosis (Hayes 1997; Mann *et al*. 2009). The morphogenesis and apoptotic tissue remodeling that characterizes metamorphosis is regulated by the release of thyroxine (T_4) from follicular cells of the thyroid gland and its subsequent conversion by 5'-deiodinase to the more active 3,5,3'-triiodothyronine (T_3) in target tissues (Brown and Cai 2007). Thyroid hormones are found in low concentrations prior to metamorphosis and peak at metamorphic climax, when the forelimbs emerge and the tail is resorbed (Galton 1992). The role of TH in metamorphosis has been experimentally demonstrated through the induction of premature metamorphosis following larval exposure to exogenous TH (Helbing *et al*. 1992) and the arrest of metamorphosis through the inhibition of TH release (Rose 2005; Gutleb *et al*. 2007).

In larval amphibians, the hypothalamus controlled secretion of thyrotropin, responsible for TH release, is promoted by corticotropin releasing hormone (Denver 1999). This hormone also mediates glucocorticoid and adrenocorticotropic hormone secretion in response to non-specific stressors (Denver 1999). Therefore, environmental conditions such as increased predation risk, decreased water quality, and limited resource availability may induce precocious metamorphosis through stimulation of TH secretion (Mann *et al*. 2009). Indeed, amphibian larvae in ephemeral pools will accelerate metamorphosis as an adaptive response to drying conditions (Denver *et al*. 1997).

Similarly, pesticides can disrupt metamorphosis either through direct effects on the hypothalamus-pituitary-thyroid (HPT) axis or through crosstalk with other hormones. Endocrine disruptors can accelerate metamorphosis by mimicking TH or acting as general environmental stressors that increase endogenous corticosterone synthesis and T_3 production (Greulich and Pflugmacher 2003; Boone *et al*. 2013). Alternatively, they can delay metamorphosis through follicular cell cytotoxicity, antagonism of thyroid hormone receptors, or disruption of 5'-deiodinase through xenoestrogen activities (Lisboa *et al*. 2001; Sullivan and Spence 2003).

Any alteration in the timing of metamorphosis can have long-term consequences on fitness and population growth (Bridges 2000). Individuals that undergo precocious metamorphosis will emerge as undersized juveniles, with greater vulnerability to predation, smaller gonad size, and decreased fecundity as adults due to lowered egg production and delayed time to reproductive maturity (Semlitsch 1988; Denver 1997; Rose 2005; Chelgren *et al*. 2006; McCoy *et al*. 2007). Additionally, accelerated metamorphosis is associated with a large reduction in lymphocyte numbers and an increased susceptibility to infectious disease (Rollins-Smith *et al*. 2009). Larvae with prolonged metamorphosis will face greater exposure to aquatic

predators and may fail to fully develop before the loss of their ephemeral pools (Sullivan and Spence 2003).

Differential susceptibilities

Endocrine disrupting pesticides can produce different types and magnitudes of effects depending upon the life stage at which the individual is exposed. These time dependent effects result from hormone receptor distribution patterns and intensities that vary across life stages and reflect distinct periods of morphogenesis (Bergman *et al.* 2012). For example, peaks in estrogen and androgen receptor levels during the late larval stage coincide with gonad differentiation and a period of demonstrated susceptibility to reproductive dysfunction following pesticide exposure (Bogi *et al.* 2002). As sex steroid receptor levels are undetectable in the embryo (Bogi *et al.* 2002), estrogenic and anti-androgenic EDC exposure at this stage may have few effects on reproductive development. Importantly, however, the inability to detect these receptors does not provide definitive evidence of their absence. Alternatively, somatic growth and development may be affected by exposure to EDCs that agonize or antagonize TH at either embryonic or larval stages. Thyroid hormone receptors are active soon after fertilization, despite a lack of TH secretion until complete development of the thyroid gland in the late larval stage (Tata 2000). Examining the differential effects of contamination during early developmental periods is critical, because whereas exposure during adulthood will produce reversible, activational effects, exposure during embryonic and larval stages can cause permanent, organizational changes (Hayes 2005). The physiological and metabolic alterations resulting from these developmental exposures may contribute to the etiology of adult disease (De Boo and Harding 2006).

Studies using pesticide exposure to examine the differential sensitivities of early amphibian life stages often find more adverse effects on larvae than embryos (Berrill *et al*. 1998, Bridges 2000, Greulich and Pflugmacher 2003, Brodeur *et al*. 2009, Yu *et al*. 2013). Bridges (2000) found that the insecticide carbaryl caused significant mortality in *Rana sphenocephala* tadpoles, yet had no effect on hatching success at any tested concentration. Additionally, individuals that were exposed to carbaryl at the egg stage were smaller at metamorphosis (Bridges 2000). This carry-over effect, in which conditions at one life stage influence fitness-related traits at the next (Stoks and Cordoba-Aguilar 2012), may reflect the ability of pesticides to bioconcentrate in amphibian eggs (Brodeur *et al*. 2009). Vulnerabilities can also be species specific, as demonstrated by LC50 values ranging from 0.41 to 127 mg/L of atrazine following exposure of embryos from multiple species (Birge *et al*. 2000). These differences in sensitivity may result from variable hormone receptor availabilities or variation in xenobiotic metabolizing systems (Pickford and Morris 2003).

Despite potential phylogenetic and life stage specific differences in susceptibility to EDCs, most research on the effects of pesticides use commonly studied species to test the lethality of brief, high-dose exposures at single life stages. Because of the mobility, persistence, and low environmental concentrations of many pesticides, these exposure scenarios are not likely ecologically relevant. To obtain an accurate measure of chemical toxicity, it is important that studies use diverse amphibian species and low-dose exposures sustained within life stages to examine sub-lethal effects throughout development.

CHAPTER 2: SPECIES AND STAGE SPECIFIC DEVELOPMENTAL TOXICITY OF ENDOSULFAN EXPOSURE IN *HYLA CINEREA* AND *RANITOMEYA IMITATOR*

INTRODUCTION

Amphibians are commonly used as biological indicators of environmental contamination because their reliance on both aquatic and terrestrial habitats increases their exposure to environmental pollutants, and because their highly permeable skin enables rapid absorption of these chemicals into the blood and distribution throughout the body without first pass metabolism (Bruhl *et al.* 2011). Tragically, amphibian populations are declining globally at an unprecedented rate (McCallum 2007). Second only to habitat destruction, environmental pollution is considered to be a major contributor to these losses (IUCN 2008). Pesticide use in particular is implicated by field surveys demonstrating reduced species richness, diminished populations, and increased incidence of developmental abnormalities in amphibians on agricultural lands (Bishop *et al.* 1999, Davidson 2004, McCoy *et al.* 2008).

Amphibian larvae are used in studies investigating environmental contamination due to their high tractability in laboratory settings and because many are obligate inhabitants of aquatic ecosystems where pollutants accumulate. Additionally, they undergo a period of metamorphic development whose onset is influenced by environmental stress and alterations to which can have negative fitness consequences resulting from decreased adult fecundity (Chelgren *et al*. 2006) and increased susceptibility to predation, disease, and desiccation (Denver 1997, Rollins-Smith *et al*. 2009). However, studies that exclusively use tadpoles in toxicity tests fail to appreciate the potential for carry-over effects from exposure across developmental stages. Amphibians have complex life cycles that include abrupt changes in morphology, physiology,

behavior, and niche use (Wilbur 1980) that ultimately influence levels of pesticide exposure. Furthermore, individuals pass through critical periods of development that create life stage specific vulnerabilities to disruptive exogenous agents. These developmental windows coincide with patterns of gene expression coordinated by the actions of endogenous hormones. Therefore, the effects of pesticide exposure will differ between endocrine regulated developmental periods, including neurulation, organogenesis and skeletal formation during embryogenesis, and the tissue growth and reproductive differentiation that occur during larval development (Birge *et al.* 2000).

Numerous studies have investigated the influence and timing of pesticide exposure on the magnitude and type of observed effects, and have often demonstrated that larvae have greater sensitivity than embryos (Berrill *et al*. 1998, Bridges 2000, Greulich and Pflugmacher 2003, Brodeur *et al*. 2009, Yu *et al*. 2013). For example, Bridges (2000) found that the insecticide carbaryl caused significant mortality in *Rana sphenocephala* tadpoles, yet had no effect on hatching success at any tested concentration. However, individuals that were exposed at the egg stage were smaller at metamorphosis (Bridges 2000). These findings highlight an underexplored topic in developmental toxicology. Few studies have tested for carry-over effects of pesticide exposure across life stage transitions, particularly between embryos and larvae (Uller *et al.* 2009). Although differential life stage susceptibility has long been recognized, most studies investigating the effects of developmental exposure overlook the interdependence of stage specific vulnerabilities, potentially leading to inaccurate assessments of toxicity.

The aim of the present study was to examine the stage specific and carry-over effects of exposure to sub-lethal pesticide doses in *Ranitomeya imitator* and *Hyla cinerea*. This study tested the hypothesis that effects on growth, rate of development, mortality, and incidence of

deformity depend on the dose and window of exposure. This was performed by treating embryonic and larval stages, either individually or in combination, with a gradient of environmentally relevant concentrations of the pesticide endosulfan.

METHODS

Study system

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) is an organochlorine pesticide used to protect commercial crops including fruits, vegetables, cotton, and tea (ATSDR 2013). It was developed as a γ-aminobutyric acid (GABA) chloride channel receptor inhibitor to induce convulsion and death in insects and acarids (Bloomquist 2003), yet has demonstrated toxic effects in non-target fish (Da Cuña *et al.* 2008), amphibians (Jones *et al.* 2009), reptiles (Rey 2009), birds (Lutz-Ostertag 1971), and mammals (Sinha *et al.* 2001), including humans (Saiyed *et al.* 2003). Additionally, endosulfan and its metabolites are genotoxic (Neuparth *et al.* 2006), teratogenic (Lemaire *et al.* 2005), and endocrine disrupting, with estrogenic and anti-androgenic properties (Soto et al. 1994, Andersen et al. 2002).

Endosulfan has numerous well-documented detrimental effects on amphibian larvae. It is highly toxic to tadpoles and even at low concentrations retards growth and development, induces deformity, and alters swimming, breathing, and feeding behaviors (Brunelli *et al*. 2009, De Jong Westman *et al*. 2010, Denoel *et al*. 2012, Bernabo *et al*. 2013, Lavorato *et al*. 2013). Previous studies have indicated that endosulfan will affect time to metamorphosis, yet the direction of the effect was not consistent. Brunelli *et al*. (2009) found that *Bufo bufo* tadpoles exposed to endosulfan experienced delayed metamorphosis in a dose dependent manner. In contrast,

Lavorato *et al*. (2013) found that while higher doses of endosulfan prolonged metamorphosis in *Rana dalmatina* tadpoles, lower doses significantly accelerated metamorphosis.

Endosulfan has been applied globally since the 1950s, used primarily in developing countries (Li and Macdonald 2005) but also widely throughout the United States and particularly eastern North Carolina (ATSDR 2013). Its residues are ubiquitous, measurable in Arctic waters (Weber *et al*. 2010), human breast milk (Cerillo *et al*. 2005), and rainforest fog (Shunthirasingham *et al*. 2011). In surface waters, its concentrations range from less than 0.5 ng/L in bromeliad water to over 2,000 ng/L in agricultural run-off (Schulz 2001). Despite its low water solubility (0.32 mg/L), endosulfan is highly persistent in aquatic environments, with a half-life of 14-18 days in freshwater (Guerin 2001). Furthermore, because of its lipophilicity, endosulfan can bioaccumulate in tissues and move throughout the food chain (Toledo and Jonsson 1992).

A wide range of pesticide sensitivities have been recorded among amphibians (Birge *et al.* 2000). The continued use of endosulfan, a pesticide with such high potential toxicity, is unwise given the paucity of studies examining its effects in amphibian species (Jones *et al.* 2009). This study uses the American green tree frog (*H. cinerea*), an abundant species in eastern North America capable of colonizing a wide variety of habitats, including those that are subject to agricultural activity, and the poison mimic frog (*R. imitator*), a small dendrobatid from north central Peru that rears its larval offspring individually in water bodies formed in plant axils that contain measurable quantities of endosulfan (Brown *et al.* 2008; Shunthirasingham *et al*. 2011). Although the early life stages of both species are likely exposed to endosulfan in their natural environments, their distinct life histories and reliance on pools with dramatically different hydroperiods may affect their susceptibility. By examining these two distantly related and

dissimilar species, we hope to draw general conclusions about the effects of ecologically relevant endosulfan exposures across amphibians.

Preparation of test solutions

A single stock solution, used for the duration of experiments on both species, was prepared by dissolving 0.1 mg of commercial grade endosulfan (purity 99%, Sigma-Aldrich, St. Louis, MO) in 1 L of reconstituted reverse-osmosis water (Provasoli; Wyngaard and Chinnappa 1982). The endosulfan was added to a plastic tube containing 1 ml of Provasoli, vortexed until there were no visible solids (approximately 3 minutes), and poured into a glass volumetric flask containing 990 ml of Provasoli. The 1 ml tube was filled with Provasoli, centrifuged, and added to the flask 9 more times to give a final stock concentration of 100,000 ng/L of endosulfan. This stock solution was divided into 12 ml aliquots held in glass mason jars and was kept at -20 °C under dark conditions to minimize degradation (USEPA 2007).

Serial dilution of the stock solution was performed to obtain 5 endosulfan test concentrations: 0.1, 1, 10, 100, and 1,000 ng/L. This series was used to determine the dose-dependent effects of endosulfan exposure and represents a spectrum of environmental concentrations encompassing those likely experienced by wild frog populations (Table 1). Furthermore, these concentrations were found to induce minimal mortality in amphibians used in other studies (De Jong Westman *et al.* 2010).

Table 1 Endosulfan test concentration series. Doses used for experiments with *H. cinerea* and *R. imitator* approximate measurements taken from the field.

Corresponding field levels	Reference	Test concentration (ng/L)
Provasoli control	(Wyngaard and Chinnappa 1982)	0
Arctic melt water	(Weber *et al.* 2010)	0.1
Rainforest fog	(Shunthirasingham *et al.* 2011)	1
Bromeliad detritus	(Shunthirasingham *et al.* 2011)	10
EPA CCC (freshwater)	(USEPA 2009)	100
Agricultural runoff	(Schulz 2001)	1000

Experimental animal collection

Hyla cinerea

Five amplexing pairs of *H. cinerea* adults were collected from an artificial wetland in Greenville, North Carolina (35.590643, -77.319299) on the night of July 4, 2014. Pairs were held separately at their point of capture in tilted polyethylene terephthalate (#1) plastic containers containing Provasoli. Adults were released at sunrise the next day and their egg clutches were transported to the laboratory at East Carolina University within 12 h of laying. Immediately upon arrival to the laboratory, the mid- to late-gastrula stage embryos (Stage 11-12, Gosner 1960) were divided into test dishes and experimental treatments were administered.

Ranitomeya imitator

Experimental *R. imitator* were acquired July 11 – September 23, 2014 from a captive breeding colony housed in the laboratory. This colony consists of 3 distinct color morphs that were collected from separate localities in Peru: Veradero, Chazuta, and Tarapoto. Breeding colony adults were paired to maintain these color morphs in the experimental offspring. Adult pairs were housed in individual glass terraria, which were checked 3 times daily for the arrival of

egg clutches. Whole clutches were transferred to glass petri dishes containing Provasoli for until they reached mid- to late-gastrula stage (Stage 11-12, Gosner 1960), when the embryos were moved to test dishes to begin treatment exposures.

Exposure protocol

To investigate the effects of the timing of endosulfan exposure, individuals of both species were treated at 1 of 3 developmental periods: egg stage only (EGG; Stages 12 – 21), tadpole stage only (TAD; Stages 21 – 46), and throughout development (EGG/TAD; Stages 12 – 46). Animals exposed to the control treatment in the egg stage either remained in the control or were switched to 1 of 5 endosulfan concentrations, resulting in 6 life stage treatments (Fig. 1). Animals exposed to endosulfan in the egg stage either remained in the same dose or were switched to the control following hatching, resulting in 2 life stage treatments for each concentration of endosulfan (Fig. 1). In *H. cinerea*, each of the 16 life stage treatments were replicated 5 times (N = 80), while in *R. imitator* each life stage treatment was replicated 3 – 4 times (N = 67).

To prevent the accumulation of ammonia and the breakdown of endosulfan, this experiment used a static renewal exposure system in which treatment containers were washed and test solutions were fully replaced 3 times every week. This frequency was chosen based on the findings by Miles and Moy (1979) that nominal endosulfan concentrations remain approximately constant after 72 h. Fresh dilutions of endosulfan were made directly before solution replacement. Solutions were changed by gently pouring each experimental animal into a treatment-coded brine shrimp net and then immediately transferring it to a clean prefilled container. Following solution replacement, the shelf position of each container was rotated.

A

EGG Treatment (ng/L)	Clutch A	Clutch B	Clutch C	Clutch D	Clutch E
0	1	9	17	25	33
0	2	10	18	26	34
0	3	11	19	27	35
0.1	4	12	20	28	36
1	5	13	21	29	37
10	6	14	22	30	38
100	7	15	23	31	39
1,000	8	16	24	32	40

B

Back left

24	32	3	25	1	31	36	13	27	9
4	22	18	28	16	20	12	34	26	23
7	14	2	10	30	39	38	6	21	40
15	37	33	29	35	17	19	8	5	11

Front right

Figure 1 *H. cinerea* random assignment scheme. Haphazardly chosen eggs from 5 clutches were systematically assigned to treatments (A) and treatments were randomly assigned to shelf positions (B).

Tadpoles were fed an ad libitum amount of crushed spirulina pellets (O.S.I., Burlingame, CA) until the start of metamorphic climax (Stage 42), when they stop feeding and metabolize energy reserves stored in their tails (Hourdry *et al.* 1996). Animals were held in a room maintained on a 12L:12D photoperiod at 23 ± 1 °C. Water quality parameters (including pH, ammonia, nitrite, and nitrate levels) were tested using API test strips before and after renewing the treatment solutions. Water quality was monitored intermittently throughout the duration of the experiment on a randomized subset of jars for each treatment. Water quality parameters were maintained at the following conditions: pH 6.0-7.0, NH_3 0-1.0 mg/L, NO_2^- ~0 mg/L, NO_3^- 0-20

mg/L. Animal use protocols were approved by the Institutional Animal Care and Use Committee (AUP #D293)

Hyla cinerea

To begin experimentation with *H. cinerea*, 40 covered glass petri dishes, color-coded to pre-designated treatments, were laid flat on a shelf along 4 rows in randomized positions (Fig. 2). The dishes were pre-filled with 60 ml of their treatments and groups of 5 haphazardly chosen eggs were assigned to their dishes in a randomized order (Fig. 2). Following hatching, 2 tadpoles from each dish were haphazardly chosen and transferred by pipette to color-coded glass mason jars filled with 150 ml of their treatments and arranged in 4 rows on 2 shelves. These treatments were pre-designated through a similarly randomized process. Tadpoles were numerically labeled to allow blind morphometric analysis. At metamorphic climax, when the first forelimb appeared, stainless steel mesh ramps were added to each jar to allow the metamorph to crawl out of the solution. After their first emergence, the jars were tilted so that each contained both wet (containing 40 ml of their treatment solution) and dry areas until completion of metamorphosis (Stage 46).

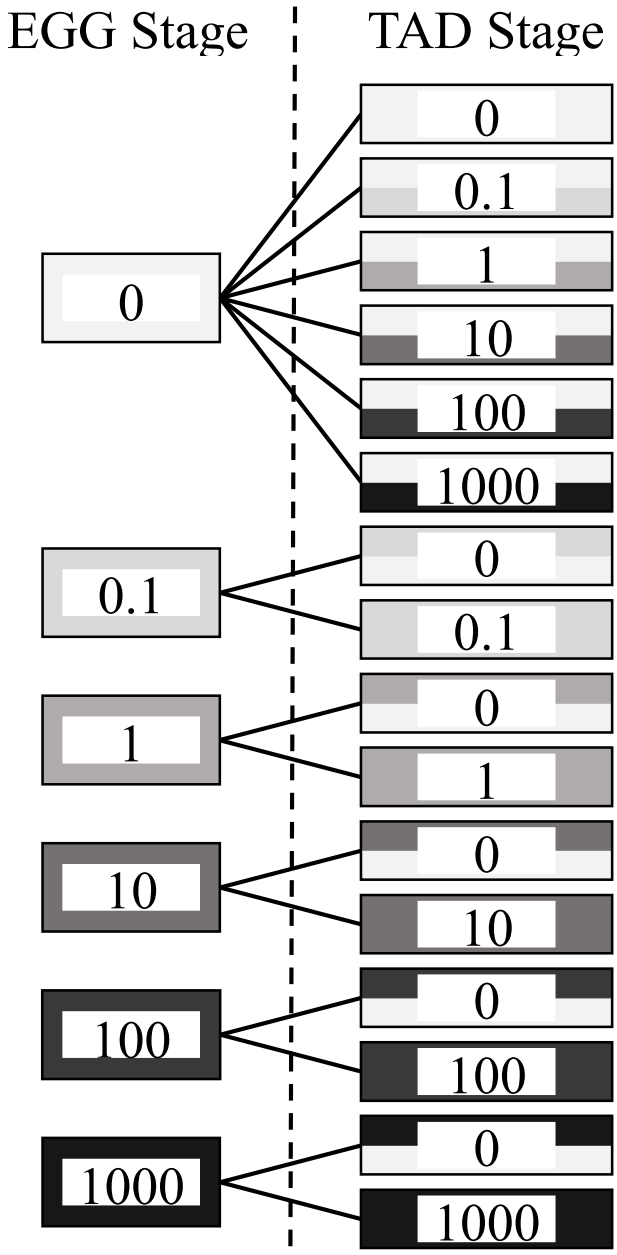


Figure 2 Life stage treatments (ng/L). At hatching, frogs either remained in their treatment (boxes on right with solid color) or were switched to another (split boxes on right with original treatment color on top and new treatment color below). Each of the 16 life stage treatments were replicated 5 times (N = 80) in *H. cinerea* and 3-4 times (N = 67) in *R. imitator*.

Ranitomeya imitator

Full clutches of *R. imitator* eggs (1 – 5 eggs/clutch) were randomly assigned to their treatments. Clutches were transferred to tilted and covered glass petri dishes containing 20 ml of treatment solution and randomly assigned shelf positions. Following hatching, tadpoles were assigned to randomly ordered and alternating treatments according to the time at which they hatched. Tadpoles were held in 150 ml of treatment solution in color-coded glass mason jars on 2 shelves. As with *H. cinerea*, a stainless steel mesh ramp was added to the jar at metamorphic climax and the tadpole was transferred to a tilted jar with 40 ml of treatment solution after first emergence and until completion of metamorphosis. Experimentation on *R. imitator* was conducted on a shelving unit separate from the unit holding experimental *H. cinerea*.

Experimental endpoints

The developmental and experimental measurement time points for both *H. cinerea* and *R. imitator* are outlined in Fig. 3. Treatment dishes were checked 3 times daily for hatched tadpoles. All tadpoles were transferred by pipette to jars containing TAD treatment within 12 h of hatching. One and 2 weeks after hatching, *R. imitator* individuals were weighed and photographed for image analysis of length. The initial measurements of body condition were delayed until these time points because of the small size and fragility of the tadpoles. Due to the sensitivity and high early mortality of the *H. cinerea* larvae, the first weighing and photographing was postponed until 23 days after TAD exposure. Subsequently, *H. cinerea* and *R. imitator* larvae were weighed and photographed at the appearance of the first forelimb, when they were first seen out of solution on the sides of the jar or on the metal ramp (first emergence), and following the completion of metamorphosis. Metamorphic completion was determined by a

tail length of less than 1 mm. The timing of each of these stages was recorded, as was the occurrence and timing of deformity and mortality.

At each experimental time point, tadpoles were poured from their jars into treatment-specific brine shrimp nets and were transferred to cut sections of stainless steel mesh through which they were blotted dry. Tadpoles were then weighed (accurate to 0.1 mg) in their respective treatment solutions. Tadpoles were subsequently placed in glass petri dishes that were filled with 60 ml of treatment solution. Rulers were attached to the bottom of the petri dishes and tadpoles were photographed from above against the ruler. The length from tail-tip to snout was measured from the digital images using a curved ruler function (accurate to 0.01 mm) on Image J software (Abràmoff *et al*. 2004). This procedure minimized disturbance of the tadpoles and allowed measurements without anesthesia or extended removal from solutions. After photographs were taken, tadpoles were transferred to jars prefilled with the appropriate treatment solution and were returned to their shelf positions.

At completion of metamorphosis, frogs were humanely euthanized by overdose of tricaine metanesulfonate (3% MS-222 buffered at pH 7 with $NaHCO_3$) until movement ceased. Excess moisture was removed by blotting before frogs were weighed and photographed. Frogs were evaluated for external malformations and surficial renal anomalies before fat bodies were dissected out and weighed. The adrenal-kidney-gonad complex was removed from each frog and was fixed in neutral buffered formalin (10%) for later histological analysis.

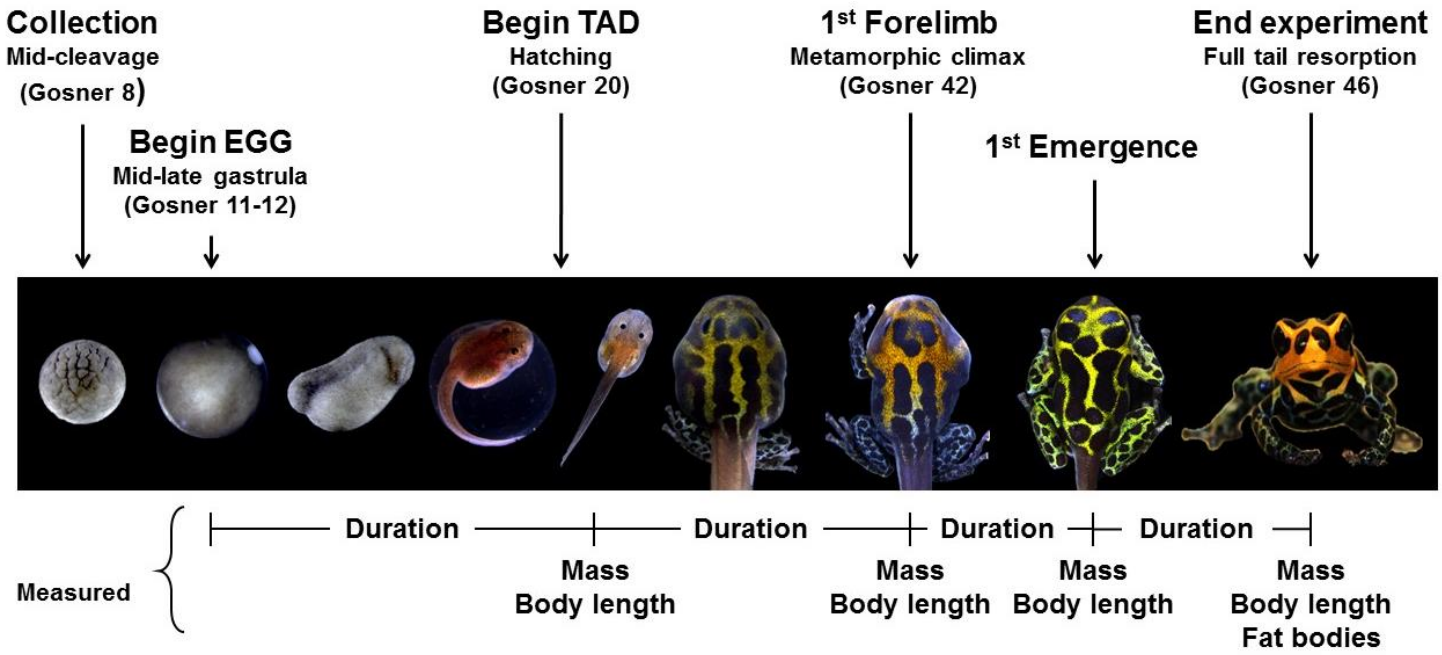


Figure 3 Experimental protocol for *H. cinerea* and *R. imitator* with developmental and measurement time points.

Statistical Analysis

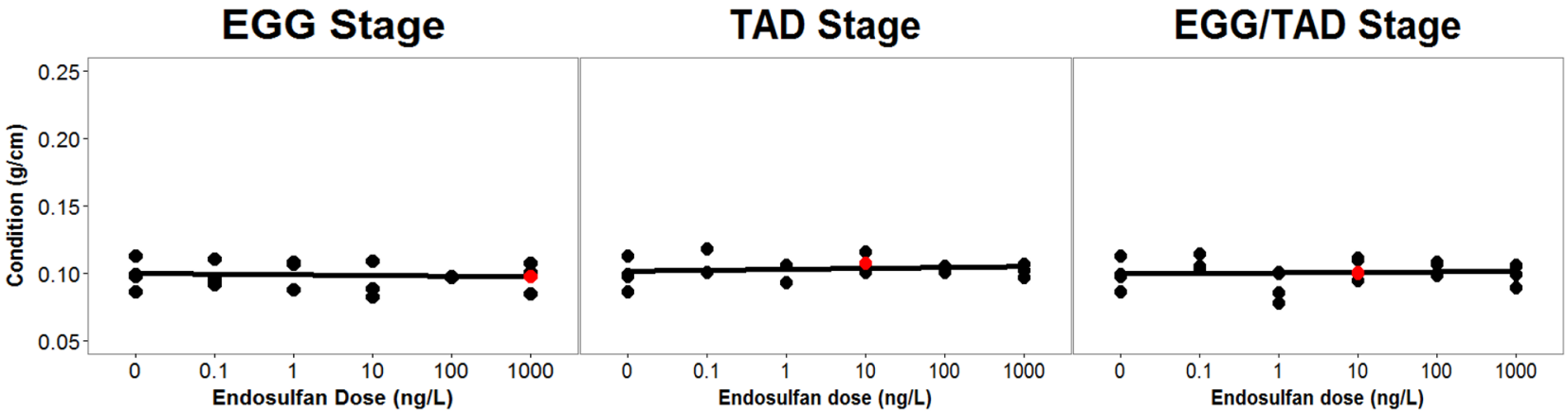
All analyses were carried out using the statistical software R v. 3.1.2 (R Core Team 2014) for Windows and differences were considered significant at $p \leq 0.05$. One-way analysis of variance (ANOVA) was used to compare the following endpoints among endosulfan treatments for each life stage (EGG, TAD, and EGG/TAD): duration of the egg stage, age at the appearance of the first forelimb (beginning of metamorphic climax), age at first emergence, age at completion of metamorphosis, duration of metamorphic climax (from the appearance of the first forelimb to metamorphic completion), growth rate, condition at each developmental stage, and fat body mass at completion of metamorphosis. The assumptions of homogeneity of variances and normal errors were validated using residuals and Q-Q plots. Mortality and incidence of deformity during the experimental period were compared between life stages using generalized mixed models (GLMM) assuming binomial error distributions.

RESULTS

Growth and development

For both species, there were no significant observed effects on any measured growth or developmental rate endpoint across endosulfan treatments and between the EGG, TAD, and EGG/TAD life stage exposures (all $p > 0.05$). However, there were differences in how variable observations of each endpoint were among species for condition at metamorphosis (Fig. 4) and duration of metamorphic climax (Fig. 5).

Hyla cinerea



Ranitomeya imitator

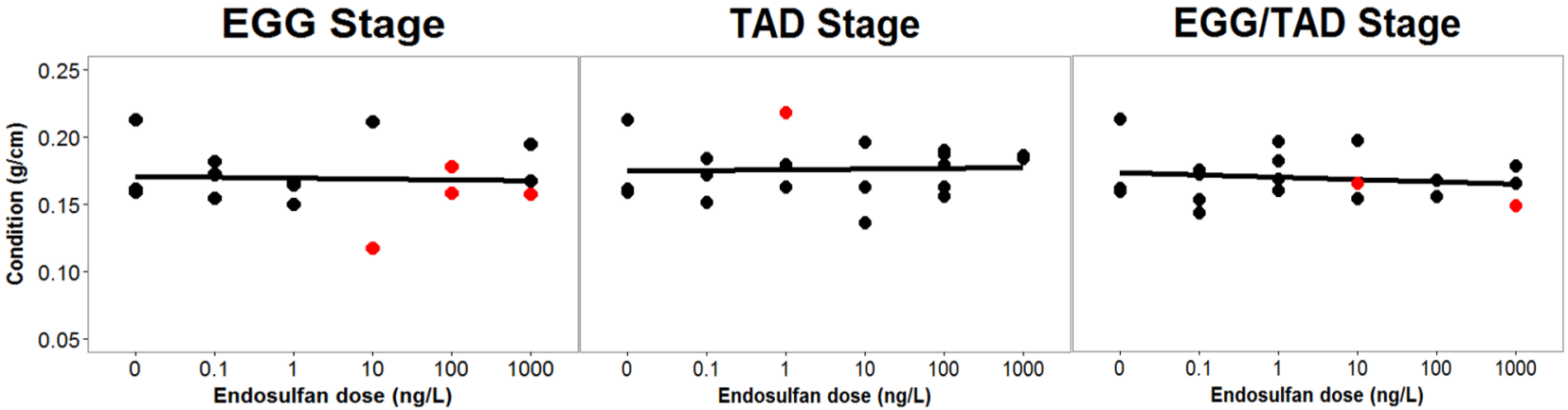
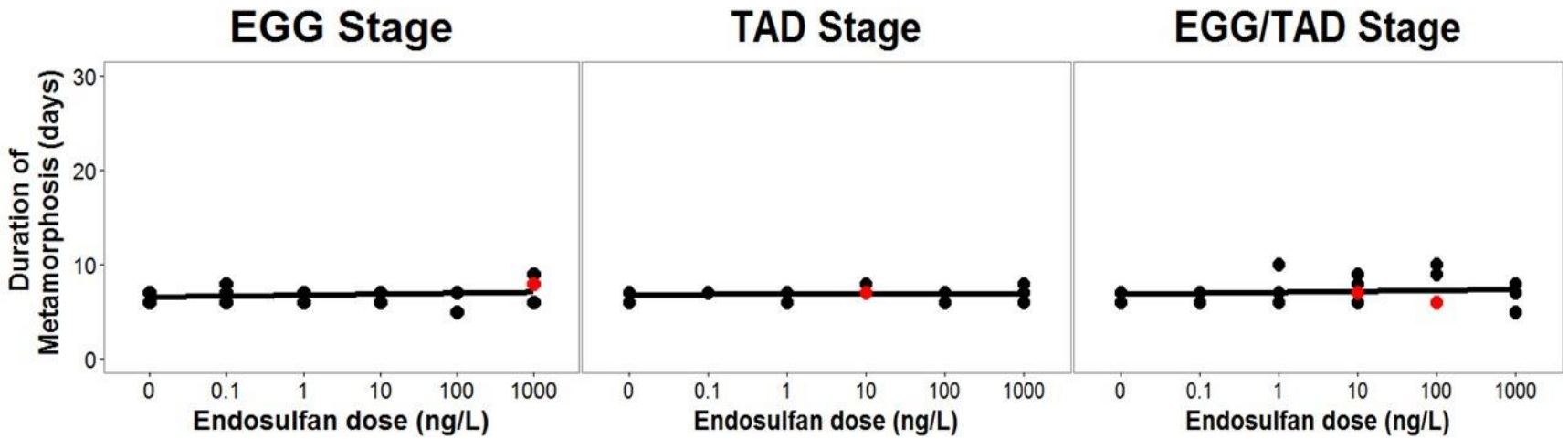


Figure 4 Condition at completion of metamorphosis in *H. cinerea* and *R. imitator* according to life stage at exposure. Condition was measured as the ratio of body mass to length corrected by the residuals of their log-log OLS regression. Condition was not different between doses, life stages, and species (all $p > 0.05$). Red points indicate individuals with morphological abnormalities.

Hyla cinerea



Ranitomeya imitator

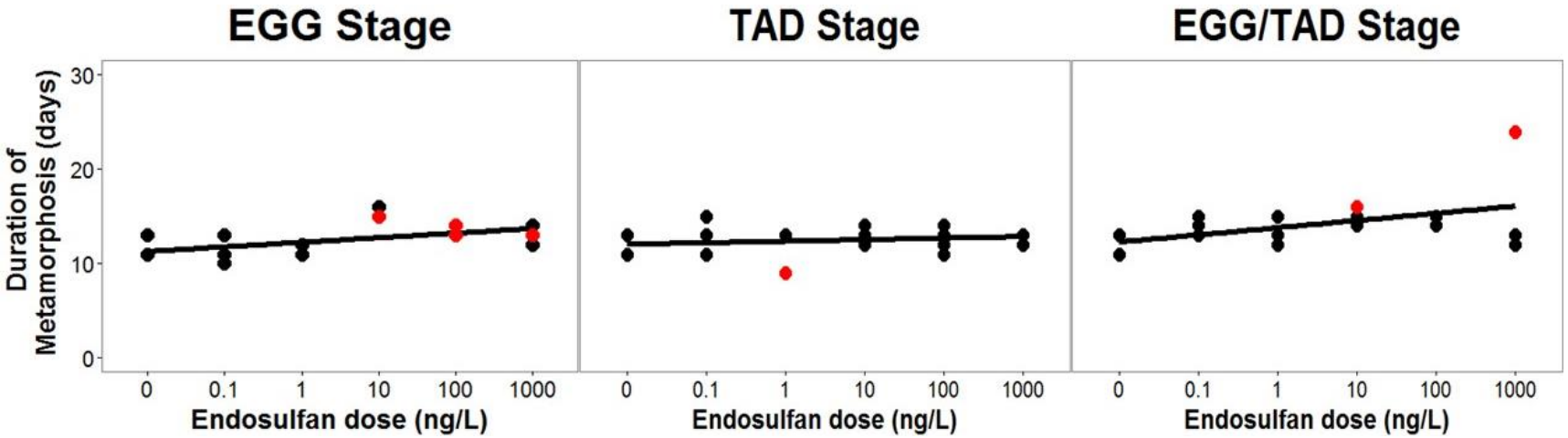


Figure 5 Duration of metamorphosis, from appearance of first forelimb to completion, in *R. imitator* and *H. cinerea* according to life stage at exposure. Duration was not different between doses, life stages, and species (all $p > 0.05$). Red points indicate individuals with morphological abnormalities.

Mortality and deformity

Mortality and incidence of morphological abnormalities varied between species and across life stages (Table 2, Fig. 6), yet there were no significant observed effects on either endpoint across endosulfan treatments and between the EGG, TAD, and EGG/TAD life stage exposures (all $p > 0.05$). Mortality occurred throughout the experiment in both species, yet was greatest in the larval stage, especially in the first 2 weeks following hatching.

Morphological abnormalities were detected in 5 (6%) *H. cinerea* and 10 (15%) *R. imitator* tadpoles. Control animals did not exhibit any noticeable deformities. Though there was no significant effect of endosulfan exposure on incidence of deformity in either species, there were more abnormal *R. imitator* from EGG (30.4%) than TAD (6.3%) and EGG/TAD (9.1%) treatments.

Abnormal phenotypes were diverse and included surficial, renal, and skeletal deformities (identified using Meteyer 2000). Exposed *H. cinerea* exhibited micromelia (proportionally smaller hind limb), hip dysplasia (Fig. 7a), hind limb amelia (missing hind limb, Fig. 7b), and lateral flexure of the tail (bent tail, Fig. 7c). Exposed *R. imitator* exhibited peripheral edema, ascites (fluid accumulation in body cavity, Fig. 8a), cloacal prolapse, unilateral renal agenesis (missing kidney lobe, Fig. 9a), forelimb amelia (missing forelimb, Fig. 10a), lateral flexure of the tail, hyperextension of the hind limbs (Fig. 11a), and tibiofibular taumelia (bone bridging, Fig. 12a).

Table 2 Stage and species-specific mortality and morphological abnormality. Incidence of mortality and abnormality in following continuous exposure of *H. cinerea* and *R. imitator* eggs and tadpoles to a series of endosulfan doses.

	Hyla cinerea						*Ranitomeya imitator*					
	Mortality			Abnormality			Mortality			Abnormality		
Endosulfan (ng/L)	EGG Stage	TAD Stage	EGG/TAD Stage	EGG Stage	TAD Stage	EGG/TAD Stage	EGG Stage	TAD Stage	EGG/TAD Stage	EGG Stage	TAD Stage	EGG/TAD Stage
0.1	2 / 5	3 / 5	2 / 5	0 / 5	0 / 5	0 / 5	1 / 5	0 / 3	0 / 4	0 / 5	0 / 3	0 / 4
1	0 / 5	2 / 5	1 / 5	0 / 5	1 / 5	0 / 5	1 / 4	0 / 3	0 / 4	0 / 4	1 / 3	0 / 4
10	2 / 5	1 / 5	1 / 5	0 / 5	1 / 5	1 / 5	3 / 5	0 / 3	3 / 6	2 / 5	0 / 3	1 / 6
100	3 / 5	3 / 5	1 / 5	0 / 5	0 / 5	1 / 5	2 / 4	0 / 4	2 / 4	3 / 4	0 / 4	0 / 4
1000	1 / 5	2 / 5	1 / 5	1 / 5	0 / 5	0 / 5	2 / 5	0 / 3	1 / 4	2 / 5	0 / 3	1 / 4
Total	8 / 25 32 %	11 / 25 44 %	6 / 25 24 %	1 / 25 4 %	2 / 25 8 %	2 / 25 8 %	9 / 23 39.1 %	0 / 16 0 %	6 / 22 27.3 %	7 / 23 30.4 %	1 / 16 6.3 %	2 / 22 9.1 %
Control		0 / 5			0 / 5			3 / 6			0 / 6	

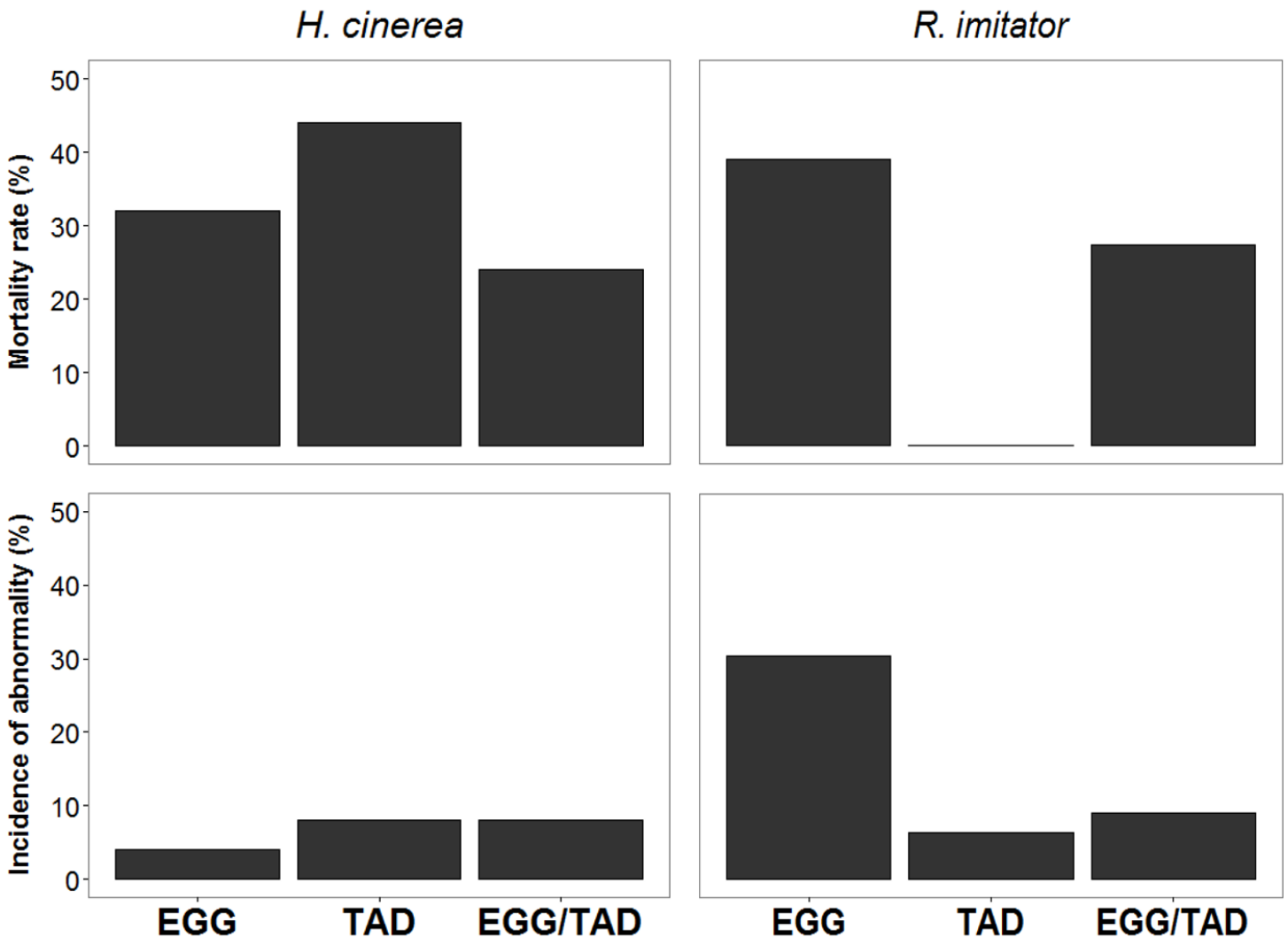


Figure 6 Mortality rate and incidence of deformity in *H. cinerea* and *R. imitator* according to life stage at exposure.

Figure 7 Example morphological abnormalities induced in endosulfan treated *H. cinerea.* (A) Left lateral view of frog treated with 1 ng/L endosulfan during TAD demonstrated hip dysplasia (arrow) that inhibited normal swimming behavior and induced mortality during metamorphic climax. (B) Dorsal view of frog treated with 1,000 ng/L endosulfan during EGG demonstrated failed development of a hind limb (hind limb amelia; arrow). (C) Dorsal view of tadpole treated with 10 ng/L endosulfan during EGG and TAD demonstrated slight kinking at the base of the tail (lateral flexure, arrow).

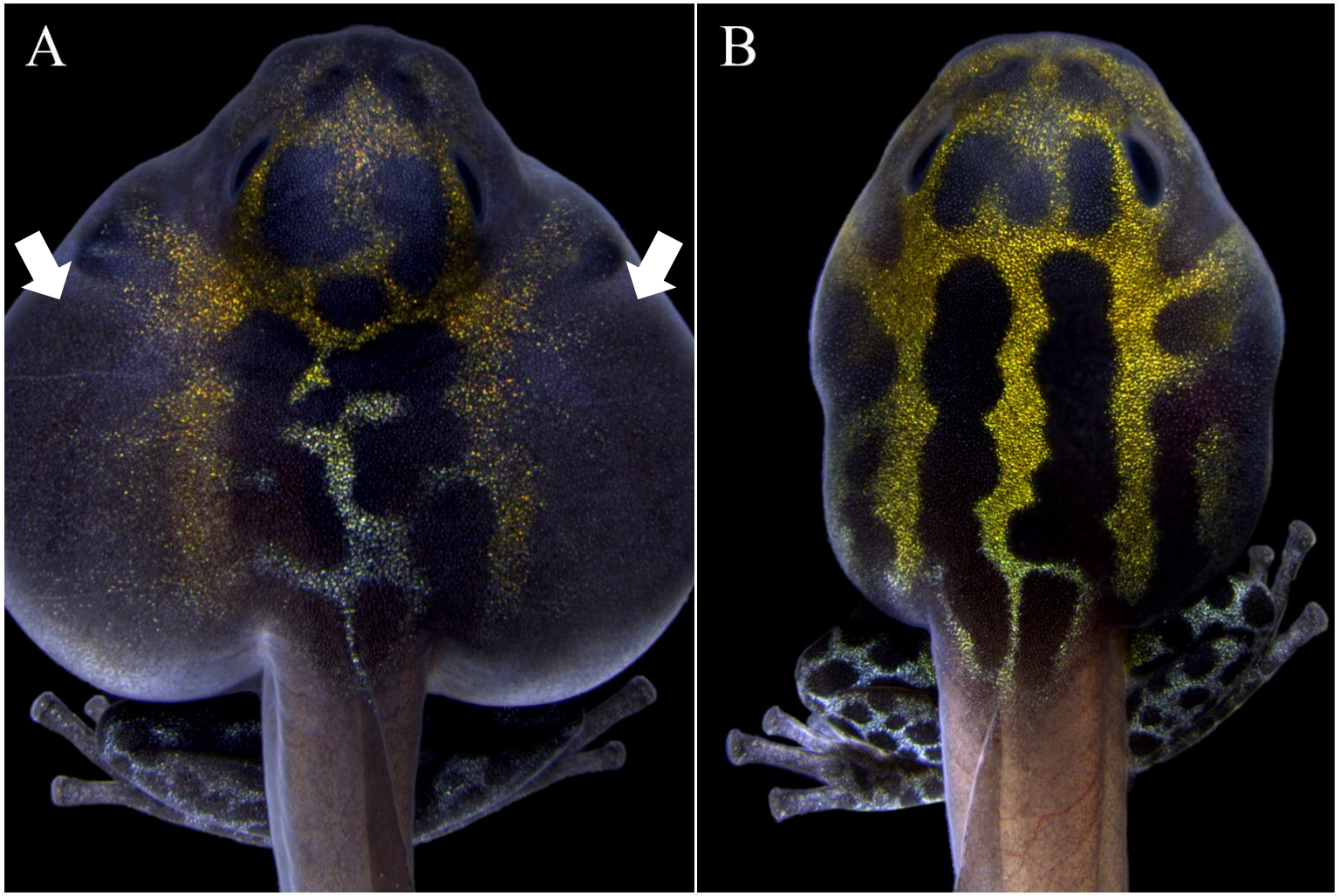


Figure 8 Endosulfan induced ascites. Dorsal view of *R. imitator* tadpoles from endosulfan (A) and control (B) treatments. Tadpole exposed to 1 ng/L endosulfan in TAD demonstrated severe bloating (ascites, arrows) that persisted throughout the larval stage and until completion of metamorphosis. This deformity inhibited normal swimming behavior.

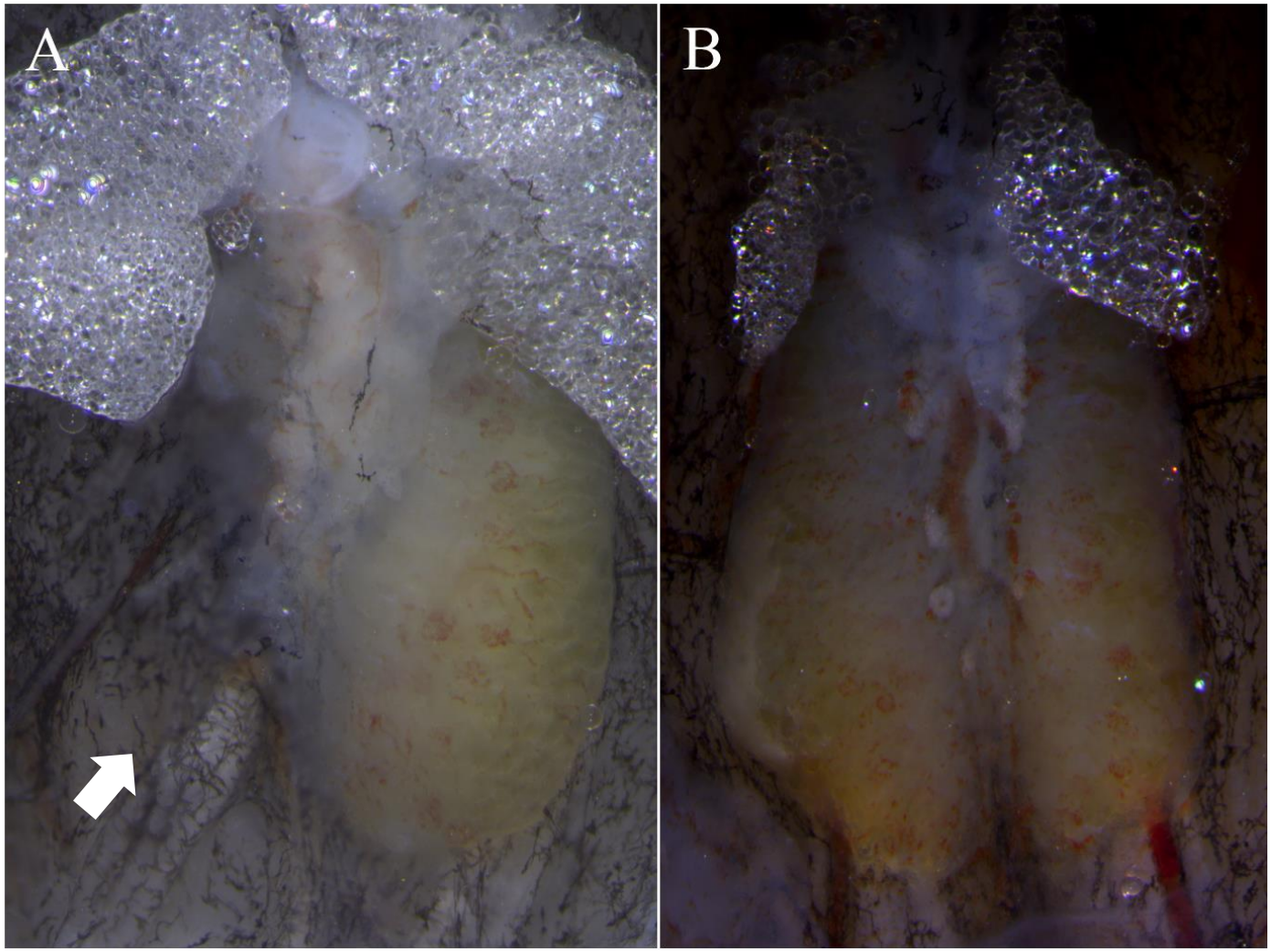


Figure 9 Endosulfan induced unilateral renal agenesis. Adrenal-kidney-gonad complex of *R. imitator* metamorphosed frogs from endosulfan (A) and control (B) treatments. Frog exposed to 100 ng/L endosulfan in EGG demonstrated failed development of a kidney lobe (unilateral renal agenesis, arrow).

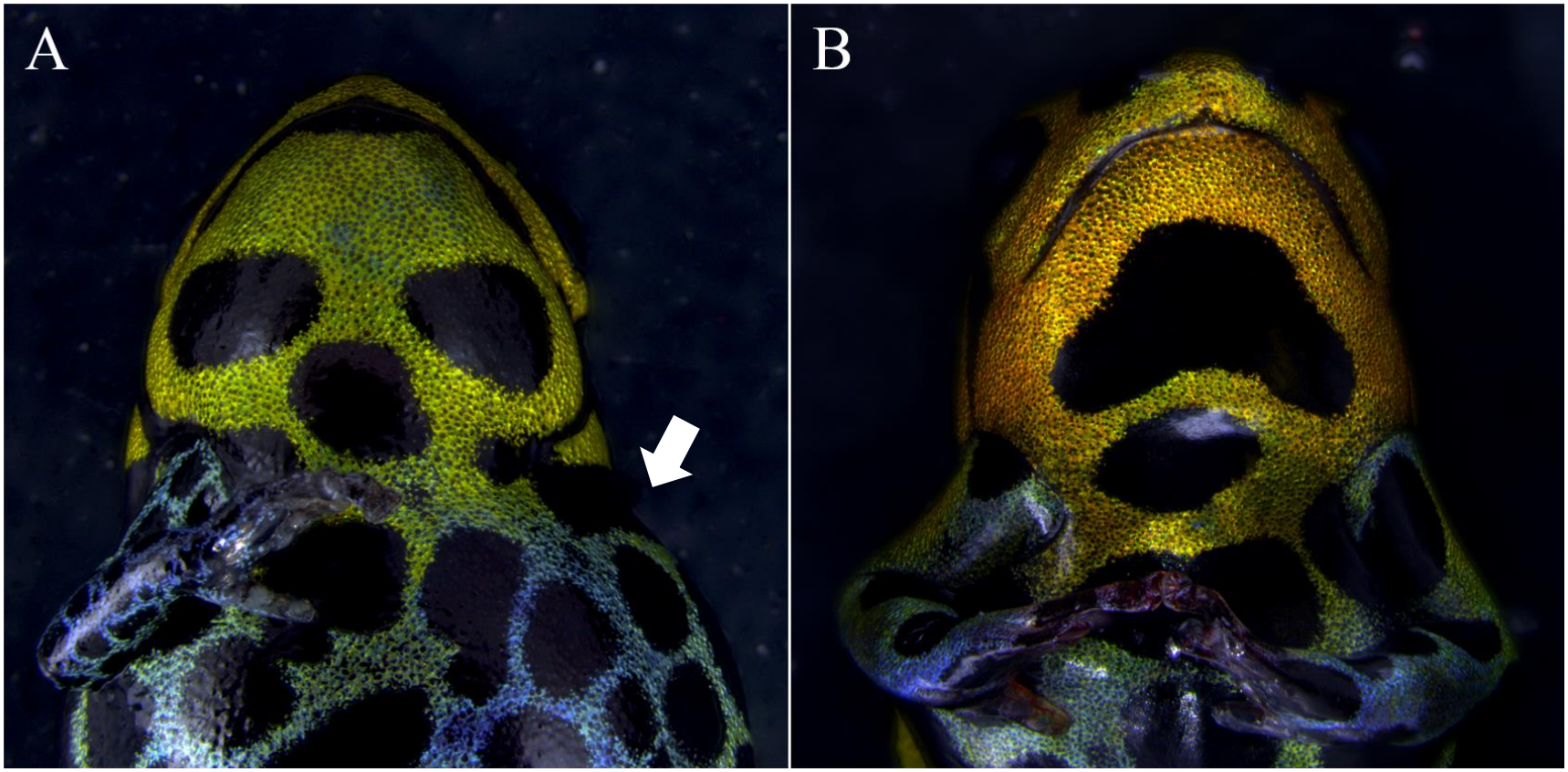


Figure 10 Endosulfan induced amelia. Ventral view of *R. imitator* metamorphosed frogs from endosulfan (A) and control (B) treatments. Frog exposed to 1,000 ng/L endosulfan in EGG demonstrated failed development of a forelimb (forelimb amelia, arrow).

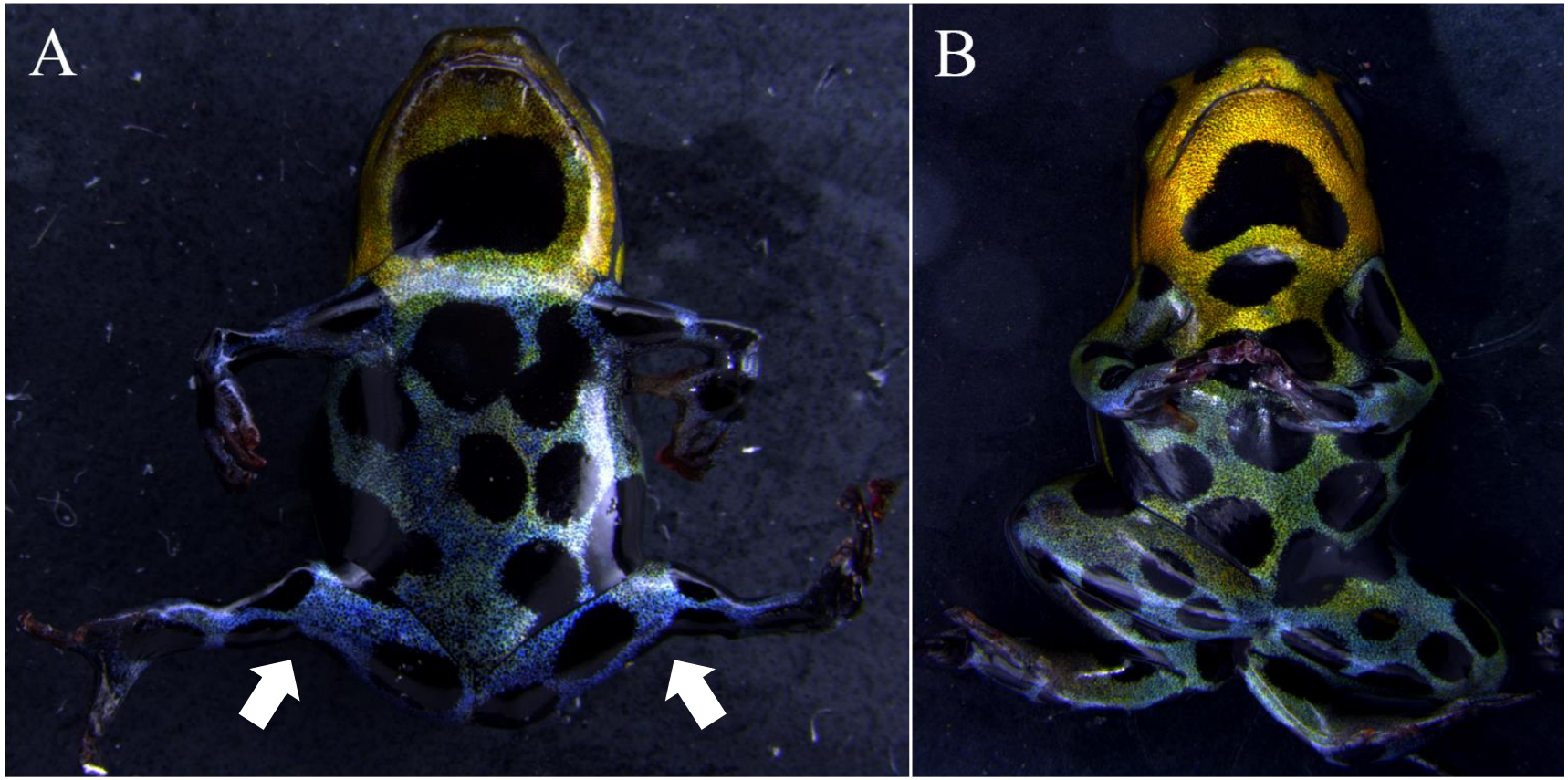


Figure 11 Endosulfan induced hyperextension. Ventral view of *R. imitator* metamorphosed frogs from endosulfan (A) and control (B) treatments. Frog exposed to 10 ng/L endosulfan in EGG demonstrated hyperextension of the hind limbs (arrows). This deformity inhibited normal swimming behavior.

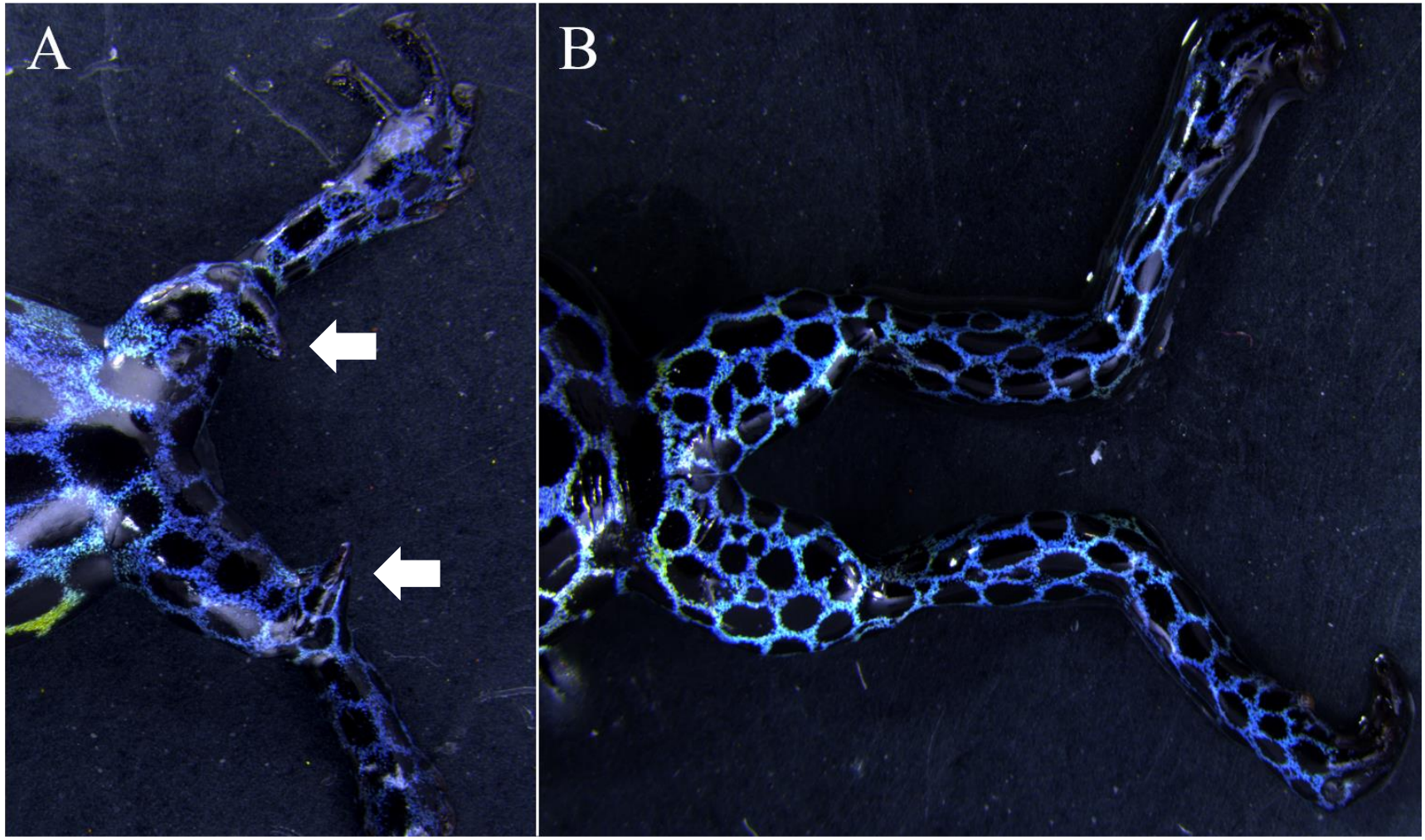


Figure 12 Endosulfan induced taumelia. Ventral view of *R. imitator* metamorphosed frogs from endosulfan (A) and control (B) treatments. Frog exposed to 1,000 ng/L endosulfan in EGG demonstrated bone bridging (tibiofibular taumelia, arrows). This deformity inhibited normal swimming behavior.

DISCUSSION

This study focuses on identifying the stage-dependent effects of exposing anuran eggs and tadpoles to a series of environmentally relevant endosulfan concentrations (from 0.1 – 1,000 ng/L), evaluating mortality, incidence of deformity, growth, and developmental rate. This is the first study to examine the effects of sustained endosulfan exposure in amphibians within and across multiple distinct developmental stages.

The findings of this study support an association between endosulfan exposure and incidence of developmental abnormality. Malformations were manifested in tadpoles exposed to concentrations of endosulfan over 2 orders of magnitude lower than those previously shown to induce deformity (Berrill *et al.* 1998, Harris *et al.* 2000, Rohr *et al.* 2003, Kang *et al.* 2008, Brunelli *et al.* 2009, De Jong Westman *et al.* 2010, Bernabo *et al.* 2013, Devi and Gupta 2013). These sub-chronic laboratory experiments report some of the characteristic abnormalities that were observed in this study, including lateral flexure of the tail and ascites. Although these effects may potentially arise through dietary insufficiency (Marshall *et al.* 1980, Martinez *et al.* 1992), the etiology of severe deformities observed in this study, such as unilateral renal agenesis and amelia, likely involves disruption of retinoid signaling pathways (Degitz *et al.* 2000, Gardiner *et al.* 2003). The occurrence of taumelia, in which the bone bends mid-shaft such that its ends fuse to form a triangular structure, is in fact diagnostic of exposure to retinoic acid and its mimics (Gardiner *et al.* 2003). These findings are supported by *in vitro* studies demonstrating disruption of retinoic acid signaling by endosulfan through RAR transactivation and induction of retinoic acid metabolizing enzymes (Lemaire *et al.* 2005),

The absence of skeletal elements and the presence of skeletal malformations suggests that both limb initiation and growth were disrupted by endosulfan (Gardiner *et al.* 2003). After

exposing mid-blastula and limb-bud stage *Xenopus laevis* and *Rana* sp. to exogenous retinoic acid, Degitz *et al.* (2000) found that hind limb malformations could only be induced by exposure at the latter stage. They concluded that anterior structures were more sensitive to teratogenesis and that embryonic exposure to retinoic acid would induce mortality before it could produce posterior abnormalities. In fact, all hind limb malformations observed in the present study were seen in individuals exposed to endosulfan as embryos. These findings suggest that endosulfan exposure during embryogenesis may have disruptive effects on limb development that carry-over into the larval stage.

Although many of the observed abnormalities were non-lethal in a laboratory setting and the condition of surviving malformed frogs at metamorphosis approximated the average effect across treatments, these deformities would undoubtedly impede foraging and predator avoidance and ultimately decrease adult survival and reproduction. Indeed, the altered swimming behavior of most deformed individuals necessitated the reduction of solution depth to prevent drowning and made them dependent on *ad libitum* feeding to avoid starvation.

Some studies have found that amphibian embryos and larvae accelerate development to complete metamorphosis early, trading off smaller size to escape from adverse environmental conditions (Denver *et al.* 1997, Bridges 2000, Boone *et al.* 2013). Alternatively, exposure to pollutants has also been found to delay development and the completion of metamorphosis, with or without effects on body condition (Sullivan and Spence 2003, Edwards *et al.* 2006). Likewise, the development and growth of some tadpoles are strongly affected by endosulfan exposure (Broomhall and Shine 2003, Lavorato *et al.* 2013). Contrasting with these findings, the results of the present study demonstrate that *H. cinerea* and *R. imitator* embryos and tadpoles are

insensitive to alterations in timing to and condition at multiple metamorphic stages following exposure to low doses of endosulfan.

Many studies use time to and condition at metamorphosis as sensitive biomarkers of amphibian health and as fitness correlates (Richard and Kendall 2003, Brunelli *et al.* 2009). These measures are chosen as generalized indicators of developmental toxicity because they are influenced by multiple physiological pathways (Mann *et al.* 2009). However, growth and rate of development are highly integrated, complex endpoints that exhibit non-monotonic responses (Lavorato *et al.* 2013) and may not be as diagnostic as assumed. This view is supported in the current study by the prevalence of morphological abnormality without significant effects on growth. This effect was also demonstrated by Tietge *et al.* (2005), after exposing larval *X. laevis* to sodium perchlorate and finding thyroid pathology at lower doses than those required to affect the timing of metamorphosis. The discrepancy in effect between the present study and those that show impairment of growth and development may reflect different experimental procedures, including continuity and dose of exposure, rearing of test subjects, and species choice.

As anticipated, we observed differences between species with measured endpoints. Across *H. cinerea* life stage treatments, mortality was high and incidence of deformity approximated frequencies expected in natural populations (Gillilland *et al.* 2001). Exposed *R. imitator*, on the other hand, exhibited exceptionally high rates of abnormality. Species differences have often been attributed to the structural diversity of egg jelly coats (Berrill *et al.* 1998, Birge *et al.* 2000, De Jong Westman *et al.* 2010) due to their ability to reduce chemical uptake rates (Licht 1985, Edginton *et al*. 2007). Nearly 30% of *R. imitator* tadpoles exposed during EGG exhibited developmental abnormalities (compared to about 6% in TAD), and thus presumably were not sufficiently protected by their jelly coats. Instead, differences in

susceptibility might be due to species specific induction of xenobiotic detoxifying enzymes (Casabar *et al.* 2010, Katagi and Ose 2014), reflecting their distinct life history strategies and potential adaptations to contaminant exposure. The observed mortality in individuals exposed as embryos may be related to the targeting of neuromuscular development. Endosulfan produces neurotoxic effects in tadpoles through inhibition of GABA activities (Bloomquist 2003), inducing disturbed breathing behavior, seizure, and mortality (Harris *et al.* 2000, Denoel *et al.* 2012).

No previous studies have investigated tadpole stage morphological abnormalities following sustained endosulfan exposure solely throughout embryonic development. The potential carry-over effect of egg stage exposure producing tadpole stage deformities was prominent in the present study and may be related to the well-documented lag effects of endosulfan (Berrill *et al*. 1998, Jones *et al*. 2009, Denoel *et al*. 2012, Bernabo *et al*. 2013). Lag effects, often recorded as mortality or abnormal behavior, manifest or persist after exposure has ended (Bernabo *et al*. 2013). For example, in Berrill *et al*. (1998), endosulfan induced low mortality to *Bufo americanus* tadpoles after 4 days of exposure, yet following transfer to clean water mortality rose to over 60% in all endosulfan treatments. It is apparent then that the expression of some detrimental effects of endosulfan require either cessation of exposure or sufficient time for the manifestation of physiological effects. Therefore, the relatively low observance of mortality and abnormality in *R. imitator* exposed only as tadpoles, compared to those exposed as eggs, may be due to lag effects rather than differential susceptibility. Because frogs were sacrificed at completion of metamorphosis, potential lag effects from TAD exposure in the adult frogs were not evaluated.

The USEPA (2009) set endosulfan's criterion continuous concentration, an estimate of the maximum chemical dose to which an aquatic community can be chronically exposed without unreasonable effects, at 112 ng/L (sum of isomers). This study observed mortality and abnormality at concentrations below this value. For example, an egg stage *R. imitator* exposed to 10 ng/L of endosulfan exhibited hyperextension and immobility of the hind limbs. Observed effects also occurred at concentrations likely experienced by amphibian populations in both agroecosystems and remote rainforests. Wild amphibians are further exposed to abiotic and biotic conditions that can act synergistically to increase pesticide toxicity (Kiesecker 2002, De Jong Westman *et al.* 2010). Indeed, Johnson *et al.* (2013) found that the lethality of endosulfan was greater in *Agalychnis callidryas* tadpoles exposed in ambient environmental conditions instead of in a climate controlled laboratory.

In conclusion, this study found rates of mortality and incidence of deformity that varied between species and life stages following sustained exposure to environmentally relevant levels of endosulfan during early development. Unexpectedly, no effects on measures of condition or timing at multiple developmental points were observed in either species at any tested concentration. Although the ultimate population level effects of endosulfan may be difficult to estimate without measures of endosulfan's effects on adults (Vonesh and de la Cruz 2002), these results provide useful insight for further toxicological studies regarding factors influencing individual susceptibility to pesticide exposure.

CHAPTER 3: GENERAL DISCUSSION

Endosulfan is an especially problematic pesticide because of its ubiquity (Weber *et al.* 2010, Shunthirasingham *et al.* 2011), its ability to bioaccumulate (Toledo and Jonsson 1992), and its toxicity to both humans and wildlife (ATSDR 2013). Even though endosulfan's isomers degrade rapidly, the resulting products are equally harmful and remain in the environment for several years after application (Rajakumar *et al.* 2012). These characteristics led to the recent addition of endosulfan to the Stockholm Convention's list of persistent organic pollutants (Bergman *et al.* 2013). Consequently, the manufacture and application of endosulfan has declined in many North American and European countries (POPRC 2010). Despite this trend, endosulfan use continues on a global scale, particularly in developing countries, and has an annual estimated production of approximately 20,000 tons (Mrema *et al*. 2013). Although the United States will conclude its phase-out of endosulfan use in 2016, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), regulated through the EPA, does not prevent pesticide companies from exporting their products (Finegan 1989). Therefore, active registrants can continue to manufacture and market endosulfan to foreign countries that grow crops for American consumption (ATSDR 2013). India is the primary producer and consumer of endosulfan, and continues its use with the justification of insufficient cost effective alternatives (Rajakumar *et al.* 2012). Understanding the nature of endosulfan's effects on non-target species is a priority of integrated pest management practices seeking to decrease reliance on this insecticide (Way and van Emden 2000, Danne *et al.* 2014).

Endosulfan accumulates in regions where amphibian declines and extinctions have been observed (Daniels 2003, Daly *et al.* 2007). Although endosulfan is highly lethal to amphibian

larvae (Jones *et al.* 2009), extinctions may not be attributable to early life mortality. Using a stage-based population dynamics model, Vonesh and De la Cruz (2002) demonstrated that the population level effects of reduced egg and larval survival relies on density dependence at each stage. For example, mortality in the egg stage may reduce larval densities, lowering competition and promoting metamorph recruitment (Vonesh and De la Cruz 2002). Population declines may result instead from the ability of pesticides to disrupt endocrine system function (McKinlay 2008, Diamanti-Kandarakis 2009, Mnif *et al.* 2011), causing growth and developmental alterations that carry over into adulthood. Induced deformity and changes in timing and condition at metamorphosis due to pesticide exposure (Lavorato *et al.* 2013, Brunelli *et al*. 2009) can delay reproductive maturity, reduce adult fecundity (Smith 1987), and ultimately decrease population growth rates (Chelgren *et al.* 2006). Resulting amphibian population declines are problematic not only because of lost ecosystem services (Valencia-Aguilar *et al.* 2013), but because of the similarities between amphibian and human physiology. These similarities make amphibians important models for determining the growth-altering and teratogenic effects of pesticides, including endosulfan, on mammalian development (Tata 1998).

Investigating the developmental roles of thyroid hormone in fetal mammals is complicated by their enclosure within the uterus and the transfer of maternal thyroid hormone across the placenta (Grimaldi *et al*. 2013). However, amphibian embryos and tadpoles are free-living and metamorphic development is dependent on thyroid hormone induced gene expression (Tata 1998). Although many vertebrates do not undergo metamorphosis, patterns of thyroid hormone signaling are highly conserved (Heimeier and Shi 2010). Peak thyroid hormone levels coincide with amphibian metamorphosis and mammalian postembryonic development, both periods of profound molecular and morphological change (Heimeier and Shi 2010).

Additionally, as reviewed in Grimaldi *et al*. (2013), the molecular mechanisms of thyroid hormone action are comparable across vertebrate taxa.

All studied vertebrates have retinoic acid receptors that exhibit similar patterns of gene expression during early development (Degitz *et al.* 2000). Therefore, both amphibians and humans may be susceptible to the teratogenic effects of endosulfan through disruption of retinoic acid signaling. The teratogenic potential of endosulfan has been demonstrated *in vitro*, through RAR agonism and increased production of retinoic acid metabolizing enzymes (Lemaire *et al.* 2005), and *in vivo*, through the induction of deformities in amphibians by this and numerous other studies.

Studies examining endosulfan effects in humans come from accidental exposures of rural communities in developing countries. A survey of Brazilian children by Freirie *et al*. (2012) found a correlation between serum concentrations of several organochlorine pesticides, including endosulfan, and elevated levels of triiodothyronine. Likewise, surveys of Indian schoolchildren living near plantations aerially sprayed with endosulfan reported significantly higher incidences of skeletal abnormalities and mental retardation (Saiyed *et al.* 2003, Embrandiri *et al.* 2012), indicative of *in utero* thyroid hormone and retinoic acid deficiencies (Haddow *et al*. 1999). These effects are made more troubling by studies of endosulfan body burden in breast feeding mothers from countries around the world. Sanghi *et al.* (2003) found high concentrations of endosulfan in the breast milk of women from Bhopal, India and estimated that infant consumption of endosulfan was 8.6 times higher than recommended by the World Health Organization. Similar levels were reported in the breast milk of mothers from South Africa (Channa *et al.* 2012) and Spain (Jimenez Torres *et al*. 2006). The highest dose of endosulfan included within the test

concentration series of this study approximated levels an order of magnitude lower than those found in human breast milk and encountered by infants.

Implications of study design

In this thesis, I have demonstrated the occurrence of morphological abnormalities at lower endosulfan concentrations than those seen to produce effects in previous studies (Harris *et al.* 2000, Devi and Gupta 2013). Surprisingly, I did not find significant effects of endosulfan on measures of body condition or developmental timing, though these effects have been observed in *Litoria freycineti* and *R. arenarum* tadpoles exposed to similar concentrations (Broomhall and Shine 2003, Svartz *et al.* 2014).

Discrepancies in findings between this and previous studies may be due to differences in study design. This study was the first to examine the developmental effects of endosulfan in amphibians following sustained exposure throughout multiple life stages. This study also used doses that were environmentally relevant, though much lower than those typically used to investigate endosulfan toxicity. Chronic exposure to extremely low concentrations of pesticides found in the environment was once generally perceived as harmless, but is now recognized as potentially more deleterious than acute exposure to high levels (Bridges 2000). This is especially true for endocrine disrupting chemicals like endosulfan, which act as exogenous modulators of development without a minimum threshold dose (Sheehan 2006, Vandenberg *et al*. 2012). Svartz e*t al.* (2014) found that the lethal and sub-lethal effects of endosulfan increased in tadpoles with greater exposure time. Therefore, the high incidence of mortality and deformity observed in this study, compared to previous studies, may reflect continuous rather than acute exposure at each developmental stage. Conversely, the absence of significant alterations to condition and

developmental timing may be attributed to the use of continuous exposure, which could prevent the expression of lag effects made evident only the cessation of treatment.

This study also differed from previous studies in the rearing of experimental animals. Tadpoles of both species were individually housed in mason jars as opposed to the standard group rearing of tadpoles in large aquaria. Although these conditions are realistic for *R. imitator* tadpoles, *H. cinerea* aggregate in the wild and may benefit through social facilitation or may experience crowding that influences the stress and thyroid axes (Griffiths and Foster 1998). Isolation can reduce growth and slow development (Griffiths and Foster 1998), causing an increase in the duration of the larval stage and thus exposure to aquatic pollutants. Therefore, rearing conditions can affect the responses of growth and development to contaminant exposure.

Research directions

Future studies should examine potential lag effects on growth and development following stage specific and continuous exposure to endosulfan. Numerous studies have demonstrated the importance of observing effects after exposure has ended (Berrill *et al*. 1998, Jones *et al*. 2009, Denoel *et al*. 2012, Bernabo *et al*. 2013) and this phenomenon merits study in the context of life stage transitions.

We have collected kidney and gonad tissues for future histological analyses. In rats, the target organs for endosulfan toxicity are the kidneys, where chronic exposure induces glomerulonephritis and nephropathy (FAO/WHO 1999). Endosulfan exposure also alters reproductive development across taxa, though its effects on gonad morphology and function have never been investigated in amphibians. Correlations of endosulfan exposure with decreased steroidogenesis and spermatogenesis in humans is controversial (Saiyed *et al.* 2003, Indulkar

2004, Saiyed 2004), and investigations into reproductive effects in amphibians may help to substantiate or refute these claims.

REFERENCES

Abràmoff MD, Magalhães PJ, Ram SJ. 2004. Image processing with ImageJ. Biophotonics International 11(7): 36-42.

Akingbemi BT, Hardy MP. 2001. Oestrogenic and antiandrogenic chemicals in the environment: effects on male reproductive health. Annals of Medicine 33: 391-403.

Andersen HR, Vinggaard AM, Rasmussen TH, Gjermandsen IM, Bonefeld-Jorgensen EC. 2002. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. Toxicology and Applied Pharmacology 179(1): 1-12.

ATSDR. 2013. Draft toxicological profile for endosulfan. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

Ballengee B, Sessions SK. 2009. Explanation for missing limbs in deformed amphibians. Journal of Experimental Zoology Part B 312B(7): 665-788.

Bergman A, Heindel JJ, Jobling S, Kidd KA, Zoeller RT. 2013. The state-of-the-science of endocrine disrupting chemicals - 2012. Geneva: UNEP/WHO.

Berrill M, Coulson D, McGillivray L, Pauli B. 1998. Toxicity of endosulfan to aquatic stages of anuran amphibians. Environmental Toxicology and Chemistry 17(9): 1738-1744.

Birge WJ, Westerman AG, Spromberg JA. 2000. Comparative toxicology and risk assessment of amphibians. In: Sparling, D.W., G. Linger and C.A. Bishop (Eds.), Ecotoxicology of Amphibians and Reptiles. Society of Environmental Toxicology and Chemistry, Pensacola, FL, 904.

Bishop CA, Mahony NA, Struger J, Ng P, Pettit KE. 1999. Anuran development, density and diversity in relation to agricultural activity in the Holland River Watershed, Ontario, Canada (1990-1992). Environmental Monitoring and Assessment 57: 21-43.

Blaustein AR, Johnson TJ. 2003. The complexity of deformed amphibians. Frontiers of Ecology and the Environment1(2): 87-94.

Bloomquist JR. 2003. Chloride channels as tools for developing selective insecticides. Archives of Insect Biochemistry and Physiology 54(4): 145-156.

Boethling R, Fenner K, Howard P, Klecka G, Madsen T, Snape JR, Whelan MJ 2009. Environmental persistence of organic pollutants: guidance for development and review of POP risk profiles. Integrated Environmental Assessment and Management 5(4): 539-556.

Bogi C, Levy G, Lutz I, Kloas W. 2002. Functional genomics and sexual differentiation in amphibians. Comparative Biochemistry and Physiology B 133: 559-570.

Boone MD, Hammond SA, Veldhoen N, Youngquist M, Helbing CC. 2013. Specific time of exposure during tadpole development influences biological effects of the insecticide carbaryl in green frogs (*Lithobates clamitans*). Aquatic Toxicology 130-131: 139-148.

Bridges CM. 2000. Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). Archives of environmental contamination and toxicology 39(1): 91-96.

Brodeur JC, Svartz G, Perez-Coll CS, Marino DJ, Herkovits J. 2009. Comparative susceptibility to atrazine of three developmental stages of *Rhinella arenarum* and influence on metamorphosis: non-monotonous acceleration of the time to climax and delayed tail resorption. Aquatic Toxicology 91(2): 161-170.

Brown DD, Cai L. 2007. Amphibian metamorphosis. Developmental biology 306(1): 20-33.

Brown JL, Twomey E, Morales V, Summers K. 2008. Phytotelm size in relation to parental care and mating strategies in two species of Peruvian poison frogs. Behaviour 145:1139-1165.

Bruhl, CA, Pieper S, Weber B. 2011. Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. Environmental Toxicology and Chemistry 30(11): 2465-2472.

Brunelli E, Bernabo I, Berg C, Lundstedt-Enkel K, Bonacci A, Tripepi S. 2009. Environmentally relevant concentrations of endosulfan impair development, metamorphosis and behaviour in *Bufo bufo* tadpoles. Aquatic Toxicology 91(2): 135-142.

Carey C, Bryant CJ. 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environmental Health Perspectives 103: 13-17.

Casabar RC, Das PC, Dekrey GK, Gardiner CS, Cao Y, Rose RL, Wallace AD. 2010. Endosulfan induces CYP2B6 and CYP3A4 by activating the pregnane X receptor. Toxicology and Applied Pharmacology 245(3): 335-343.

Cerrillo I, Granada A, Lopez-Espinosa MJ, Olmos B, Jimenez M, Cano A, Olea N, Olea-Serrano MF. 2005. Endosulfan and its metabolites in fertile women, placenta, cord blood, and human milk. Environmental Research 98(2): 233-239.

Channa KR, Rollin HB, Wilson KS, Nost TH, Odland JO, Naik I, Sandanger TM. 2012. Regional variation in pesticide concentrations in plasma of delivering women residing in rural Indian Ocean coastal regions of South Africa. Journal of Environmental Monitoring 14(11): 2952-2960.

Chelgren ND, Rosenberg DK, Heppell SS, Gitelman AI. 2006. Carryover aquatic effects on survival of metamorphic frogs during pond emigration. Ecological Applications 16: 250-261.

Collins JP, Storfer A. 2003. Global amphibian declines: sorting the hypotheses. Diversity and Distributions 9: 89-98.

Crews D, Willingham E, Skipper JK. 2000. Endocrine disruptors: present issues, future directions. The Quarterly Review of Biology 75(3): 243-260.

Da Cuna RH, Pandolfi M, Genovese G, Piazza Y, Ansaldo M, Lo Nostro FL. 2013. Endocrine disruptive potential of endosulfan on the reproductive axis of *Cichlasoma dimerus* (Perciformes, Cichlidae). Aquatic Toxicology 126: 299-305.

Daly GL, Lei YD, Teixeira C, Muir DCG, Castillo LE, Wania F. Accumulation of current-use pesticides in neotropical montane forests. Environmental Science and Technology 41:1118-1123.

Daniels RJR. 2003. Impact of tea cultivation on anurans in the Western Ghats. Current Science 85(10): 1415-1422.

Danne AW, Llewellyn R, Huwer RK, Furlong M. 2014. Fruitspotting bugs, *Amblypelta nitida* Stal and *A. lutescens lutescens* Distant (Hemiptera: Coreidae): A review of the potential for integrated management practices. Austral Entomology 53: 112-123.

Daszak P, Cunninham AA, Hyatt AD. 2003. Infectious disease and amphibian population declines. Diversity and Distributions 9: 141-150.

Davidson C. 2004. Declining downwind: amphibian population declines in California and historical pesticide use. Ecological Applications 14(6): 1892-1902.

De Boo HA, Harding JE. 2006. The developmental origins of adult disease (Barker) hypothesis. Australian and New Zealand Journal of Obstetrics and Gynaecology 46: 4-14.

De Jong Westman A, Elliott J, Cheng K, van Aggelen G, Bishop CA. 2010. Effects of environmentally relevant concentrations of endosulfan, azinphosmethyl, and diazinon on Great Basin spadefoot (*Spea intermontana*) and Pacific treefrog (*Pseudacris regilla*). Environmental Toxicology and Chemistry / SETAC 29(7): 1604-1612.

Degitz SJ, Kosian PA, Makynen EA, Jensen JM, Ankley GT. 2000. Stage- and species-specific developmental toxicity of all-trans retinoic acid in four native North American ranids and *Xenopus laevis*. Toxicological Sciences 57: 264-274.

Denoel M, D'Hooghe B, Ficetola GF, Brasseur C, De Pauw E, Thome JP, Kestemont P. 2012. Using sets of behavioral biomarkers to assess short-term effects of pesticide: a study case with endosulfan on frog tadpoles. Ecotoxicology 21(4): 1240-1250.

Denver RJ. 1997. Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis. American Zoologist 37(2): 172-184.

Denver RJ. 1999. Evolution of the corticotropin-releasing hormone signaling system and its role in stress-induced phenotypic plasticity. Annals of the New York Academy of Sciences 897: 46-53.

Devi NN, Gupta A. 2013. Toxicity of endosulfan to tadpoles of Fejervarya spp. (Anura: Dicroglossidae): mortality and morphological deformities. Ecotoxicology 22(9): 1395-1402.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocrine reviews 30(4): 293-342.

Dunson WA, Wyman RL, Corbett ES. 1992. A symposium on amphibian declines and habitat acidification. Journal of Herpetology 26(4): 349-352.

Edginton AN, Rouleau C, Stephenson GR, Boermans HJ. 2007. 2,4-D butoxyethyl ester kinetics in embryos of *Xenopus laevis*: the role of the embryonic jelly coat in reducing chemical absorption. Archives of Environmental Contamination and Toxicology 52(1): 113-120.

Edwards TM, McCoy KA, Barbeau T, McCoy MW, Thro JM, Guillette LJ, Jr. 2006. Environmental context determines nitrate toxicity in Southern toad (*Bufo terrestris*) tadpoles. Aquatic Toxicology 78(1): 50-58.

Embrandiri A, Singh RP, Ibrahim HM, Khan AB. 2012. An epidemiological study on the health effects of endosulfan spraying on cashew plantations in Kasaragod District, Kerala, India. Asia Journal of Epidemiology 5(1): 22-31.

FAO/WHO. 1999. Pesticide residues in food — 1998 evaluations. Part II — Toxicological. Geneva, World Health Organization, Joint FAO/WHO Meeting on Pesticide Residues (WHO/PCS/99.18).

Fenner-Crisp PA. 1997. Endocrine disruptor risk characterization: an EPA perspective. Regulatory Toxicology and Pharmacology 26: 70-73.

Finegan PA. 1989. FIFRA lite: A regulatory solution or part of the pesticide problem? Pace Environmental Law Review 6(2) 615-641.

Freed VH, Haque R, Schmedding D. 1972. Vaporization and environmental contamination by DDT. Chemosphere 2: 61-66.

Freire C, Koifman RJ, Sarcinelli P, Rosa AC, Prada R, Clapauch R, Koifman S. 2012. Long term exposure to organochlorine pesticides and thyroid function in children from Cidade dos Meninos, Rio de Janeiro, Brazil. Environmental Research 117: 68-74.

Galton VA. 1992. The role of thyroid hormone in amphibian metamorphosis. Trends in

Endocrinology and Metabolism 3(3): 96-100.

Gardiner D, Ndayibagira A, Grun F, Blumberg B. 2003. Deformed frogs and environmental retinoids. Pure Applied Chemistry 75: 2263-2273.

Gillilland CD, Summer CL, Gillilland MG, Kannan K, Villeneuve D, Coady KK, et al. 2001. Organochlorine insecticides, polychlorinated biphenyls, and metals in water, sediment, and green frogs from southwestern Michigan. Chemosphere 44: 327-339.

Grimaldi A, Buisine N, Miller T, Shi YB, Sachs LM. 2013. Mechanisms of thyroid hormone receptor action during development: lessons from amphibian studies. Biochimica et Biophysica Acta 1830(7): 3882-3892.

Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16: 183-190.

Greulich K, Pflugmacher S. 2003. Differences in susceptibility of various life stages of amphibians to pesticide exposure. Aquatic Toxicology 65(3): 329-336.

Griffiths RA, Foster JP. 1998. The effect of social interactions on tadpole activity and growth in the British anuran amphibians (*Bufo bufo, B. calamita, and Rana temporaria*). Journal of Zoology, London 245: 431-437.

Guerin TG. 2001. Abiological loss of endosulfan and related chlorinated organic compounds from aqueous systems in the presence and absence of oxygen. Environmental Pollution 115: 219-230.

Gutleb AC, Schriks M, Mossink L, Berg JH, Murk AJ. 2007. A synchronized amphibian metamorphosis assay as an improved tool to detect thyroid hormone disturbance by endocrine disruptors and apolar sediment extracts. Chemosphere 70(1): 93-100.

Haczkiewicz K, Ogielska M. 2013. Gonadal sex differentiation in frogs: how testes become shorter than ovaries. Zoological science 30(2): 125-134.

Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gangnon J, et al. 1999. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. The New England Journal of Medicine 341(8): 549-555.

Hall, R.J., Henry, P.F.P., 1992. Assessing effects of pesticides on amphibians and reptiles: Status and needs. Herpetological Journal 2: 65-71.

Harris ML, Chora L, Bishop CA, Bogart JP. 2000. Species- and Age-Related Differences in Susceptibility to Pesticide Exposure for Two Amphibians, *Rana pipiens*, and *Bufo americanus*. Bulletin of Environmental Contamination and Toxicology 64(2): 263-270.

Hayes TB. 1997. Steroids as potential modulators of thyroid hormone activity in anuran

metamorphosis. American Zoology 37: 185-194.

Hayes TB. 2005. Welcome to the revolution: integrative biology and assessing the impact of endocrine disruptors on environmental and public health. Integrative and Comparative Biology 45: 321-329.

Hayes TB, Case P, Chui S, Chung D, Haeffele C, Haston K, et al. 2006. Pesticide Mixtures, Endocrine Disruption, and Amphibian Declines: Are We Underestimating the Impact? Environmental Health Perspectives 114(S-1): 40-50.

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, et al. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences of the United States of America 99(8): 5476-5480.

Heimeier RA, Shi YB. 2010. Amphibian metamorphosis as a model for studying endocrine disruption on vertebrate development: effect of bisphenol A on thyroid hormone action. General and Comparative Endocrinology 168(2): 181-189.

Helbing CC, Gergely G, Atkinson BG. 1992. Sequential up-regulation of thyroid hormone b receptor, ornithine transcarbamylase and carbamyl phosphate synthetase mRNAs in the liver of *Rana catesbeiana* tadpoles during spontaneous and thyroid hormone-induced metamorphosis. Developmental Genetics 13: 289–301.

Hopkins WA. 2007. Amphibians as models for studying environmental change. Institute of Laboratory Animal Resources 48: 270-277.

Hourdry J, L'Hermite A, Ferrand R. 1996. Changes in the digestive tract and feeding behavior of anuran amphibians during metamorphosis. Physiological Zoology 69(2): 219-251.

Indulkar AS. 2004. Endosulfan's effects: Inaccurate data. Environmental Health Perspectives 112(10): A538-A539.

Inoue D, Sei K, Ike M. 2010. Disruption of reintoic acid receptor signaling by environmental pollutants. Journal Health Science 56(3): 221-230.

IUCN. 2008. Red list of endangered species of 2008, amphibians. Available: http://www.iucnredlist.org/initiatives/amphibians/analysis [accessed 30 October 2013].

Jimenez Torres M, Campoy Folgoso C, Canabate Reche F, Rivas Velasco A, Cerrillo Garcia I, Mariscal Arcas M, et al. 2006. Organochlorine pesticides in serum and adipose tissue of pregnant women in Southern Spain giving birth by cesarean section. The Science of the Total Environment 372(1): 32-38.

Johnson LA, Welch B, Whitfield SM. 2013. Interactive effects of pesticide mixtures, predators, and environmental regimes on the toxicity of two pesticides to red-eyed tree frog larvae.

Environmental Toxicology and Chemistry / SETAC 32(10): 2379-2386.

Jones DK, Hammond JI, Relyea RA. 2009. Very highly toxic effects of endosulfan across nine species of tadpoles: lag effects and family-level sensitivity. Environmental Toxicology and Chemistry / SETAC 28(9): 1939-1945.

Kang HS, Gye MC, Kim MK. 2008. Effects of endosulfan on survival and development of *Bombina orientalis* (Boulenger) embryos. Bulletin of Environmental Contamination and Toxicology 81(3): 262-265.

Katagi T, Ose K. 2014. Bioconcentration and metabolism of pesticides and industrial chemicals in the frog. Journal of Pesticide Science 39(2): 55-68.

Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp PA, Gray LE, Kaattari S, et al. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environmental Health Perspectives 104: 1-26.

Kiesecker JM. 2002. Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? Proceedings of the National Academy of Sciences 99(15): 9900-9904.

Kiesecker JM, Blaustein AR, Belden LK. 2001. Complex causes of amphibian population declines. Nature 410: 681-684.

Kloas W, Urbatzka R, Opitz R, Wurtz S, Behrends T, Hermelink B, et al. 2009. Endocrine disruption in aquatic vertebrates. Annals of the New York Academy of Sciences 1163: 187-200.

Lavorato M, Bernabo I, Crescente A, Denoel M, Tripepi S, Brunelli E. 2013. Endosulfan effects on *Rana dalmatina* tadpoles: quantitative developmental and behavioural analysis. Archives of Environmental Contamination and Toxicology 64(2): 253-262.

Lemaire G, Balaguer P, Michel S, Rahmani R. 2005. Activation of retinoic acid receptor-dependent transcription by organochlorine pesticides. Toxicology and Applied Pharmacology 202(1): 38-49.

Li YF, Macdonald RW. 2005. Sources and pathways of selected organochlorine pesticides to the Arctic and the effect of pathway divergence on HCH trends in biota: a review. The Science of the Total Environment 342(1-3): 87-106.

Licht LE. 1985. Uptake of 14C DDT by wood frog embryos after short term exposure. Comparative Biochemistry and Physiology B 81C(1): 117-119.

Lisboa PC, Curty FH, Moreira RMO, K.J., Pazos-Moura CC. 2001. Sex steroids modulate rat anterior pituitary and liver iodothyronine deiodinase activities. Hormone and Metabolic

Research 33: 532-535.

Loeffler IK, Stocum DL, Fallon JF, Meteyer CU. 2001. Leaping lopsided: a review of the current hypotheses regarding etiologies of limb malformations in frogs. The Anatomical Record 265: 228-245.

Lutz I, Kloas W. 1999. Amphibians as a model to study endocrine disruptors: I. environmental pollution and estrogen receptor binding. The Science of the Total Environment 225: 49-57.

Lutz-Ostertag Y, Kantelip JP. 1971. Sterilizing action of endosulfan (Thiodan) on the gonads of chick and quail embryos in vivo and in vitro. Comptes Rendus des Seances de la Societe de Biologie et des ses Filiales 165(4): 844-848.

Mann RM, Hyne RV, Choung CB, Wilson SP. 2009. Amphibians and agricultural chemicals: review of the risks in a complex environment. Environmental Pollution 157(11): 2903-2927.

Marshall H, Morrison A, Studer M, Popperi H, Krumlauf R. 1996. Retinoids and hox genes. FASEB Journal 10(9): 969-978.

Martinez I, Alvarez R, Herraez I, Herraez P. 1992. Skeletal malformations in hatchery reared *Rana perezi* tadpoles. The Anatomical Record 233: 314-320.

McCallum ML. 2007. Amphibian decline or extinction? Current declines dwarf background extinction rate. Journal of Herpetology 41(3): 483-491.

McCoy KA, McCoy MW, Amick A, Guillettte LJ Jr., St. Mary CM. 2007. Tradeoffs between somatic and gonadal investments during development in the African clawed frog (*Xenopus laevis*). Journal of Experimental Zoology 307A: 637-646.

McCoy KA, Bortnick LJ, Campbell CM, Hamlin HJ, Guillette LJ, Jr., St. Mary CM. 2008. Agriculture alters gonadal form and function in the toad *Bufo marinus*. Environmental Health Perspectives 116(11): 1526-1532.

McCoy MW, Barfield M, Holt RD. 2009. Predator shadows: complex life histories as generators of spatially patterned indirect interactions across ecosystems. Oikos 118(1): 87-100.

McKinlay R, Plant JA, Bell JN, Voulvoulis N. 2008. Endocrine disrupting pesticides: implications for risk assessment. Environment International 34(2): 168-183.

Meteyer CU. 2000. Field guide to malformations of frogs and toads with radiographic interpretations. Biological Science Report USGS/BRD/BSR-2000-0005.

Miles JRW, Moy P. 1979. Degradation of endosulfan and its metabolites by a mixed culture of soil microorganisms. Bulletin of Environmental Contamination and Toxicology 23: 13-

19.

Mnif W, Hassine AI, Bouaziz A, Bartegi A, Thomas O, Roig B. 2011. Effect of endocrine disruptor pesticides: a review. International Journal of Environmental Research and Public Health 8(6): 2265-2303.

Mrema EJ, Rubino FM, Brambilla G, Moretto A, Tsatsakis AM, Colosio C. 2013. Persistent organochlorinated pesticides and mechanisms of their toxicity. Toxicology 307: 74-88.

Neuparth T, Bickham JW, Theodorakis CW, Costa FO, Costa MH. 2006. Endosulfan-induced genotoxicity detected in the Gilthead Seabream, *Sparus aurata* L., by means of flow cytometry and micronuclei assays. Bulletin of Environmental Contamination and Toxicology 76(2): 242-248.

Noriega NC, Hayes TB. 2000. DDT congener effects on secondary sex coloration in the reed frog *Hyperolius argus*: a partial evaluation of the *Hyperolius argus* endocrine screen. Comparative Biochemistry and Physiology B 126: 231-237.

Novak J, Benisek M, Hilscherova K. 2008. Disruption of retinoid transport, metabolism and signaling by environmental pollutants. Environment International 34(6): 898-913.

Ouellet M, Bonin J, Rodrigue J, DesGranges J, Lair S. 1997. Hindlimb deformities (ectromelia, ectrodactyly) in free-living anurans from agricultural habitats. Journal of Wildlife Diseases 33: 95-104.

Palmer BD, Huth LK, Pieto DL, Selcer KW. 1998. Vitellogenin as a biomarker for xenobiotic estrogens in an amphibian model system. Environmental Toxicology and Chemistry / SETAC 17(1): 30-36.

Park BJ, Kidd KA. 2005. Effects of the synthetic estrogen ethinylestradiol on early life stages of mink frogs and green frogs in the wild and in situ. Environmental Toxicology and Chemistry / SETAC 24(8): 2027-2036.

Peterle TJ. 1969. DDT in Antarctic snow. Nature 224: 620.

Pickford DB, Morris ID. 2003. Inhibition of gonadotropin-induced oviposition and ovarian steroidogenesis in the African clawed frog (*Xenopus laevis*) by the pesticide methoxychlor. Aquatic Toxicology 62: 179-194.

POPRC (Persistent Organic Pollutants Review Committee). 2010. Recommendation of the POPRC on endosulfan. Available: http://chm.pops.int/ [accessed 30 October 2013].

Pounds JA, Crump ML. 1994. Amphibian declines and climate disturbance: the case of the golden toad the harlequin frog. Conservation Biology 8(1): 72-85.

Propper CR. 2005. The study of endocrine-disrupting compounds: past approaches and new

directions. Integrative and Comparative Biology 45: 194-200.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available: http:/www.R-project.org/.

Rajakumar A, Singh R, Chakrabarty S, Murugananthkumar R, Laldinsangi C, Prathibha Y, et al. 2012. Endosulfan and flutamide impair testicular development in the juvenile Asian catfish,4 *Clarias batrachus*. Aquatic Toxicology 110-111: 123-132.

Rey F, Gonzalez M, Zayas MA, Stoker C, Durando M, Luque EH, et al. 2009. Prenatal exposure to pesticides disrupts testicular histoarchitecture and alters testosterone levels in male *Caiman latirostris*. General and Comparative Endocrinology 162(3): 286-292.

Richards S, Kendall R. 2003. Physical effects of chlorpyrifos on two stages of *Xenopus laevis*. J Toxicology and Environmental Health A 66: 75-91.

Rohr JR, Elskus AA, Shepherd BS, Crowley PH, McCarthy TM, Niedzwiecki JH, et al. 2003. Lethal and sublethal effects of atrazine, carbaryl, endosulfan, and octylphenol on the streamside salamander (*Ambystoma barbouri*). Environmental Toxicology and Chemistry/ SETAC 22(10): 2385-2392.

Rollins-Smith LA, Ramsey JP, Pask JD, Reinert LK, Woodhams DC. 2011. Amphibian immune defenses against chytridiomycosis: impacts of changing environments. Integrative and Comparative Biology 51(4): 552-562.

Rose CS. 2005. Integrating ecology and developmental biology to explain the timing of frog metamorphosis. Trends in Ecology & Evolution 20(3): 129-135.

Saiyed H, Dewan A, Bhatnagar V, Shenoy U, Shenoy R, Rajmohan H, et al. 2003. Effect of endosulfan on male reproductive development. Environmental Health Perspectives 111(16): 1958-1962.

Saiyed H. 2004. Endosulfan's effects: Saiyed's response. Environmental Health Perspectives 112(10): A539-A541.

Sanghi R, Pillai MKK, Jayalekshmi TR, Nair A. 2003. Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India. Human & Experimental Toxicology 22(2): 73-76.

Schulz R. 2001. Rainfall-induced sediment and pesticide input from orchards into the Lourens River, Western Cape, South Africa: importance of a single event. Water Research 35(8): 1869-1876.

Semlitsch RD, Scott DE, Pechmann JHK. 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. Ecology 63: 184-192.

Sheehan DM. 2006. No-threshold dose-response curves for nongenotoxic chemicals: Findings and applications for risk assessment. Environmental Research 100(1): 93-99.

Shunthirasingham C, Gouin T, Lei YD, Ruepert C, Castillo LE, Wania F. 2011. Current-use pesticide transport to Costa Rica's high-altitude tropical cloud forest. Environmental Toxicology and Chemistry / SETAC 30(12): 2709-2717.

Simonich SL, Hites RA. 1995. Global distribution of persistent organochlorine compounds. Science 269(5232): 1851-1854.

Sinha N, Adhikari N, Saxena DK. 2001. Effect of endosulfan during fetal gonadal differentiation on spermatogenesis in rats. Environmental Toxicology and Pharmacology 10: 29-32.

Smith DC. 1987. Adult recruitment in chorus frogs: Effects of size and date at metamorphosis. Ecology 68(2): 344-350.

Smith MA, Green DM. 2005. Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations. Ecography 28(1): 110-128.

Soto AM, Chung KL, Sonnenschein C. 1994. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environmental Health Perspectives 102(4): 380-383.

Stocum DL. 2000. Frog limb deformities: an "eco-devo" riddle wrapped in multiple hypotheses surrounded by insufficient data. Teratology 62: 147-150.

Stoks R, Cordoba-Aguilar A. 2012. Evolutionary ecology of Odonata: A complex life cycle perspective. Annual Review of Entomology 57: 249-265.

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, et al. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306(5702): 1783-1786.

Sullivan KB, Spence KM. 2003. Effects of sublethal concentrations of atrazine and nitrate on metamorphosis of the African clawed frog. Environmental Toxicology and Chemistry / SETAC 22(3): 627-635.

Svartz GV, Wolkowicz IR, Coll CS. 2014. Toxicity of endosulfan on embryo-larval development of the South American toad *Rhinella arenarum*. Environmental Toxicology and Chemistry / SETAC 33(4): 875-881.

Tanabe S, Tatsukawa R, Kawano M, Hidaka H. 1982. Global distributions and atmospheric transport of chlorinated hydrocarbons: HCH (BHC) isomers and DDT compounds in the Western Pacific, Eastern Indian, and Antarctic oceans. Journal of the Oceanographical Society Japan 38: 137-148.

Tata JR. 1998. Amphibian metamorphosis as a model for studying the developmental actions of thyroid hormone. Cell Research 8: 259-272.

Tata JR. 2000. Autoinduction of nuclear hormone receptors during metamorphosis and its significance. Insect Biochemistry and Molecular Biology 30: 645-651.

Tavera-Mendoza L, Ruby S, Broussea P, Fournier MC, D., Marcogliese D. 2002. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. Environmental Toxicology and Chemistry / SETAC 21(3): 527-531.

Tietge JE, Holcombe GW, Flynn KM, Kosian PA, Korte JJ, Anderson LE, et al. 2005. Metamorphic inhibition of *Xenopus laevis* by sodium perchlorate: effects on development and thyroid histology. Environmental Toxicology and Chemistry / SETAC 24(4): 926-933.

Toledo MC, Jonsson CM. 1992. Bioaccumulation and elimination of endosulfan in zebra fish (*Brachydanio rerio*). Journal of Pesticide Science 36: 207-211.

Uller T, Sagvik J, Olsson M. 2009. Pre-hatching exposure to water mold reduces size at metamorphosis in the moor frog. Oecologia 160(1): 9-14.

USEPA. 2007. Method 8081B: Organochlorine pesticides by gas chromatography. Available: http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/8081b.pdf [accessed 30 March 2014]

USEPA. 2009. National recommended water quality criteria. Available: http://water.epa.gov/scitech/swguidance/standards/current/ [accessed 30 March 2014].

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Jr., Lee DH, et al. 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocrine Reviews 33(3): 378-455.

Valencia-Aguilar A, Cortés-Gómez AM, Ruiz-Agudelo CA. 2013. Ecosystem services provided by amphibians and reptiles in Neotropical ecosystems. International Journal of Biodiversity Science, Ecosystem Services & Management 9(3): 257-272.

Vonesh JR, De la Cruz O. 2002. Complex life cycles and density dependence: assessing the contribution of egg mortality to amphibian declines. Oecologia 113(3): 325-333.

Wallace H, Badawy GM, Wallace BM. 1999. Amphibian sex determination and sex reversal. Cellular and Molecular Life Sciences 55(6-7): 901-909.

Way MJ, van Emden HF. 2000. Integrated pest management in practice - pathways towards successful application. Crop Protection 19: 81-103.

Weber J, Halsall CJ, Muir D, Teixeira C, Small J, Solomon K, et al. 2010. Endosulfan, a global pesticide: A review of its fate in the environment and occurrence in the Arctic. The Science of the Total Environment 408(15): 2966-2984.

Wilbur HM. 1980. Complex life cycles. Annual Review of Ecology and Systematics 11(6-7): 67-93.

Wyngaard GA, Chinnappa CC. 1982. General biology and cytology of cyclopoids. *In*: Harrison RW, Cowden RC (eds.), Developmental Biology Freshwater Invertebrates. Liss AR, NY, NY, 495-533.

Yu S, Wages MR, Cai Q, Maul JD, Cobb GP. 2013. Lethal and sublethal effects of three insecticides on two developmental stages of *Xenopus laevis* and comparison with other amphibians. Environmental Toxicology and Chemistry / SETAC 32(9): 2056-2064.

Zoeller RT, Brown TR, Doan LL, Gore AC, Skakkebaek NE, Soto AM, et al. 2012. Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. Endocrinology 153(9): 4097-4110.

APPENDIX: IACUC APPROVAL LETTER

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

July 22, 2013

Krista McCoy, Ph.D.
Department of Biology
Howell Science Building
East Carolina University

Dear Dr. McCoy:

Your Animal Use Protocol entitled, "Understanding the Effects of Endocrine Disrupting Chemicals Across the Amphibian Lifecycle" (AUP #D293) was reviewed by this institution's Animal Care and Use Committee on 7/22/13. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure