EVIDENCE OF ENDOCRINE DISRUPTION IN AMPHIBIANS DUE TO AGRICULTURAL CHEMICAL EXPOSURE

By

MARISOL MARIA GUTIERREZ

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ABSTRACT OF THE DISSERTATION

Evidence of Endocrine Disruption in Amphibians
Due to Agricultural Chemical Exposure

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Dissertation Director:
Professor Mark G. Robson

It is hypothesized that atrazine acts as an endocrine disruptor in amphibians, targeting male reproduction. To determine the impact of environmental atrazine on amphibians, we undertook a comprehensive study utilizing field and laboratory experimentation. In field studies utilizing *Rana catesbeiana* and *R. clamitans melanota*, we found evidence that secondary sexual traits are altered by environmental contamination. We also found that testes weights in both bullfrogs and green frogs were reduced at sites with chemical contamination compared to reference sites. At the histological level, renal and testicular dysgenesis were more prevalent at medium-contaminated sites than low or high. Ovotestes occurred most frequently (16.7%) at a site containing atrazine. The frequencies of renal parasites increased, while metacercarial cysts decreased with site contamination. Prevalence of hepatic and renal inflammation was higher in captures from medium and high contaminated sites compared to low. Bullfrog females from high

contamination sites had reduced hematocrit than low and medium sites. In our field locations, there was evidence of endocrine disruption in frog populations. However, atrazine did not correlate well with observed effects; methoxychlor and metolachlor were associated with various endpoints.

To further identify the role of atrazine in developmental effects, we exposed *Xenopus laevis* tadpoles to environmental atrazine concentrations (0, 1, 5, 20, 60, 120 ppb) during their larval period. Many endpoints examined presented with non-monotonic responses. Tadpole survival was reduced only at 5 ppb atrazine. At 5 and 60 ppb atrazine, tadpoles reached metamorphosis earlier than controls. Atrazine concentrations 5, 20 and 60 ppb yielded smaller metamorphs than controls, and metamorphs from 5 ppb atrazine also had shorter body lengths, limbs and abdominal girth. Malformations of the spine and limbs were highest at 5 and 20 ppb atrazine. We also observed a lowest observable adverse effect level (LOAEL) of 1 ppb atrazine for testicular dysgenesis, which is within environmental levels and supports concerns regarding the herbicide’s ability to hinder amphibian reproductive output. Overall, our research suggests that low environmentally relevant doses of atrazine have the ability to impact wild amphibians by altering endocrine processes.

Dedication

In Your name, Father, I dedicate this work in thanksgiving to the family You have given me: Mom (Yolanda), Dad (Arnaldo), Lizzette, Arnaldo, Madeline, Ciara, Coby and Richard. I ask for continual blessings upon my family through whom You have blessed me. Amen.

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Table of Contents

List of Tables

List of Figures

Introduction

Chapter 1

Global amphibian loss

Many species of amphibians are undergoing population declines across a variety of habitats worldwide. Since 1980, 113 (~2%) amphibian species are believed to have gone extinct (Hayes 2005), with 48% of amphibian species in decline (Alford and Richards 1999; Stuart et al. 2004). An ecological decline is characterized as the state where the loss of populations across the normal range of a species exceeds the rate at which populations of that species can be established, resulting in a sustained downward trend in population numbers (Green 1997). This trend has been most felt by the ranid frogs which have experienced the greatest extent of decline (Sparling et al. 2000). What is puzzling is that some declines are occurring in relatively pristine environments and within national parks, and that some species are declining alongside healthy ones (Sparling et al. 2000).

These amphibian declines have occurred on almost every continent. In North America, declines have occurred in the mostly agricultural and urban areas of Canada and the western United States, as exemplified by the decline of the once

abundant northern leopard frog, ***Rana pipiens*** (Hecnar and M'Closkey 1996). However, declines have also been reported in protected habitats, as in the case of the foothill yellow-legged frog, ***Rana boylii***, of the Sierra Nevadas (Drost and Fellers 1996). In South America, declines have been most pronounced in pristine areas. In the Monteverde Cloud Forest of Costa Rica, half of the native species have disappeared, most notably the golden toad (***Bufo periglenes***) and the harlequin frog (***Atelopus varius***), while others have been reduced in number (Pounds and Crump 1994). In Las Tablas, another protected region of Costa Rica, the loss or reduction of seven anuran species has been observed (Lips 1998). Even in Puerto Rico, known for its coquís (or "little frogs"), several species of the much beloved frog have undergone decline or extinction (Woolbright 1997). In Australia, the epidemic has predominantly affected frogs dwelling in streams and high elevations, including the unique gastric brooding frog, ***Rheobatrachus silus***, which appears to have been extirpated (Laurance et al. 1996).

Though most reports of amphibian losses have been from North America, South America and Australia, these only reflect the large number of surveys that have taken place in those areas (Sparling et al. 2000). Europe, Asia and Africa are not immune to amphibian declines, but surveys have been few and those

surveys are not well documented. Even so, European declines have been described for both the common toad (*Bufo bufo*) and the common frog (*Rana temporaria*) as well as other anurans and salamanders (Sparling et al. 2000). While amphibians are not the only vertebrates experiencing declines, anthropogenic habitat changes are thought to be the main causes in the declines of other wildlife (Sparling et al. 2000). Even so, amphibian declines are greater than those among mammals or birds (Hayes 2005). In the case of amphibians, habitat alterations are also responsible for many of the population declines (Blaustein and Wake 1995); however, other factors that are both anthropogenic and natural have been observed and/or proposed as playing roles in the wide spread population losses of amphibians (Sparling et al. 2000).

Amphibians as environmental sentinels

Amphibians are abundant, residing in a diverse range of habitats (Sparling et al. 2000). In addition, these animals are sensitive to their environment compared to other taxa (Sparling et al. 2000; Sanders 2002). Amphibians readily respond to changes in their habitat, including changes that are physical, chemical and biotic, particularly if hormonal effects are induced (Veldhoen and Helbing 2001). This sensitivity exists due to their moist permeable skin, gills and eggs lacking

protective coverings (Blaustein and Wake 1995; Clark et al. 1998; Howe et al. 1998; Lips 1998; Pickford and Morris 1999; Mattoon 2000; Rohr et al. 2003; Storrs and Kiesecker 2004). This morphology leaves amphibians exposed to soil, water and air, from which substances are readily absorbed (Blaustein and Wake 1995; Howe et al. 1998; Hatch and Blaustein 2000; Mattoon 2000; Blaustein and Kiesecker 2002; Roy 2002). The permeability of their skin could mean that levels of contaminants within the frog match those of its environment (Sparling et al. 2000). Furthermore, because amphibians display high habitat fidelity with limited ranges, it can be expected that amphibians present in contaminated mediums have experienced life time exposures. Following dispersal events, when newly metamorphed individuals move out form the breeding waters, frogs establish a territory and remain there (Martof 1953). Amphibians display high site fidelity because of their need for water. Frogs cannot tolerate much water loss (Martof 1953), and therefore, do not go far from permanent bodies of water with a typical home range of a couple meters (Currie and Bellis 1969). Ranges are also limited because breeding, egg development, foraging and hibernation are all tied to the habitat (Sparling et al. 2000).

Due to amphibian life history, egg to larvae to adult, each life stage is a separate entity in respect to its interaction with

the environment (Berrill et al. 1993). The biphasic life style (aquatic as larvae, terrestrial or semiaquatic as adults) presents distinct opportunities for direct and indirect chemical effects, which results in varying response and effect patterns to chemical exposures (Saber and Dunson 1978; Cooke 1981; Duellman and Trueb 1994). The embryo is the most sensitive life stage as eggs are typically laid atop surface waters (for most frog species) in a gelatinous mass without any parental care (Lips 1998). In this state, frog embryos cannot avoid stressors/chemicals in their environment. The jelly coating may provide some protection from mechanical stressors (like temperature fluctuations and mild vibrations in the water); however, egg masses swell once laid as the jelly coating imbibes water (Sparling et al. 2000). This flux into the egg mass can potentially increase exposure levels. Second in sensitivity to chemical or physical harm are developing larvae, which encounter environmental insults without the benefit of full immune defenses or metabolizing enzymes (Sparling et al. 2000). Post-metamorphs are more susceptible to chemical toxicity than larger adults due to their larger surface to volume ratio, higher uptake rates and incomplete immune system development (Carey and Bryant 1995).

Because of their interactions with the environment, amphibians are at increased risk to experience multiple stressors and the synergistic and/or additive effects multiple stressors bring (Sparling et al. 2000). With greatly vascularized and highly porous skin, frogs are most likely to dermally absorb lipophilic compounds (Datta et al. 1998) even from the air (Barinaga 1990). Another reason amphibians are so sensitive is that chemicals are able to enter the bloodstream and reach target organs without first passing through the liver and undergoing first pass metabolism (Sohoni et al. 2001). These characteristics put amphibians at high risk for exposure to endocrine-disrupting chemicals (Mackenzie et al. 2003).

This risk of exposure also exists because the main environmental sinks for these compounds are where amphibians reside, wetland habitats (Cohen Jr. 2001), due to chemical accumulation and distribution in sediments (Sower et al. 2000; Reeder et al. 2005). Consequently, sediments act as both a sink for and a source of contamination (Burkhart et al. 2000). Additionally, the residence times for these compounds are likely to be long due to slow degradation in sediments (Johannesen and Aamand 2003). This phenomenon makes aquatic vertebrates, like amphibians, the most susceptible organisms (Storrs and Kiesecker 2004) and therefore the most appropriate in which to study

endocrine disruption effects (Iguchi et al. 2001; Kloas 2002). Furthermore, amphibians are consistently more sensitive to toxicants in general compared to fish species typically used in exposure studies (Howe et al. 1998). The presence of agro-chemicals in the aquatic environment gives these endocrine disruptors the potential to impact the health of humans as well as wildlife (Palmer et al. 1998).

Amphibians are particularly important to study as their decline can impact other organisms. Amphibians provide a significant contribution to an ecosystem's biomass (Clark et al. 1998) and they also occupy a critical place in the food chain (Barinaga 1990). Amphibians contribute greatly to trophic dynamics in many aquatic and terrestrial ecosystems as important predators and prey (Blaustein and Wake 1995; Blaustein and Kiesecker 2002; Reeder et al. 2005). This trophic input includes tadpoles that as herbivores act to transport the aquatic plant biomass of an ecosystem to the upper levels of the food chain through predation. Accordingly, contaminants that affect amphibians, either directly or through prey items, will in turn affect their predators, which includes bioconcentration of chemicals, since amphibians serve as conduits for contaminants and connect the aquatic and terrestrial habitats (Sparling et al. 2000). As a result of the importance that amphibians have to ecosystems,

their reduction or loss would have consequences for the rest of the ecological community (Blaustein and Kiesecker 2002).

With close contact to both land as adults (for most species) and water as larvae, amphibians experience both aquatic, terrestrial and air stressors (Noriega and Hayes 2000), making these animals important indicators of ecological integrity (Reeder et al. 2005). For this reason, amphibians appropriately serve as early sentinels of significant environmental changes and/or decline (Roy 2002) that have the potential to impact human, as well as environmental, health (Carey 2000; Kiesecker 2002; Bails 2005), which are integrally connected (Kendall and Smith 2003).

Implicated factors in amphibian declines

The drop in amphibian diversity is thought to be atypical because it is occurring among a variety of species with different reproductive strategies and life histories (Lips 1998). Moreover, amphibian declines are characterized by sudden coincidental declines of several species over large areas (Wake 1991). The probability of losing such a large number of populations in this short time period over these wide distances is extremely small (Pounds et al. 1997). Houlahan et al. (2000) looked at 936 populations between the years 1950 to the late 1990s and demonstrated steady declines with periods of rapid

decline coinciding with industrialization. Consequently, the declines are due to something other than natural variation, though population fluctuation certainly plays a role (Wake 1991). A typical ranid population cycle is three to five years (Pauley and Lannoo 2006). A number of factors have been identified as possible explanations in these global declines and can be categorized as either direct habitat alteration, global anthropogenic influences or natural causes.

Habitat alteration includes fragmentation, deforestation (Blaustein et al. 1994c; Blaustein and Wake 1995), simplification (e.g., drainage of wetlands in the Midwest for recreational fisheries), changes in water use, and outright destruction for industrial, urban and/or agricultural development (Sparling et al. 2000). For example, Appalachian timber harvesting has resulted in annual losses of salamanders, most notably Jordan's red-cheeked salamander, ***Plethodon jordani*** (Petranka et al. 1993). In addition, introduction of non-native species negatively alters ecosystems (Drost and Fellers 1996). Bullfrogs (***Rana catesbeiana***) introduced to the western United States prey on other frogs and have contributed to declines in leopard frogs, ***Rana pipiens*** of Arizona and California red-legged frogs, ***Rana aurora draytonii*** (Hayes and Jennings 1986). Game fish stocked in ponds and lakes are predators to all stages of

amphibians (Semlitsch and Gibbons 1988) and have been implicated in the declines of the mountain-yellow legged frog, ***Rana muscosa*** {Blaustein, 1995 #1}. Humans have exploited certain species as food sources and have contributed to the loss of ***Leptodactylus fallax*** (the giant ditch frog) populations (Sparling et al. 2000). These changes in habitat dramatically disrupt the balance of an ecosystem and its members, and in some cases displace populations.

A host of global factors contributing to amphibian declines can be attributed to human activity. Increased acid rain has led to the acidification of amphibian habitats (Hatch and Blaustein 2000). Environmental acidification can delay or arrest development and prevent hatching of embryos (Pough 1976), as well as alter sodium balance in larvae (Freda and Dunson 1984). In Europe, changes in habitat structure and the acidification of habitats have been implicated as the root causes of declines in the common frog, ***Rana temporaria*** (Sparling et al. 2000).

Chemical contamination of our water, soil and air has been ubiquitous due to industrial, urban and agricultural efforts, and has also been implicated in the disappearance of amphibians {Lips, 1998 #829;Reeder, 2005 #553;Relyea, 2005 #558;Hayes, 2006 #302;Russell, 1997 #870;Boone, 1999 #763;Blaustein, 1995 #1}.

Russell et al. (1997) demonstrated that high agricultural contamination in green frogs (*Rana clamitans melanota*) coincided with changes in population numbers. Herbicides specific for aquatic use, runoff and over spray from aerial applications of agricultural chemicals, the increased use of lawn products and the presence of pharmaceuticals all contribute to the complex milieu of exogenous agents experienced by amphibians in breeding waters (Sparling et al. 2000).

In addition, increased exposure to ultraviolet B (UVB) radiation has resulted from stratospheric ozone depletion (Blaustein et al. 1994c). UVB causes reduced embryo viability in certain species, like the Cascades frog (*Rana cascadae*) and western toad, *Bufo boreas* (Blaustein et al. 1994a; Blaustein et al. 1995). UVB produces mutagenic photoproducts which are repaired by the enzyme photolyase, an enzyme with reduced activities (by two orders of magnitude) in those species which had the greatest reduction in embryo hatching success when exposed to UVB {Blaustein, 1994 #47;Blaustein, 1995 #1;Kiesecker, 2001 #380}. UVB also displays synergistic interactions with habitat acidification and contamination, enhancing their toxicities (Hatch and Blaustein 2000).

Natural causes attributed to amphibian declines include global climate change and disease. As the Earth's temperature increases and the climate becomes drier, conditions for favorable amphibian development decrease (Lips 1998). With world climate change, extreme weather events like flash flooding, cold spells producing frost, and drought, become more frequent and can precipitate amphibian die offs (Heyer et al. 1988; Pounds and Crump 1994; Boone and Bridges 1999; Kiesecker et al. 2001). Both the golden toad (*Bufo periglenes*) and the harlequin frog (*Atelopus varius*) suddenly declined following unusually warm and dry conditions in Costa Rica (Pounds and Crump 1994). However, where declines due to extreme weather events occur, rebounds would be expected during times of normal conditions (Sparling et al. 2000). For example, following hurricane Hugo in 1989, the Puerto Rican coquí frog, *Eleutherodactylus coqui*, increased in numbers four fold (Woolbright 1991). Although the losses and declines in the Monteverde Cloud Forest seem to have also coincided with weather changes (increased temperature with decreased precipitation), only a few of the missing species (e.g., the glass frog, *Centrolenilla fleischmanni*) have come back as conditions returned to normal (Pounds and Crump 1994).

Another factor in declines by natural causes is the range of infections that lead to disease. Amphibian disease can result from any number of infectious agents which include viruses. Iridoviruses (Laurance et al. 1996), erythrocytic and similar viruses (Gruia-Gray and Desser 1992), and herpesviruses (Bennati et al. 1994) have all been isolated from wild amphibians and implicated in their declines. Laurance et al. (1996) implicate the Bohle virus as the causative agent in the declines of montane stream-dwelling amphibians in Australia, like the ornate burrowing frog (*Limnodynastes ornatus*). Disease may also be bacterial; red-leg disease results from infection by a collection of bacteria, most likely due to immune suppression following stress (Carey 1993). Other bacterial diseases include tuberculosis, caused by *Mycobacterium*, and chlamydia (Sparling et al. 2000). *Pseudomonas aeruginosa* has been shown to cause high mortality in leopard frogs, *Rana pipiens* (Pounds and Crump 1994). In addition to viruses and bacteria, amphibians are also susceptible to fungal infections. *Saprolegnia* is a fungus which has been linked to embryo mortality following UVB exposure in western toads, *Bufo boreas* (Blaustein et al. 1994b). This fungus is a secondary infection that is normally preceded by bacterial infestation (Sparling et al. 2000). *Saprolegnia* is not the only fungus that has been the subject of study. The chytrid fungus causes thickening of amphibian skin and prevents

dermal respiration (Berger et al. 1998). The chytrid fungus has been detected in histological sections of skin in dead green tree frogs (*Litoria caerulea*) from Queensland, Australia (Berger et al. 1998). While amphibians are hosts to a wide variety of parasites (protozoans, nematodes, cestodes, trematodes, etc.), these parasites may present an additional burden, but are rarely a cause of mortality (Sparling et al. 2000). However, the protozoan *Plistophora myotrophica* can cause mortality during warm conditions (Pounds and Crump 1994). Because the same infectious agents are being identified and associated with amphibian mortality at vastly separate locales, a global phenomenon is suspected (Carey 1993; Lips 1998; Kiesecker et al. 2001). However, increases in the virulence of infectious agents could be the result of stresses arising from previously discussed factors that weaken the frog's immune system (Carey 1993). Indeed, the declines at Las Tablas are thought to be due to a combination of habitat contamination and disease (Lips 1998).

While amphibian population declines may result from any one factor (particularly within a single geographical area), a complex combination is a more probable explanation. Combinations include environmental conditions and other factors, like disease, acting synergistically to bring about population

declines. These factor combinations are not consistent and/or predictable, but nonetheless act in concert to result in population losses. Consequently, local factors, such as agricultural chemical contamination, can interact with other influences in quite complex ways. Therefore, when studying amphibian (or any ecological) decline, it is very difficult to identify individual effects of different and overlapping causes.

Habitat alteration is by far the primary cause of amphibian declines; however, the indirect and subtle effects of less obvious influences may pose greater threats to long-term survival of amphibians (Sparling et al. 2000). For instance, the most pronounced losses have been in aquatic or semi-aquatic species, those with extended aquatic larval development, and species which retain close ties to water bodies throughout their life stages (Pounds and Crump 1994; Drost and Fellers 1996; Laurance et al. 1996). These trends would indicate that there is a global influence present in the water habitat of these animals. Furthermore, ranid population declines have been most severe in urban and agricultural areas (Sparling et al. 2000). Davidson et al. (2002) found a strong association between amphibian declines in the Sierra Nevadas and upwind agricultural use in the central valley of California. Additionally, pesticides have been found in Sierra Nevada frog tissues (Ribick

et al. 1982). Therefore, amphibian losses may reflect a global decline in environmental quality associated with environmental contamination (Russell et al. 1997; Clark et al. 1998; Howe et al. 1998; Burkhart et al. 2000; Davidson et al. 2002).

Acute toxicity in amphibians due to pesticide exposure has been extensively studied (Hall and Kolbe 1980; Hall and Swineford 1981; Feng et al. 2004). In contrast, studies on the low-level effects of these compounds have been largely ignored. These doses are of interest because low dose exposures are what amphibians encounter in their environment. Within this range of exposure, pesticides may exert endocrine disruptive effects. These endocrine disruptors elicit responses on their own and also interact with other stressors encountered in the environment (Hayes 2005). These exposures may have larger influences on amphibian populations than hypothesized due to lack of investigation.

Metamorphosis is the best studied endocrine-directed process in amphibians, and these studies demonstrate that all changes are stimulated by thyroid hormones, while corticoids and sex steroids affect the timing of metamorphosis (Sparling et al. 2000). An endocrine-disrupting chemical is any exogenous agent with the potential to interfere with an animal's normal

endocrine signaling and communication mechanisms (Figure 1.1), ultimately leading to an altered cellular response (Palmer et al. 1998; Chen 2001). Endocrine disruptors typically interfere with sex steroid and thyroid hormone actions, which direct several important developmental and physiological processes in the amphibian (Sparling et al. 2000). Sex steroids mediate the development and function of the reproduction system, including development of secondary sex characteristics (Crain et al. 1997). Therefore, endocrine disruptors have the ability to alter reproductive events and primary sex ratios (Akingbemi and Hardy 2001). These events include gender determination of the gonads and brain during development, which in turn affect response activation, both endocrine and behavioral, during sexual maturation (Noriega et al. 1997). Any altered response potentially causes changes in function and/or structure of biological systems, resulting in adverse effects such as altered reproductive capacity and infertility (Bigsby et al. 1999). This interference can be caused by a chemical's ability to mimic, modulate or antagonize the activity of a particular system (Welshons et al. 2003) by affecting the synthesis, secretion, transport, binding or elimination of natural hormones responsible for homeostasis, reproduction, development and/or behavior (ATSDR 2003). Generally, the effects of endocrine disruptors are normal events that are either induced in the

wrong sex or at the wrong time (Sparling et al. 2000). Since amphibians employ diverse reproductive strategies, the role of regulating hormones (estrogens, prolactin and progesterone) will differ with the species being investigated (Sparling et al. 2000). Endocrine disruption from anthropogenic sources has been implicated as a contributing factor in population declines and/or disappearances in frogs and other species like birds and fish (Noriega and Hayes 2000; Watts et al. 2001). For example, Reeder et al. (2005) found marked reductions in cricket frog numbers in the most industrialized and urbanized portions of Illinois with male-skewed sex ratios.

Atrazine's potential role in amphibian declines

Herbicides comprise about 80% of all pesticide use worldwide (Pennington et al. 2001) and atrazine is one of the most widely utilized herbicides (Solomon et al. 1996; Müller et al. 1997; Miller et al. 2000). Wild frog populations are a non-target species in a unique position for exposure to atrazine as their spawning season coincides with peak atrazine applications and spring rains (Withgott 2002; Rohr et al. 2003). Atrazine may enter amphibian breeding sites via direct aerial spraying, aerial drift, postapplication volatilization and/or erosion, but terrestrial runoff is the dominant route of aquatic exposure (LeNoir et al. 1999; Relyea and Mills 2001; Coady et al. 2004;

Giddings et al. 2005). In the United States, 60% of atrazine application occurs in the Midwest, which receives high levels of rainfall during the planting season (Giddings et al. 2005). Figure 1.2 illustrates the distributions of both atrazine use and rainfall across the United States. Atrazine is applied early in the season and it is estimated that 2-5% of applied atrazine is lost through runoff events (Seybold et al. 1999), exposing frogs (and other aquatic wildlife) to elevated doses of atrazine (Coady et al. 2004). Figure 1.3 identifies those areas of the country vulnerable to atrazine runoff events based on the herbicide's use and precipitation patterns (Giddings et al. 2005). As a result of timing, surface water concentrations of atrazine are at their highest during this period, ranging from 5 ppb to 1 ppm (Detenbeck et al. 1996). Consequently, frogs are getting sufficient doses of atrazine during breeding and larval development to result in adverse effects (Renner 2002). While typical environmental concentrations of atrazine may not cause mortality or changes in developmental rates in amphibians (Renner 2002), they may contribute to population declines by affecting the reproductive capacity, and therefore the fitness, of an exposed population (Crain et al. 1997; Kendall and Smith 2003). It is these sublethal effects, possibly through endocrine interference, that may induce critical changes in the survival of a population (Sparling et al. 2000).

Use and properties of atrazine

Atrazine was patented in Switzerland by Geigy Agricultural Chemicals in 1958 and registered for commercial use in the United States in 1959 (Kaufman and Kearney 1970; Giddings et al. 2005). The herbicide is used world-wide under more than thirty trade names, including Aatrex, Candex, Geigy 30027, Primaze and Weedex A (Markwell and Namuth 2003). Atrazine (see Figure 1.4 for structure of atrazine and metabolites) is one of the most commonly used herbicides in the United States (Weiss 2004; Giddings et al. 2005; Rohr et al. 2006), particularly in the Midwest (Coady et al. 2004), with an annual usage of over 60 million pounds (1993, Sanderson et al. 2001; Renner 2002; Yoon 2002). Though atrazine's use patterns vary from crop to crop, it is usually applied as a pre-emergent agent (pre-plant and post-emergent applications are also used) on principal row crops like corn, sorghum and sugarcane, as well as on pineapples, macadamia nuts, evergreens, and highways and railways to control annual broadleaf and grass weeds (ATSDR 2003; Coady et al. 2004; Giddings et al. 2005). The majority of atrazine use (85%) in the United States is on corn, again 60% of which is applied in the Midwest alone (Giddings et al. 2005). Total atrazine use in the United States on sorghum is 9%, a major crop in Kansas and

Texas, while Florida and Louisiana utilize 3% of U.S. atrazine on sugarcane (Giddings et al. 2005).

Susceptible weeds, such as chickweed, cocklebur, foxtails, mustards and ragweed (Davidson and University 1995), are exposed to applied atrazine mainly through roots and foliage, as well as across cell surfaces (Giddings et al. 2005). As Figure 1.5 illustrates, once atrazine enters the cell, it blocks the electron transport of photosystem II by competing with plastoquinone II binding (Markwell and Namuth 2003; Giddings et al. 2005). The result is inhibition of carbohydrate synthesis, reduction of the carbon pool and a build up of carbon dioxide that damages chlorophyll molecules via oxidative stress and radical formation (Markwell and Namuth 2003; Giddings et al. 2005).

Crops are tolerant of atrazine action because they are able to biochemically convert the herbicide to non-toxic molecules, while weeds cannot (Sterling et al. 2006). In the roots of atrazine-resistant plants (Figure 1.6) and in the presence of DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one acid, Bailey et al. 1988), a natural product, and water, a nucleophilic substitution of a hydroxyl group for the chloride atom occurs at position two on the triazine ring yielding

hydroxyatrazine (Sterling et al. 2006). Compared to the parent compound, hydroxyatrazine is less toxic and has greater affinity for conjugation with glutathione, which can then be sequestered in the cell's vacuole and eventually incorporated into the cell wall (Markwell and Namuth 2003; Sterling et al. 2006). Since these metabolic pathways are only found in plants, it was hypothesized that atrazine would not be toxic to animals (Sparling et al. 2000; Giddings et al. 2005). However, although the components differ, electron transport chains do exist in animals and may potentially be a target for atrazine action.

Properties

Atrazine is a weak base with a pKa of 1.68, moderate water solubility of 33 mg/L, low vapor pressure (2.89 x 10^{-7} mm Hg) and a low Henry's law constant (2.48 x 10-9 atm·m3/mol, TOXNET; ATSDR 2003; Johannesen and Aamand 2003; Giddings et al. 2005). (Table 1.1 lists additional properties of atrazine.) With the low vapor pressure and Henry's law constant, volatilization from either soil or water is negligible (Giddings et al. 2005). Due to atrazine's moderate water solubility and its relatively low K_d (3.08 mL/g) and K_{oc} (170.68), the herbicide will move in surface or subsurface waters, especially in the dissolved state, during irrigation and/or rainfall because it does not adsorb strongly to sediments (Giddings et al. 2005).

Atrazine is relatively stable, its *S*-triazine ring renders it resistant to both biotic and abiotic breakdown (Howe et al. 1998; Zhang et al. 2003), including microbial breakdown (Kaufman and Kearney 1970; Solomon et al. 1996; Seybold et al. 1999). However, atrazine can undergo environmental transformation, the predominant pathway for degradation being microbial metabolism with products containing the triazine ring (Giddings et al. 2005). Chemical degradation of the herbicide occurs via hydrolysis at carbon two, N-dealkylation at carbon four or six (Shimabukuro 1967; Huber 1993; Du Preez et al. 2005) and splitting of the triazine ring (Kaufman and Kearney 1970; Giddings et al. 2005). The hydrolysis of atrazine produces hydroxyatrazine (Figure 1.7), the most frequent metabolite present in the field (Giddings et al. 2005). Figure 1.8 shows that N-dealkylation produces either deethylatrazine (at carbon 4) or deisopropylatrazine (at carbon 6), and didealkylatrazine if both reactions occur. Although cleavage of the triazine ring may occur following hydrolysis (Figure 1.9), it is limited and not considered a major pathway of degradation (Kaufman and Kearney 1970). Hydroxyatrazine is the least mobile of the metabolites because it binds to suspended particulate matter and is therefore the most frequent environmental atrazine transformation product, while diethylatrazine is more mobile

than its parent compound (Giddings et al. 2005). In Figure 1.10, the maximum concentrations of atrazine metabolites over time under aqueous photolysis are shown as a percentage of the parent compound.

Half-life

Half-life estimates for atrazine are highly variable due to the influences of environmental conditions like temperature, pH and medium type (Giddings et al. 2005). For example, as temperature increases atrazine's half-life shortens and it begins to decompose at temperatures above 70°C (TOXNET). Between pH 5 to 9, atrazine is stable with little change after 30 days. However, when the pH falls below 4 atrazine's half-life decreases to just days due to increased hydrolysis (Giddings et al. 2005). This shorter half-life indicates that hydrolysis can be catalyzed, and this transformation occurs in either strong acids or bases (TOXNET). The average half-life of the herbicide under aerobic metabolism is 44 days (20-146 day range), while the average half-life in anaerobic soil and sediment is 228 days (58-547 day range, Giddings et al. 2005). Aerobic aquatic degradation of atrazine averages 159 days (41-237 day range), while atrazine's half-life in water is between three days and five years (Solomon et al. 1996; Schottler and Eisenreich 1997; Diana et al. 2000; Relyea and Mills 2001; ATSDR 2003; Coady et

al. 2004; Giddings et al. 2005). Total atrazine dissipation from the field (encompassing all routes of disappearance: degradation, soil sorption, volatilization and leaching) occurs at a half-life between 8.2 and 99 days (average of 39.2 days, Giddings et al. 2005). Atrazine breaks down in soil in generally one growing season, most likely due to its mobility, but is slower in river and lake surface waters and is persistent in groundwater (ATSDR 2003).

Due to its heavy use, mobility (Rohr et al. 2006), moderate solubility (Zhang et al. 2003) and stability (Diana et al. 2000), atrazine is the most prevalent herbicide found in the aquatic environment (Kadoum and Mock 1978; Goolsby et al. 1997; Sparling et al. 2000; Friedmann 2002; Rohr et al. 2003; Rohr et al. 2006). Atrazine residues are also frequently found in drinking water (Graebing et al. 2003), necessitating its ban in some countries (Dalton 2002; Sanders 2002). In fact, the European Union banned atrazine use in 2005 (Weiss 2004) based on human health concerns (Marcus 2002). Syngenta Crop Protection (formerly Ciba-Geigy Corporation and Norvartis Crop Protection), atrazine's principle manufacturer (Giddings et al. 2005), replaced the banned herbicide with terbuthylazine (Green 2003), the structure of which can be found in Figure 1.11. In contrast, in 2003 the United States Environmental Protection

Agency (US EPA) renewed atrazine as a restricted-use pesticide with no additional restrictions.

Environmental persistence

Because of its widespread (Sanders 2002; Rohr et al. 2006) and persistent use (applied annually at relatively consistent times and quantities), trace atrazine concentrations can be maintained or even increased over time. Atrazine trace concentrations are slowly increasing in the Great Lakes due to its long half-life (2-5 years) in these bodies of water (Schottler and Eisenreich 1997). Many agrochemicals persist in the environment (Lips 1998), a phenomenon of particular concern since small shallow volume systems (where concentrations become disproportionately large) are where amphibians tend to breed (Howe et al. 1998; Renner 2005). In salt marshes, detectable levels of atrazine persisted 71 days after application, indicating atrazine has the potential for long term residence in the environment (Giddings et al. 2005). Atrazine has also been shown to persist in rivers and streams of the midwestern United States with spring concentrations five to twenty times higher than the US EPA maximum contamination level of 3 ppb (Thurman et al. 1992; Rohr et al. 2006). The herbicide can be routinely found at 50 ppb (typically 15 to 75 ppb, Detenbeck et al. 1996) in the Midwest, but levels can reach as high as 200 ppb in streams, 500 ppb in

ponds (Rohr et al. 2006), and even several ppm (40 mg/L, Sanders 2002) in agricultural runoff (Howe et al. 1998; Dalton 2002; Sanders 2002). Generally, atrazine is not found at concentrations greater than 20 ppb (six times the EPA standard), but can persist at low ppb for long periods. For example, Hayes et al. (2003) detected 0.1 to 6.7 ppb atrazine in various Midwest breeding ponds in mid-to-late July. In addition, Pennington et al. (2001) found 2 ppb atrazine in Texas estuaries with an average concentration of 0.92 ppb atrazine for the year.

Global impact

Pesticides, like atrazine, are used locally, but are readily spread regionally and even globally through atmospheric deposition (Sparling et al. 2000; Storrs and Kiesecker 2004). In this manner, pesticides may potentially harm organisms in remote, relatively pristine environments (Rice and Chernyak 1997; Datta et al. 1998; LeNoir et al. 1999). Atmospheric deposition renders no habitat, including mountain areas, free of anthropogenic compounds (Wake 1991; Drost and Fellers 1996). As chemicals vaporize, they are transported through the atmosphere globally to other areas (Lips 1998). Pesticides have been detected in precipitation (Goolsby et al. 1997; McConnell et al. 1998), which acquires chemicals through flux into the atmosphere from farmed lands (Mattoon 2000; ATSDR 2003). Atrazine has been

found as high as 40 ppb in rain near agricultural areas (Storrs and Kiesecker 2004; Rohr et al. 2006). Such high concentrations would result when a rain event immediately follows application and atrazine sorbed to particles in the air is flushed from the atmosphere (Nations and Hallberg 1992). The herbicide has also been detected at 1 ppb in rainwater in areas where it is not regularly used (Roberts 2006). Furthermore, through surface interactions, pesticides can become concentrated in fog, sorbed to particulates (Rice and Chernyak 1997; ATSDR 2003). This atmospheric deposition phenomenon is not limited to spring and summer when chemicals are typically used; wintertime precipitation has also been shown to contain pesticides (LeNoir et al. 1999). In Lake Superior, 95% of the atrazine present is reported to arise from atmospheric inputs (Schottler and Eisenreich 1997). Once in the atmosphere, such persistent compounds may travel great distances through air currents before deposition into water or onto land (Goolsby et al. 1997). This transport includes chemicals carried to arctic regions where residues can become trapped, allowing them to reach high concentrations in marine mammals and fish (Gregor and Gummer 1989; Rice and Chernyak 1997; McConnell et al. 1998).

Though the concentrations resulting from air transportation are typically low (though atrazine concentrations as high as 21 ppb

have been detected in ground waters considered pristine), they may still impair developing organisms, like amphibian larvae, under certain conditions (Drost and Fellers 1996; Blaustein and Kiesecker 2002). Pesticides have been found in frogs from areas lacking historic pesticide use (Storrs and Kiesecker 2004), areas unprotected from airborne contaminants (Lips 1998). Thus, atrazine can contribute to the degradation of ecosystems that are remote to application areas (LeNoir et al. 1999). These facts, coupled with atrazine's use in over 80 countries, make this issue important for both environmental and public health.

Proposed ecological effects of atrazine

Atrazine has been shown to accumulate in fatty tissues (Khan and Foster 1976; Takai et al. 2000; Sanderson et al. 2001), as well as in tadpoles which store lipids (Allran and Karasov 2000). Lipophilic chemicals may become sequestered in the tadpole only to become mobilized and redistributed during the process of metamorphosis (Honrubia et al. 1993). Compounds that reside in tissues are the ones with the greatest potential to disrupt endocrine function (Noriega and Hayes 2000). In addition, lipid-soluble compounds can be transferred to offspring through deposition into egg yolk (Metcalfe et al. 2000). Although atrazine is not expected to bioconcentrate or biomagnify because of its low K_{ow} and susceptibility to metabolism (Giddings et al.

2005), approximately 24 hours following exposure only 1-2% of the atrazine dose experienced by manufacturer workers was excreted in urine (Catenacci et al. 1993). Of the excreted products, the majority were atrazine metabolites (80% of which was didealkylatrazine) with only 2% unmetabolized atrazine (Catenacci et al. 1993). The major pathway for atrazine metabolism in humans is via cytochrome P_{450} 1A2, which oxidizes atrazine at either alkyl group (Lang et al. 1997). This study shows that atrazine has the potential to reside long-term in biological tissues.

Though not overtly toxic at typical environmentally relevant concentrations (Eldridge et al. 1999a), atrazine may disrupt critical endocrine mechanisms, like steroid hormone balance, and do so during a sensitive developmental period in amphibians (Renner 2002). Such exposures would lead to a variety of developmental and reproductive anomalies (Akingbemi and Hardy 2001). These effects include reduced survival (Storrs and Kiesecker 2004), reduction in testosterone levels (Hayes et al. 2002b) and injury to the testis (Tavera-Mendoza et al. 2002a; Hayes et al. 2003). Exposure to endocrine-disrupting chemicals at critical life stages could lead to permanent, adverse effects to the reproductive potential and fitness of any species (Veldhoen and Helbing 2001; Schönfelder et al. 2002a). However,

it is unclear if these effects result in reduced fertility, contributing to population declines (Clark et al. 1998). The adverse consequences of hormonally active agents have been demonstrated during development (Delclos et al. 2001) as differentiating tissues are most vulnerable to changes in its cellular environment, particularly its hormone content (Akingbemi and Hardy 2001). Therefore, concern over the adverse effects of atrazine arises because development, the function of both male and female reproductive tracts and their regulation by hormones (Danzo 1997), are conserved among vertebrates (Akingbemi and Hardy 2001; Cohen Jr. 2001; Veldhoen and Helbing 2001; Tavera-Mendoza et al. 2002a), giving research in amphibians far-reaching application. Therefore, amphibians are a great model for development, the role of the endocrine system in development and the impact of endocrine disruptors on development. Though evolutionarily distant, humans and amphibians are closely related as to their biochemical, metabolic, physiological, embryological and developmental processes (Sparling et al. 2000). Therefore, disruption of development and endocrine function in wildlife may indicate a danger of the same in humans (Colborn et al. 1993; LeBlanc and Bain 1997). Indeed, the tadpole is considered the classic vertebrate model for hormone action during development, due to the important role hormones play during metamorphosis (Denver et

al. 1997) and because their endocrine system is similar to most vertebrates (Hayes 1997). Furthermore, during this period of extensive remodeling, the effects of hormones (and hormone interference) can be correlated with functional changes in development (Denver et al. 1997).

Mammalian toxicity

Rodent literature

There exists a substantial body of literature in rodents detailing the adverse effects of atrazine, a documented endocrine disruptor (Rohr et al. 2006), now classified as an endocrine modulator (MacLennan et al. 2003). LD_{50}s in rats are as follows: 1471-1212 mg/kg in males, 737-672 mg/kg in females and 2310 mg/kg in young males (ATSDR 2003). Treatment with atrazine in rat model systems has yielded reproductive effects in both the male and female. These effects include antiandrogenic reduction of testosterone hormone levels. Friedmann (2002) reported a 50% reduction in testosterone serum and intratesticular levels following 50 ppm atrazine exposure to juvenile male rats by gavage. These results are similar to those shown by Trentacoste et al. (2001) who saw declines in the hormone following 100 and 200 ppm atrazine. Sperm number and motility have been shown to decrease following intraperitoneal injections of 60 and 120 ppm atrazine to adult male Fischer rats

(Kniewald et al. 2000). Histologically, cells of the testis were disorganized with irregular Leydig cells and vacuolated cytoplasm (Kniewald et al. 2000). Stoker et al. (2000) found dose-dependent increases in estrone and estradiol following gavage administration of atrazine. In addition, 25 and 50 ppm atrazine resulted in prostatitis when administered prior to postnatal day 9 (Stoker et al. 1999). It has also been shown that 5α-reductase (the enzyme that converts testosterone to dihydrotestosterone) activity declined 34% after a single subcutaneous dose of 1 ppm atrazine (Kniewald et al. 1979; Kniewald et al. 1987; Babic-Gojmerac et al. 1989). Researchers have shown the inhibition of 5α-reductase to be noncompetitive and reversible (Kniewald et al. 1995). Reduction in 5α-reductase activity and change in steroid secretion indicate a delay in the gonadotropic system's maturation process (Kniewald et al. 1987; Stoker et al. 2000). Changes in tissue weights have been contradictory, even within the same strain. Kniewald et al. (2000) showed decreased pituitary and prostate weights in male Fischer adults following 60 and 120 ppm atrazine administration. Similar results have been shown in Wistar rats with 50-200 ppm atrazine (Stoker et al. 2000). However, others have shown transient increased pituitary and prostate weights in Fischer rats with reports of pituitary hypertrophy (Babic-Gojmerac et al. 1989; Simic et al. 1994). These studies were

done in postnatal rats and the reversibility of effects only refers to exposure at this time and is not necessarily characteristic of exposure consequences earlier in development, such as during gestation.

In female rats disruptions in puberty have also been seen with delayed vaginal opening following postnatal 200 ppm atrazine exposure of Wistar female rats (Laws et al. 2000), with similar results using atrazine byproducts didealkylatrazine and hydroxyatrazine (Laws et al. 2003). Atrazine has been reported to disrupt the estrus cycle with prolonged estrus in some strains, Fischer 344, Long Evans hooded and Sprague-Dawley (Eldridge et al. 1994a; Wetzel et al. 1994; Cooper et al. 1996; Eldridge et al. 1999a) and extended diestrus in others, Wistar (Simic et al. 1994; Laws et al. 2000). Hormone analyses have shown increased estradiol, decreased progesterone and an inhibition of the luteinizing hormone prolactin surge, altering the estrus cycle (Eldridge et al. 1994b; Cooper et al. 2000; Cummings et al. 2000). This disruption of the ovarian cycle has led to reduced mating success (Simic et al. 1994). Other reproductive effects include regressed ovaries (300 ppm atrazine, Cooper et al. 1996) and reduced ovarian and uterine weight (Eldridge et al. 1994a). Cummings et al. (2000) has shown increased preimplantation loss in pregnant Fischer 344

rats, and increased postimplantation loss in Holtzman rats following gavage administration of 100 and 200 ppm atrazine. In addition, increases in uterine carcinomas, mammary and pituitary tumors, lymphomas and leukemias have been shown in Fischer 344 and Sprague-Dawley female rats given atrazine concentrations ranging from 375 to 750 ppm (Pinter et al. 1990; Eldridge et al. 1994b; Stevens et al. 1994; Wetzel et al. 1994; Eldridge et al. 1999a).

Other consequences of atrazine exposure in rats have included hyperactivity in females (Peruzovic et al. 1995), as well as reduced hemopoietic progenitors and reticulocytes (Mencoboni et al. 1992). *In utero* exposure of Sprague-Dawley rats to 35 ppm atrazine resulted in immune suppression in males only, leading to impaired primary antibody response (Rooney et al. 2003). Because effects in rats were seen at such high doses (the only doses investigated), it was hypothesized that risk to human health was negligible (Eldridge et al. 1999b) though human exposure levels were not tested. Investigation of low-dose effects is especially warranted since many of the adverse observations were hypothesized to result from hormonal interaction (Stevens et al. 1994; Eldridge et al. 1999a; Rooney et al. 2003), including tumorigenesis (Eldridge et al. 1994b).

Human data

Atrazine's toxicity may extend to humans. Because atrazine contaminates drinking water, particularly in the Midwest, there is concern about its effects on children and infants (as well as on the fetus), with its potential to affect sex steroids (Sanders 2002). Epidemiological studies have uncovered associations between atrazine and multiple endpoints. Data obtained on incidence of cancer types and atrazine drinking water contamination in Ontario, Canada revealed a positive association ($p < 0.05$) with stomach cancer incidence and atrazine contamination levels (Van Leeuwen et al. 1999). Swan et al. (2003) collected semen and urine samples from men residing in Missouri. Semen quality parameters (concentration, percent normal morphology and sperm motility) and urine concentrations of current-use pesticides were measured. The researchers found that men with detectable levels of atrazine were significantly more likely (odds ratio = 11.3) to have low semen quality than unexposed men (Swan et al. 2003). Kettles et al. (1997) performed a regression analysis between Kentucky counties classified as low, medium or high triazine exposure and uncovered statistically significant increases in breast cancer risk for medium and high exposure counties (odds ratio = 1.14, 1.2 respectively). In a population-based case versus referent study, female farmers previously exposed to triazine herbicides

showed a significant relative risk (2.7) for ovarian neoplasms compared to unexposed female farmers (Donna et al. 1989). In a study comparing Iowa communities defined by drinking water sources, the community with elevated levels of triazines in its water source had a greater risk (relative risk = 1.8) of intrauterine growth retardation (Munger et al. 1997). Furthermore, atrazine levels were the greatest predictor of community intrauterine growth retardation rates (Munger et al. 1997). In addition, miscarriages and premature births have been associated with communities regularly exposed to triazines (ATSDR 2003). Reports are beginning to surface concerning heart damage, eye trouble, muscle breakdown and weight loss related to atrazine exposure in humans (Marcus 2002). Also, Syngenta, the producer of atrazine, has uncovered increased rates of prostate cancer in its manufacturing employees (Weiss 2004). These studies suggest relationships between atrazine exposure and the various cancer and reproductive endpoints; however, epidemiological studies cannot imply causality.

It is important to note that over the last 50 years, there has been a significant decrease in mean sperm count and seminal volume (Sharpe and Skakkebaek 1993; Takai et al. 2001; Honma et al. 2002). There has also been a doubling of male reproductive disorders, including infertility (Akingbemi and Hardy 2001),

indicating an impact on gonadal function which may be a reflection of increased exposure to estrogens *in utero* (Carlsen et al. 1992; Sharpe and Skakkebaek 1993; Newbold 1995). Testicular cancer is now the most common malignancy among 21-35 year old men (Akingbemi and Hardy 2001). With increased reproductive abnormalities in wildlife mirroring that in humans, there is growing concern that environmental contaminants may be adversely affecting human reproductive health (Akingbemi and Hardy 2001). The increase in genital abnormalities in boys, earlier sexual maturation in girls and increase in breast cancer may reflect an increase in endocrine-disrupting chemical exposures in the environment (Howdeshell et al. 1999; Markey et al. 2001; Sohoni et al. 2001; Takai et al. 2001). Estrogen exposure in women is a risk factor for breast cancer (Markey et al. 2001); while in contrast, preeclampsia, characterized by low estrogen production in the placenta, is correlated with decreased incidences of breast cancer in daughters and decreased prostate cancer in sons (Markey et al. 2001). Endocrine disruptors are likely contributing to both male and female cancers of the reproductive tract (Newbold 1995; Elswick et al. 2000). In addition, it is possible that atrazine exposure is contributing to the declining trends of reproductive health in humans.

Study overview

A three-year field study was conducted as well as a single laboratory experiment to test the effects of sublethal concentrations of the herbicide atrazine on amphibians. These experiments primarily explored atrazine's capacity to alter reproductive processes in order to assess atrazine's potential to impact natural amphibian populations.

New Jersey is in a unique situation as it has extensive pesticide use data that have been collected on a three-year cycle since 1985. Detailed information on application rates and crops are available from these records. In addition, corn, a principal row crop on which atrazine is applied, is the largest single agricultural crop planted in New Jersey (USDA 2003). Figures 1.2 and 1.3 indicate that New Jersey's atrazine use and rainfall input are both in the moderate ranges. Therefore, the state is mildly vulnerable to atrazine runoff to its surface waters. Thus, the state of New Jersey is a suitable location in which to study the effects of atrazine.

Furthermore, as endocrine disruption is a major environmental and human health issue, NJ DEP's Division of Science, Research and Technology (DSRT) and other agencies have a suite of projects that focus on endocrine burdens and their implications

for human health. This field study was an indicator study that may be useful in identifying environmentally significant and relevant levels of atrazine in the ecosystem that can have adverse effects on wildlife and possibly humans.

Study hypotheses and specific aims

The purposes of the three-year correlational study were to assess levels of atrazine and related compounds in New Jersey surface waters, survey the resident frog populations for gross morphogenic and histopathologic changes, and evaluate potential endocrine-mediated adverse effects comparing contaminated sites to reference sites. The goal was to determine the consequences of atrazine exposure and whether atrazine poses an environmental health concern in the state. To do so, atrazine's ability to act as an endocrine disruptor was evaluated in frogs, utilizing the bullfrog (*Rana catesbeiana*) and green frog (*Rana clamitans melanota*) as the target species. Our hypothesis was that there would be a greater incidence of gonadal and tissue abnormalities among frog populations from high exposure sites in comparison to those from low exposure sites through histological examination. In addition, we (1) evaluated the expression of secondary sexual traits to determine if expression of these characteristics decreased with increased environmental contamination, (2) calculated plasma testosterone levels to see in increased

pesticide exposure correlated with depressed testosterone levels, (3) determined if shifts in blood components were associated with agricultural chemical presence, and (4) recorded differences in parasite, malformation and inflammation prevalence between sites to observe whether frequencies increased with contamination.

The laboratory-based experiment was designed to assess the effects of sublethal atrazine exposures on *Xenopus laevis*, the African-clawed frog. The purpose of this study was to corroborate effects in the field with effects in a controlled setting. In the laboratory, atrazine's actions were evaluated without the confounding variables present in the field. Effects seen in the laboratory were then compared to effects from the field in order to determine atrazine's role in field observed pathologies. Our hypothesis was that atrazine exposure would result in abnormal testicular development through morphological and histological evaluation. In addition, we (1) recorded survival and growth measurements to determine changes due to atrazine exposure, (2) determined whole body testosterone and corticosterone levels to see if testosterone decreased and corticosterone increased with increased doses of atrazine, and (3) observed any malformations to determine whether frequencies of deformities increased with atrazine concentrations.

Overall, these experiments, and resulting analyses, were designed to evaluate atrazine's ability to act as an endocrine disruptor in amphibians.

Figure 1.1. Environment and amphibian hormone system interactions
External factors (temperature, rainfall, food, density, predators, etc.) are taken in by the central nervous system, which controls releasing hormones by the hypothalamus, which then act on the pituitary to release tropic hormones, which control the endocrine tissues of the periphery. CNS = central nervous system; GnRH = gonadotropin-releasing hormone; TRH = thyrotropin-releasing hormone; CRH = corticotropin-releasing hormone; GTH = gonadotropic hormone; TSH = thyroid-stimulating hormone; ACTH = adrenocorticotropin hormone; T = testosterone; E_2 = estradiol; T_4 = thyroxine; T_3 = triiodothyronine; CORT = cortisosterone (Hayes 1997; Sparling et al. 2000).

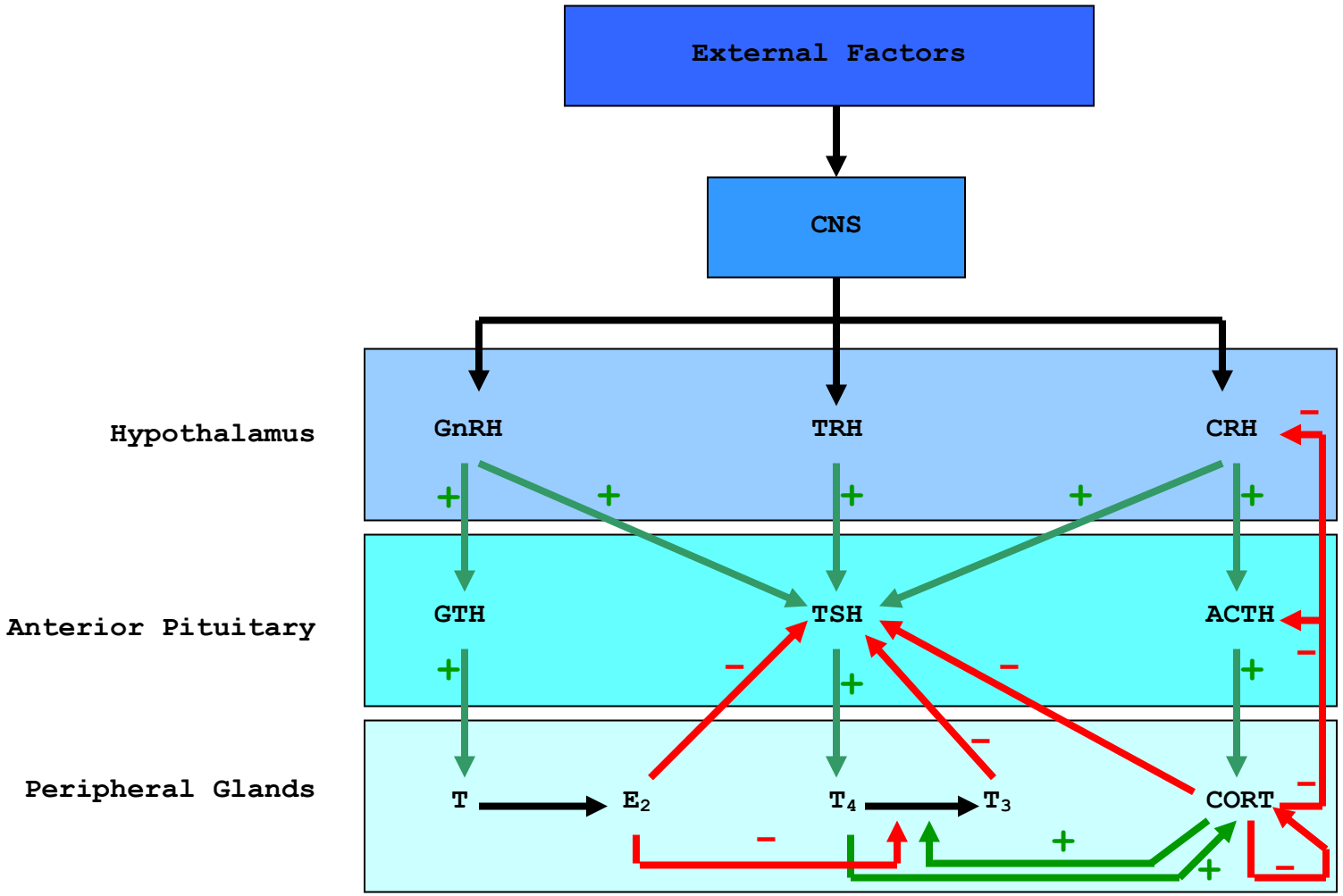
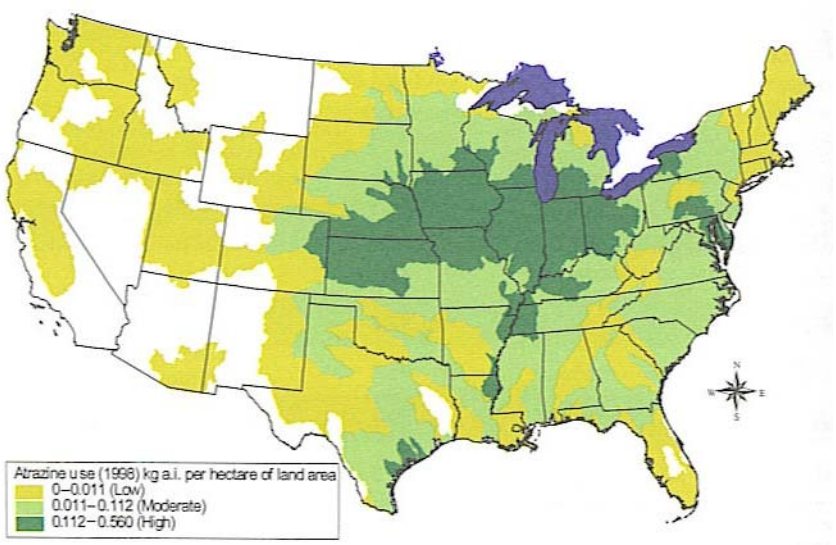

External Factors
CNS
Hypothalamus
GnRH
TRH
CRH
Anterior Pituitary
GTH
TSH
ACTH
Peripheral Glands
T
E2
T4
T3
CORT

Figure 1.2. Atrazine use and rain geography in the United States
A, map of the continental US showing atrazine use. The highest use is mostly concentrated in the cornbelt region of the Midwest. B, map of the continental US showing rainfall following typical planting. Much of the Midwest receives moderate to high levels of rainfall during the planting season (Giddings et al. 2005).

A.



B.

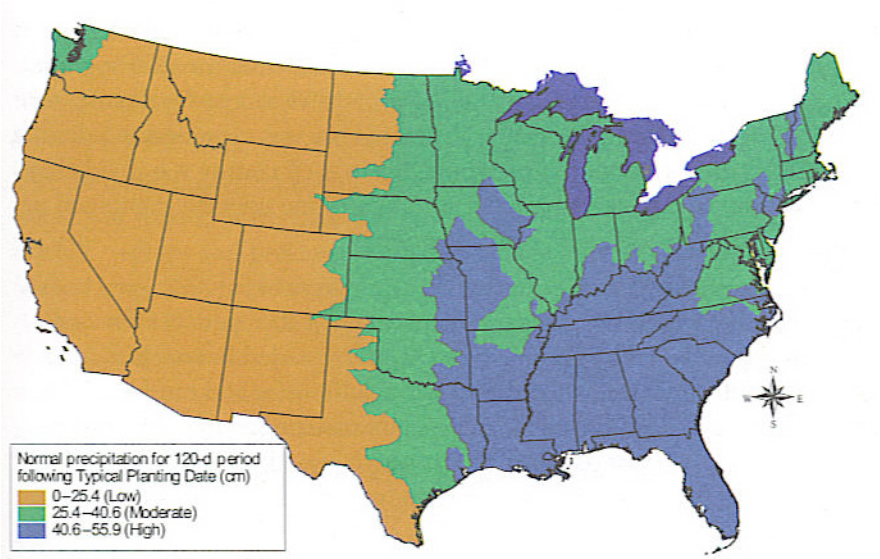


Figure 1.3. Climate-atrazine use across the United States Map of the US integrating atrazine and rainfall data in order to identify those areas most vulnerable to runoff events carrying atrazine, since runoff is the dominant route of aquatic exposure. This map shows that the Midwest has the greatest potential for aquatic exposure to atrazine. Note that the areas of greatest risk are those areas adjacent to three major rivers (Mississippi, Missouri and Ohio), which lends to the transport of atrazine to areas where it is not used. This analysis does not integrate potential runoff from irrigation events (Giddings et al. 2005).

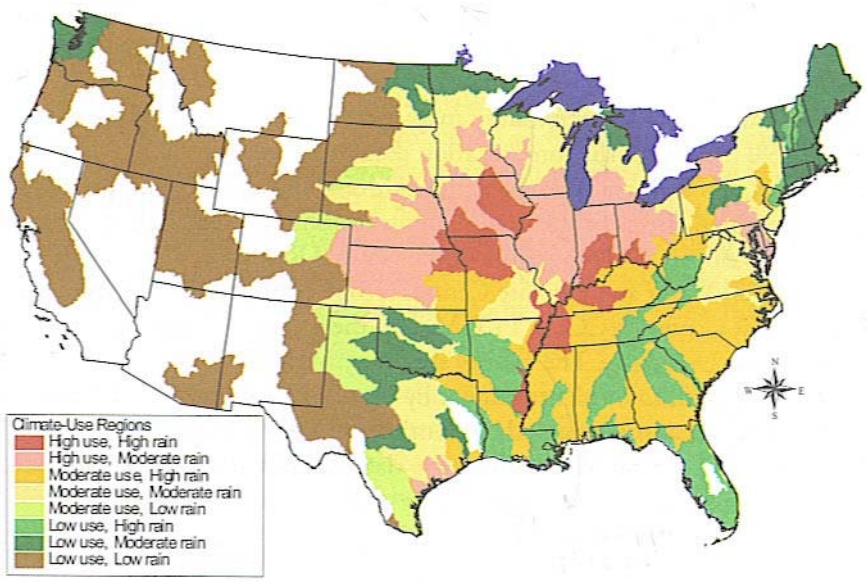
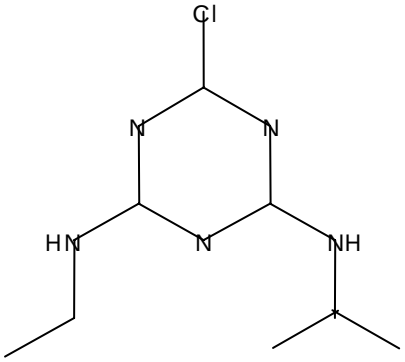
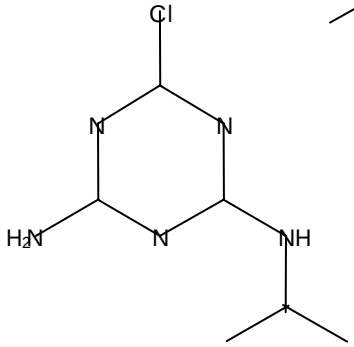


Figure 1.4. Structures of atrazine and its metabolites

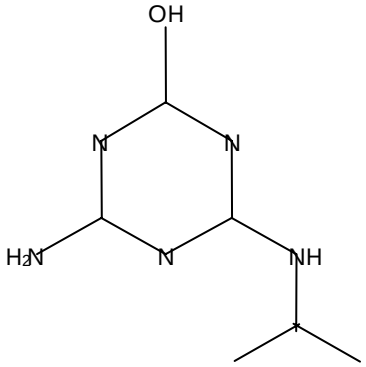
Modified from (Kruger et al. 1996; Stein et al. 2002; Giddings et al. 2005).



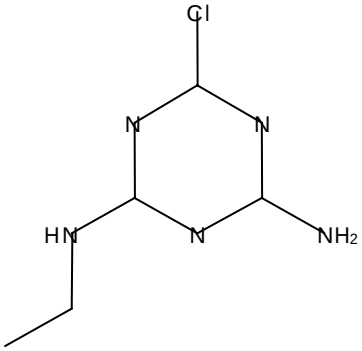
Atrazine (ATR)



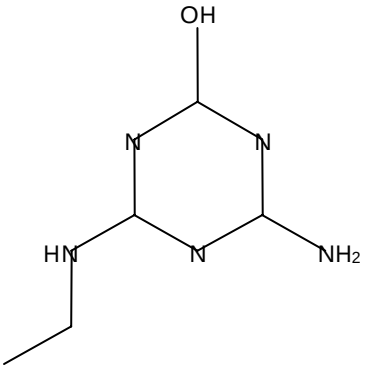
Deethylatrazine (DEA)



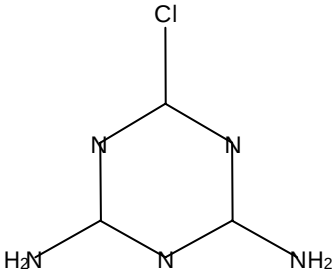
Deethylhydroxyatrazine (DEHA)



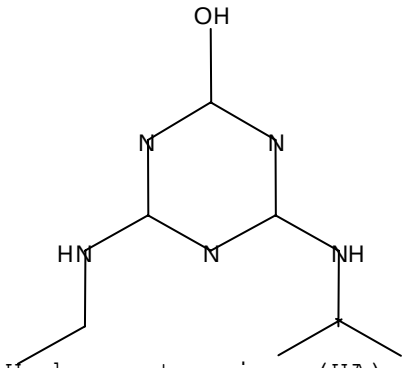
Deisopropylatrazine (DIA)



Deisopropylhydroxyatrazine (DIHA)



Didealkylatrazine (DDA)



Hydroxyatrazine (HA)

Figure 1.5. Atrazine's herbicidal action
Atrazine, and its triazine relatives, competively block electron transfer to plastoquinone in the redox chain of photosystem II. ATR = atrazine; Chl = chlorophyll; PS II = photosystem II; Pheo = pheophytin; PQ = plastoquinone; Cyt = cytochrome; PC = plstocyanin; PS I = photosystem I; Fd = ferredoxin (Davidson and University 1995; Giddings et al. 2005).

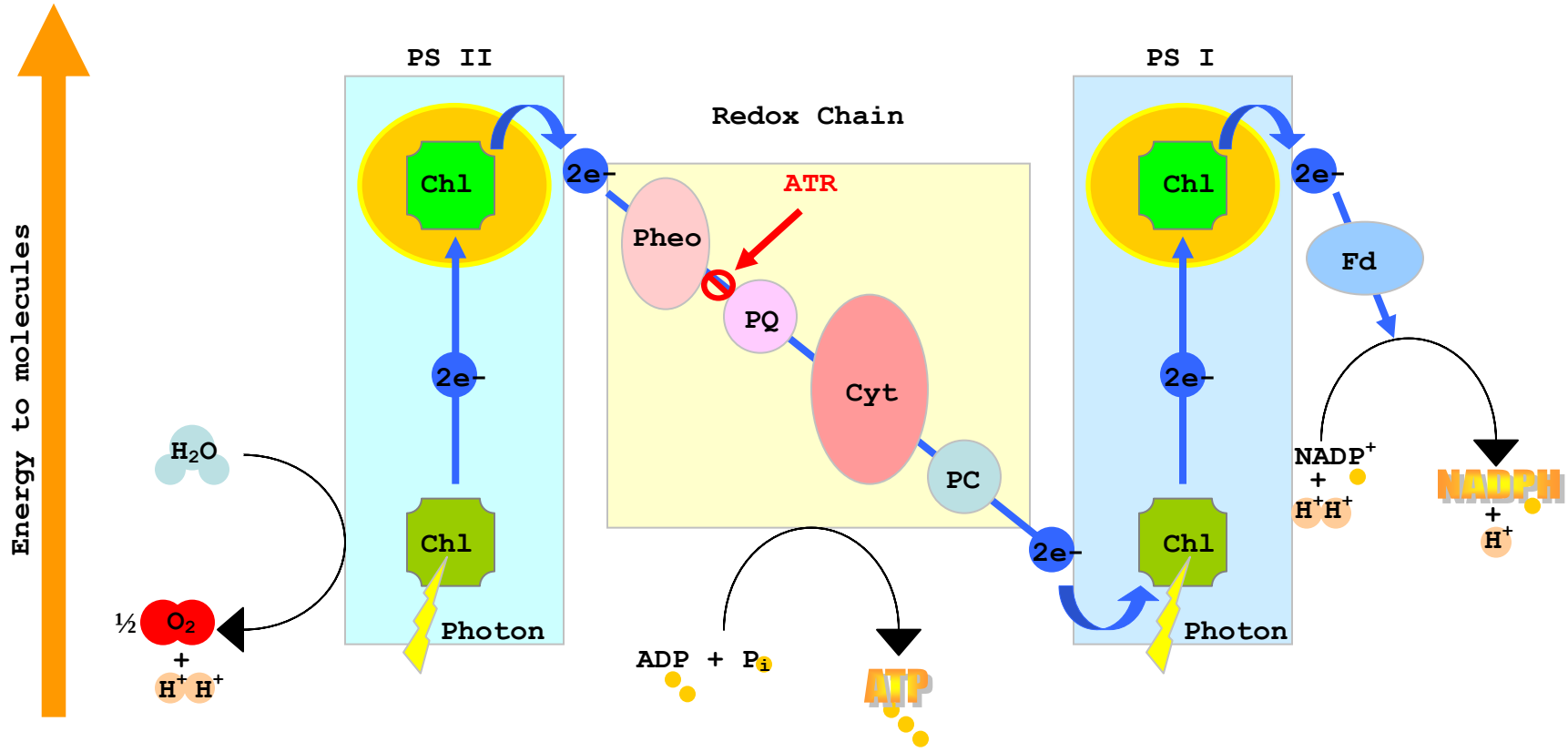


Figure 1.6. Atrazine metabolism in higher plants
1, the natural product DIMBOA catalyzes the hydrolysis of atrazine to hydroxyatrazine. 2, using the hydroxyl as a functional group, glutathione-S-transferase performs the conjugation of hydroxyatrazine and glutathione. 3, a pump, which recognizes xenobiotic-glutathione conjugates, transports the molecule into the cell's vacuole. 4, the molecule is further processed in the vacuole. 5, eventually it is sequestered by incorporation into the cell wall. DIMBOA = 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one acid; GST = glutathione-S-transferase; R = xenobiotic-glutathione congjugate recognition receptor (Griffin; Bailey et al.; Markwell and Namuth 2003; Sterling et al. 2006).

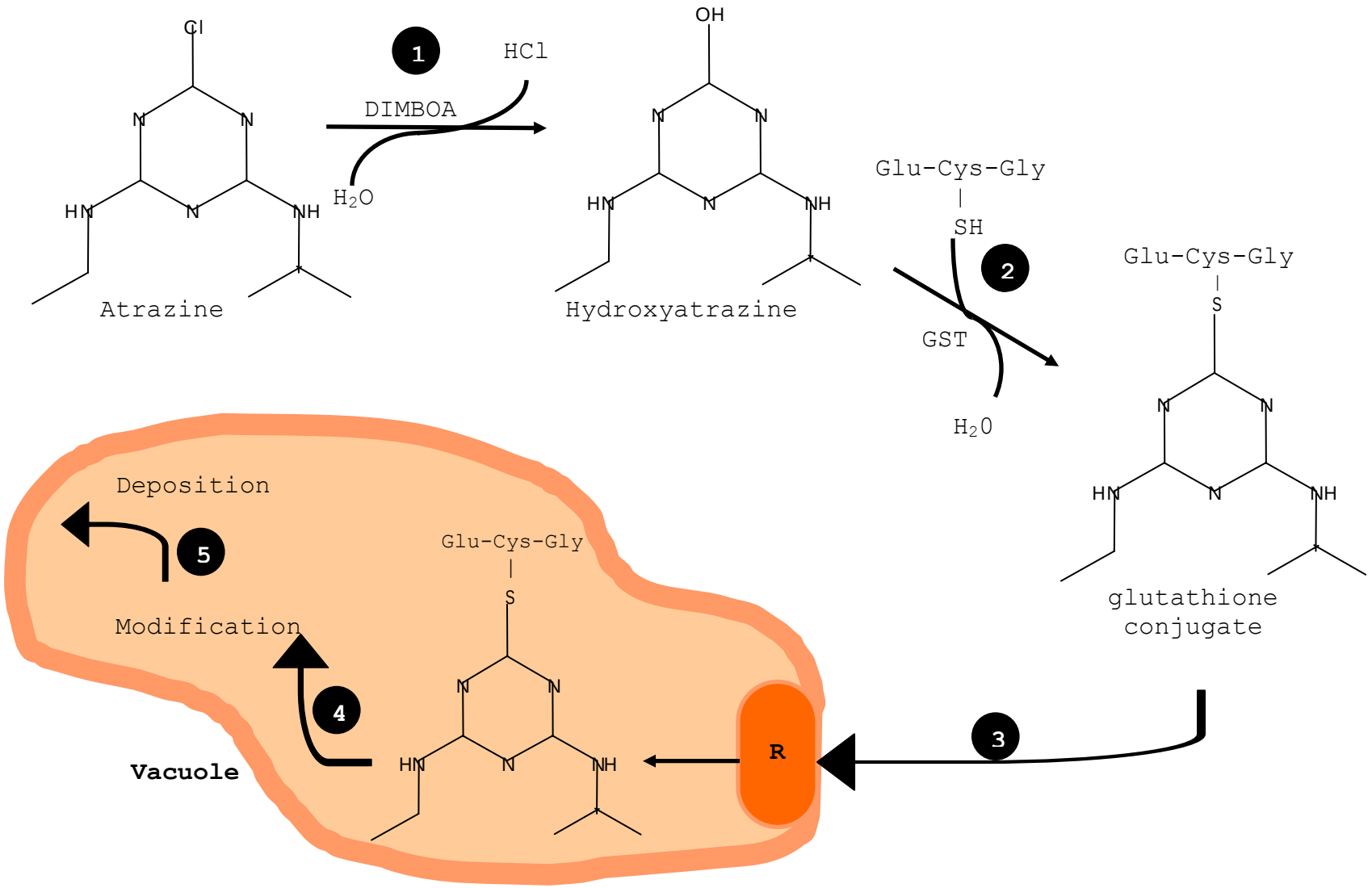
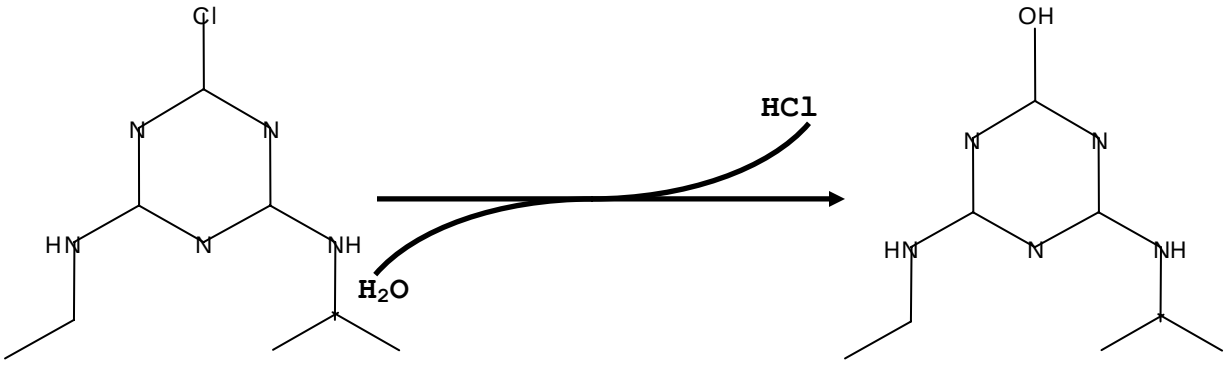

Cl
N
N
HN
N
NH
Atrazine
1
HCl
DIMBOA
H_2O
OH
Hydroxyatrazine
Glu-Cys-Gly
SH
2
GST
H_2O
Glu-Cys-Gly
S
glutathione
conjugate
3
R
Vacuole
4
Modification
5
Deposition
Glu-Cys-Gly
S

Table 1.1. Physical and chemical properties of atrazine Modified from (TOXNET; Pelish et al. 2003; Giddings et al. 2005).

CAS Number	1912-24-9
Chemical Name	2-chloro-4-ethylamino-6-isopropylamino-***S***-triazine
Molecular Weight	215.7 g/mole
Molecular Formula	$C_8H_{14}N_5Cl$
Melting Point	171-177°C
Boiling Point	unstable, decomposes
Water Solubility	33 mg/L (pH 7, 25°C)
Vapor Pressure	2.89×10^{-7} mmHg (25°C)
Henry's Law Constant	2.48×10^{-9} atm m^3/mol
pK_a	1.68, very weak base
Log K_{OW}	2.68 (25°C)
K_d (average)	3.08 mL/g
K_{OC} (average)	170.68
Half-life	3 days – 5 years
Density	1.187 g/cm^3 (20°C)
Apparent Color	White crystalline, colorless powder
Odor	Odorless

Figure 1.7. Atrazine hydrolysis
Hydrolysis of atrazine occurs at the two position on the triazine ring and is a nonenzymatic reaction involving the nucleophilic substitution of the cloride with a hydroxide ion in the presence of water to form hydroxyatrazine (Griffin; Shimabukuro 1967; Klaassen 1996; Stein et al. 2002; Rodriguez et al. 2004; Giddings et al. 2005; Sterling et al. 2006).



Atrazine Hydroxyatrazine

Figure 1.8. N-dealkylation
N-dealkylation can occur either at position 4 or 6 on the triazine ring. DEA, deethylatrazine, results from N-dealkylation at position 4, DIA, deisopropylatrazine, at position 6 and DDA, didealkylatrazine, forms when both N-dealkylation reactions occur (Griffin; Shimabukuro 1967; Huber 1993; Stein et al. 2002; Rodriguez et al. 2004; Du Preez et al. 2005; Sterling et al. 2006).

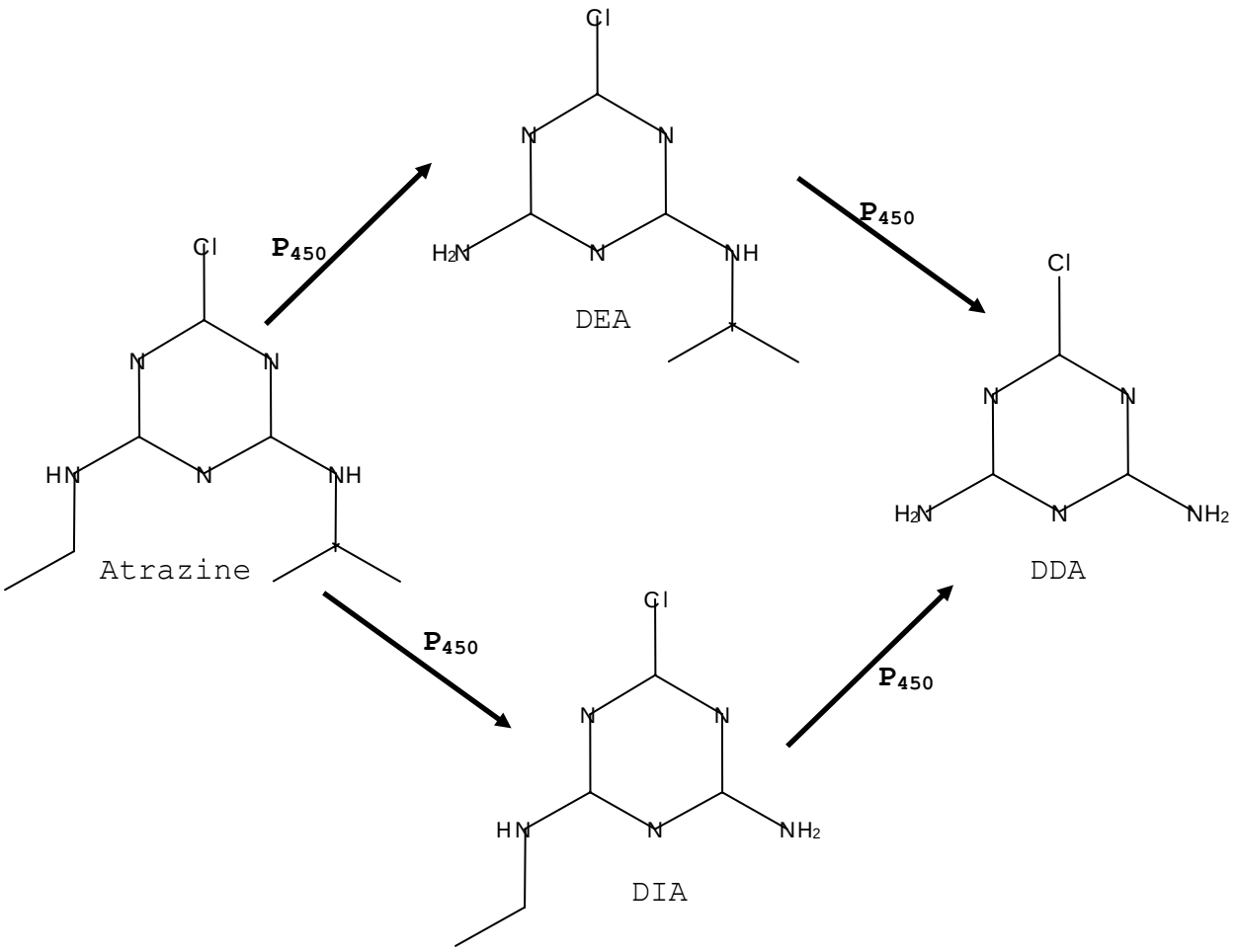


Figure 1.9. Splitting of the triazine ring
After the formation of hydroxyatrazine, rearrangement may occur due to the electronegativity of the oxygen at position two, which pulls electrons from the carbon atom. This rearrangement leaves the ring less stable and vulnerable to splitting between positions one and two with a reduction reaction (Kaufman and Kearney 1970; Klaassen 1996; Stein et al. 2002; Giddings et al. 2005).

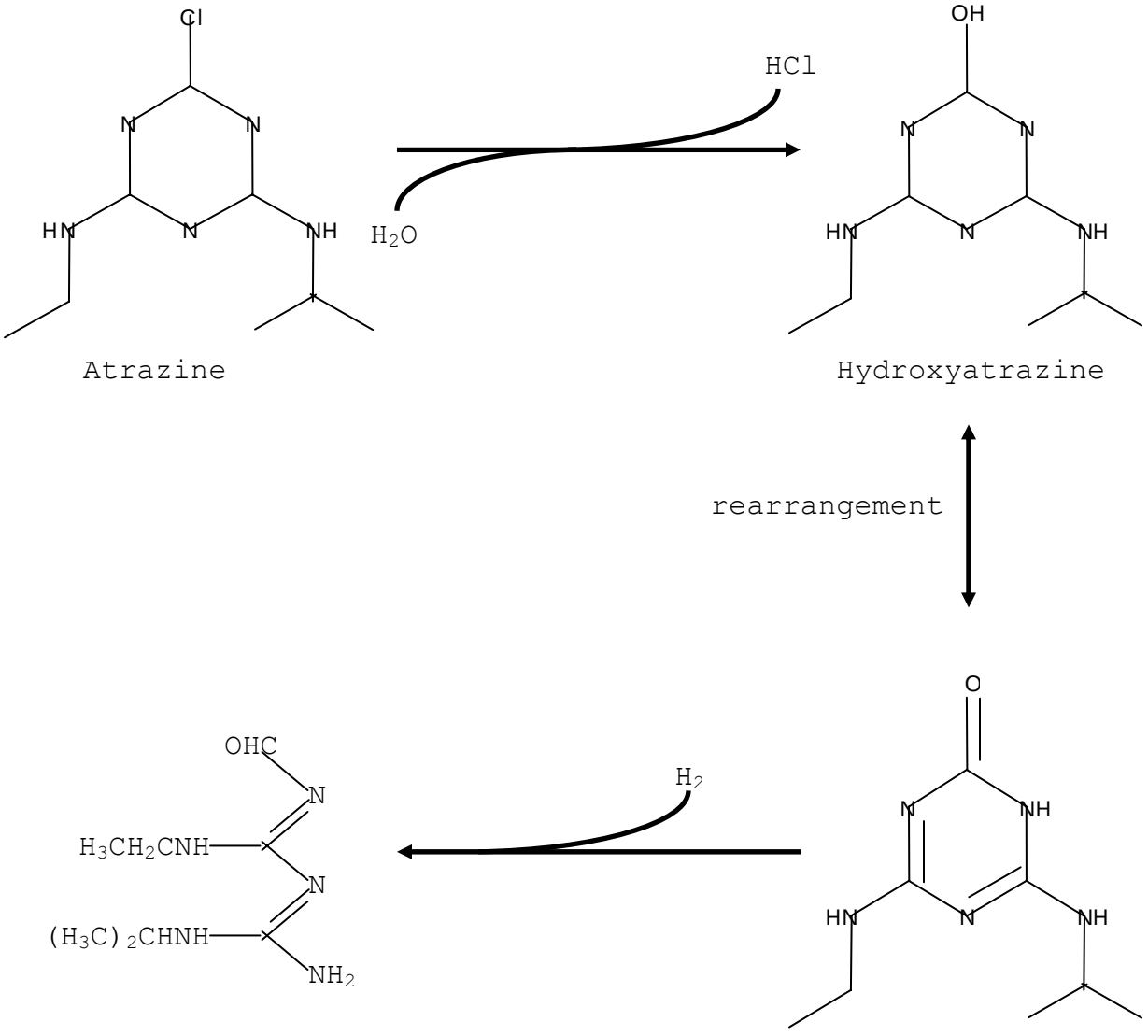
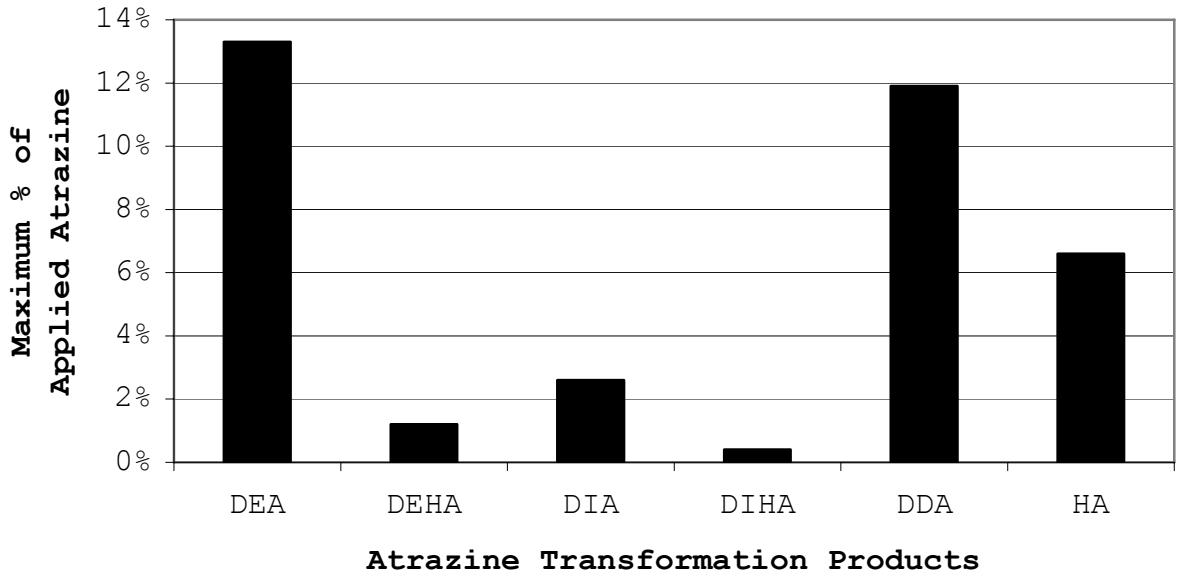


Figure 1.10. Levels of atrazine transformation products A, maximum concentration of transformation products (as a percentage of initial atrazine conentration) under aqueous photolysis. B, raw data with day of maximum concentration in parantheses (Giddings et al. 2005).

A.

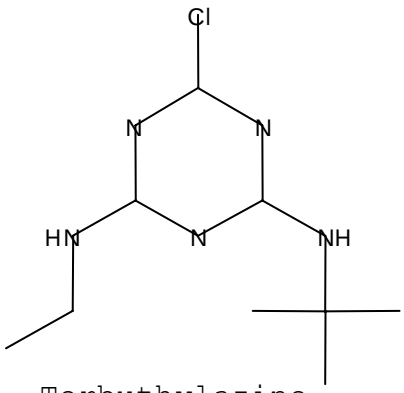


B.

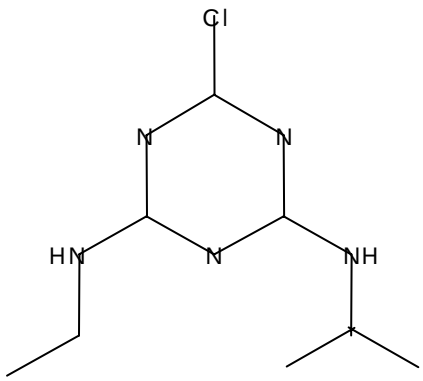
DEA	DEHA	DIA	DIHA	DDA	HA
13.3 (30)	1.2 (6.9)	2.6 (275)	0.4 (15)	11.9 (30)	6.6 (183)

DEA: Deethylatrazine
DEHA: Deethylhydroxyatrazine
DIA: Deisopropylatrazine
DIHA: Deisopropyhydroxyatrazine
DDA: Didealkylatrazine
HA: Hydroxyatrazine

Figure 1.11. Terbuthylazine
Manufacturers replaced atrazine (right) with terbuthylazine (left) in European countries due to a ban on atrazine beginning in 2005 (Marcus 2002; Green 2003; Weiss 2004). The isopropylamine group at position 6 was replaced with a terbuthylamine. Structures were obtained and/or modified from ChemSketch (Stein et al. 2002).



Terbuthylazine



Atrazine

Disruption of secondary sexual characteristics in the New Jersey bullfrog (*Rana catesbeiana*) from agricultural chemical exposure in the field

Chapter 2

Abstract

Male secondary sex traits may be altered by exposure to environmental chemical contamination. To determine the sex of a frog externally while in the field, it is a common practice to compare the size of the eye and the tympanic membrane (eardrum). It has been generally accepted that in bullfrog (*Rana catesbeiana*) and green frog (*Rana clamitans melanota*) males, the tympanic membrane is larger than the eye, while in females of both species, the eardrum is smaller or about the same size as the eye. Our observations during field studies between 2003 and 2005 indicate that this technique is unreliable for sexing bullfrogs. Several bullfrogs marked as female by this assessment turned out to be male, confirmed by the presence of testes upon dissection. Use of the technique yielded a 14.8% error rate in bullfrogs, while the technique was 100% accurate for green frogs. It was unclear if these observations indicated an altered expression of secondary sexual characteristics or were a result of the extended length of time needed for

bullfrogs to reach sexual maturity. Therefore, the diameters of the eardrum and of the eye were recorded in order to test the validity of this common sexing method. The resultant analyses indicate that the ear to eye ratio is highly variable in bullfrog males and this variability increases with agricultural contamination. Such a technique that relies on traits that are affected by changes in the environment may lead to artificially skewed sex ratios in population surveys.

Introduction

Exposure to chemical contamination has been listed as one of several potential causes contributing to amphibian declines over recent years (Hayes et al. 2003). A variety of agrochemicals disrupts endocrine function and exposure to such compounds may contribute to population declines (Reeder et al. 2005; Hayes et al. 2006). In an effort to determine whether endocrine disruption was a risk factor to native New Jersey populations of bullfrogs (*Rana catesbeiana*) and/or green frogs (*Rana clamitans melanota*), we engaged in a field study to examine endpoints of endocrine disruption in populations residing on agricultural land. Evidence of endocrine disruption may be reflected in the expression of secondary sex traits, like tympanum membrane size. Therefore, our hypothesis was that the expression of male

secondary sex characteristics would decrease with increasing exposure to environmental chemical contamination.

Our primary chemical of concern during the field studies was atrazine, an herbicide used in New Jersey and a frequent contaminant of surface waters worldwide. Since reported effects of atrazine exposure were most apparent in males (Hayes et al. 2002b; Tavera-Mendoza et al. 2002a; Hayes et al. 2003), our field efforts focused on male frogs. When in the field we located males by their mating calls and concentrated on capturing frogs displaying the secondary sexual characteristics of males: yellow throats and large tympanic membranes. However, when transported back to the laboratory for analyses requiring dissection, the gonads within did not always match the secondary traits presented externally.

Depending on timing, effects of general contaminant exposure can lead to shorter body lengths, decreased weight, intersex gonads, immune suppression, limb and/or organ malformation and other lesions that can lead to mortality (Sparling et al. 2000). Our interests were not in lethal effects, but rather in sublethal consequences such as reduced reproductive success. Therefore, the goal of this analysis was to determine the validity of a popular and well-utilized field sexing technique and to see if

its decreased effectiveness correlated with environmental contamination, thus indicating alteration in secondary sex characteristic display, possibly through chemical contamination.

Materials and Methods

Study area. This study was carried out in Jacobstown, Burlington County, New Jersey during the years of 2003, 2004 and 2005. All collection sites were ponds located on agricultural land and were chosen based on pesticide use histories and presence of anuran populations. Nine pond locations were sampled and these were identified as Durr II, Bruch II, Bruch I, Croshaw, Robson East, Robson West, Durr, Hopkins and Rahilly.

Frog collection. Wearing chest waders, investigators collected frogs by hand and netting primarily at night (some collection events did occur during the day) using headlamps to allow hands-free searching. Frogs were located by the reflection of the light from their eyes, and males were specifically located by their calls. Targeted animals would freeze and activity cease when light shined upon them. Animals were then approached and when close enough, a net was typically placed over the frog until it jumped into the net either because it was spooked by the net itself or because it was gently nudged. Sometimes debris would not permit the access of the net to the frog and

hands were used to quickly and gently capture the animal. Frogs retrieved from the net were placed in a bag with a cinch closure, allowing multiple captures at a time. When frogs were removed from the bag, they were placed in a half gallon glass jar containing the frog's pond water. The jars were labeled to identify site and order of capture, allowing individual identification. At the end of the collection, jars and specimens were transported back to the laboratory for further analyses.

Calculation of growth, hind limb length (HLL)/snout vent length (SVL), index. Each frog was measured for HLL, the length of each leg from anus to toes. The average HLL between the left and right legs was obtained in each individual. SVL was measured anteroposteriorly between the frog's mouth and its anus and recorded for each frog. The HLL/SVL index was calculated for each frog as follows: average HLL divided by SVL. Inclusion of juveniles did not affect analyses.

Calculation of ear/eye index (EEI). For each frog, the diameters of the tympanic membranes and eyes were measured using digital calipers (± 0.01 mm accuracy). Diameters were obtained at their shortest (dorsoventral) and longest (anteroposterior) points, then averages obtained. The average ear diameter for

each frog was then divided by that frog's average eye diameter to come up with the frog's "ear/eye index," or ratio. Juvenile frogs were excluded from this analysis since they are sexually immature and do not display secondary sex traits at this stage (Casper and Hendricks 2006).

Statistics. Comparisons of means were performed by one-way analysis of variance (ANOVA) with Bonferroni correction for body weight, SVL, HLL/SVL indices across sites; and by independent t-test for HLL/SVL indices between bullfrogs and green frogs, as well as between males and females, within individual sites, and EEI between males and females within each species. Significance for each test was set at $p < 0.05$. Data points that are 1.5 to 2 standard deviations from the mean are denoted by "o" in all graphs. Data points greater than two standard deviations from the mean were excluded from analyses.

Results

Site characteristics. Figure 2.1 illustrates the locations of the study ponds in relation to one another. The entire study area is within eight kilometers. With the exception of Bruch I and II, all sites lie within a kilometer radius. Bruch I and II are located about a quarter kilometer from one another. Figures 2.2 through 2.4 give detailed geographical information about the

sites. Table 2.1 lists the main crops and pesticides for each site, which remained constant over all collection years. Based on the pesticide use histories and the water chemical analyses (see Chapter 3), each pond was characterized as low, medium or high in regards to level of chemical contamination. The categories in increasing contamination were: low – Durr II, Bruch II and Bruch I; medium – Croshaw, Robson East and Robson West; high – Durr, Hopkins and Rahilly.

Durr II sits on the edge of an agricultural plot (Figure 2.2A) and due to its positioning and the land's topography, not expected to incur any direct agrochemical input and none was seen in chemical analyses. Vegetation at the site includes reeds and grass. At Durr II both species were found, with the smaller green frog out-numbering the larger bullfrog. Observed predators at this site included snakes and owls. Bruch II is located on the property of a Christmas tree farmer. The site sits upland of agricultural activity within a wooded area (Figure 2.2B) and is a shallow man-made pond intended for recreation (ice skating). The pond is fed from underground sources and chemical contamination was not expected. This was confirmed by our inability to detect contaminants in water samples (see Chapter 3). Also at this site is a much smaller depression that also fills with water due to upwelling.

Depending on the water table level, this depression would form a separate pond or form a continuous pond with the Bruch II site. While both species inhabit this pond, the green frog was more abundant. In contrast to Bruch II, Bruch I lies at the center of the agricultural activity, with a few established trees and grass surrounding the pond (Figure 2.2B). Bruch I sits lower than the surrounding land, and consequently, receives agricultural input primarily from simazine, a selective triazine herbicide. In addition, this pond is stocked with predatory fish (bass and bluegill) for recreational purposes. In contrast to its sister pond, bullfrogs dominate Bruch I. Both sites are included in the ranges of raccoons, deer, owls, snapping turtles and the American toad (*Bufo americanus*), while the northern spring peeper (*Pseudacris crucifer crucifer*) was only observed at Bruch II. Tadpoles of both species were present at both sites and newly metamorphed bullfrogs were observed at Bruch I. Bruch I allows for wading, while the sediments of Bruch II are too soft for wading beyond the edges. Consequently, frogs were more easily accessed at Bruch I, than Bruch II. In addition, the softer sediments of Bruch II would adsorb contaminants present in the water column, which is lacking at Bruch I. This situation would result in a shorter water half-life for contaminants (if any were present) at Bruch II compared to Bruch

I, but would conversely increase winter exposures to these animals when they overwinter in the ponds' sediments.

The sites Croshaw, Robson East and Robson West are all interconnected with water flow from Robson East to Robson West to Croshaw (Figure 2.3). All three locales are irrigation ponds and subject to both agricultural runoff and pumping for irrigation of surrounding crops when needed. Most of the banks at these ponds are very steep with few access points, particularly at Robson West. This topography makes wading prohibitive and collections difficult. Consequently, our collection numbers do not accurately reflect the abundance of frogs at these sites. Both species were very abundant, with egg masses, tadpoles and newly metamorphed frogs observed at all three sites. These sites also house other wildlife including raccoons, snapping turtles, ducks and other birds. Robson East also contains honeybee colonies near the north end and leeches exist in the pond. The northern gray tree frog (*Hyla versicolor*) also inhabits Robson West and Croshaw. Croshaw's surrounding land contains mostly grass and reeds with forest at the east end. The major crop near the Croshaw pond is strawberries. The herbicides napropamide and clomazone are used at this site. Both Robson East and West have thick, high reeds (mainly *Spartina*) surrounding most of the ponds. Robson East

has a peach orchard on the west side of it, and the pesticides used include the herbicides simazine and napropamide and the insecticide chlorpyrifos. Asparagus, cole crops (e.g., broccoli, cauliflower and cabbage), and chrysanthemums are grown near Robson West, with the herbicides triflurilan and norflurazon applied. (For a complete list of crops and pesticides, see Table 2.1.)

Durr is a pond situated in a wetland with multiple established trees on a flower farm (Figure 2.4A) where methoxychlor is the predominant contaminant. The pond is home to a host of animals besides bullfrogs and green frogs (the latter being more abundant), including the pickerel frog (*Rana palustris*) and the Fowler's toad (*Bufo fowleri*). In addition, Eastern painted turtles, geese, and ducks utilize this habitat, and duck weed dominates the more stagnant portions at the east end of the water. The pond is also stocked with fish (bass and bluegill), while horses and cows graze on adjacent land. Three-quarters of the surrounding upland from the Hopkins site is surrounded by sweet corn upon which atrazine is applied and likely finds its way to the pond via runoff events. The southwest portion of the site contains trees (Figure 2.4B), with high grass covering other portions. Both species are present at the site though in small numbers. Horses reside on adjacent land, pickerel frogs

have been observed and minnows are present in the pond. Rahilly is an irrigation pond much like Croshaw, and Robson East and West. It also is fitted with a pump that is used when needed to irrigate the crops that lie to one side of the pond. Major crops of this site include cantaloupe, eggplant, tomatoes and field corn. Atrazine, metalochlor, napropamide and metribuzin are herbicides utilized at this site. Opposite the pump, is a hedgerow where numerous birds reside, whereas meadow surrounds the rest of the site (Figure 2.4C). Snapping turtles and sunfish also occupy this site. In addition, the banks are very steep precluding wading and making collections very difficult. Bullfrogs dominate this site, with only a single male green frog heard calling during one collection event and a single female green frog captured. No evidence of breeding was seen at this site and it is believed that its residents are a result of dispersal events from other ponds (like Durr II, see map in Figure 2.1). (For a complete list of crops and pesticides, see Table 2.1.)

Other than the pesticides used on crops, no additional pesticides were utilized on undergrowth, which, if managed, were generally mowed. We cannot definitively state where captures underwent development, as movement during dispersal events was possible between sites (refer to Figure 2.1 for relative

locations). Possible movements include between Bruch I and II, between all three medium-contaminated sites (Croshaw, Robson East and Robson West), as well as from Durr II to Rahilly (as there was no evidence of active breeding at Rahilly, dispersal events from this pond would not be expected).

Captures. Table 2.2 lists the distribution of captures across sites, species and sex. Sex listed was determined by identification of gonads. When using the field sexing technique (Table 2.3A), we experienced a 14.8% error rate, while the method was 100% accurate in green frogs. Consequently, although we targeted male frogs of both species, our capture events only yielded 58.8% bullfrog males (Table 2.3B). In contrast, green frog captures consisted of 81.8% males. Frog populations were noticeably smaller at the two most contaminated sites, Hopkins and Rahilly. Meanwhile, the medium-contaminated sites (Croshaw, Robson East and Robson West) seemed to have the largest frog populations, though comparable in size to other ponds. Numbers in Table 2.2 only refer to those frogs included in the ear/eye index analysis. For several reasons frogs were excluded from this analysis. First, validation of the field sexing technique was not part of the original purposes of the field study. Measurements of the eye and ear diameters began after the first collection event when it was noticed that the technique was not

accurate for bullfrogs. Therefore, the diameters of frogs collected prior were not measured. Second, any animals that expired en route to the laboratory (fourteen frogs out of 391, see Table 2.3C) were not measured due to the difficulty of measuring eye diameters in dead frogs (the eyes tend to sink into the sockets making measurements unreliable). In addition, juveniles were not included because they have yet to reach sexual maturity with full display of secondary sex traits and to do so biases the data by artificially decreasing EEI (Casper and Hendricks 2006).

Body weight. Figure 2.5 illustrates the distribution of body weight in bullfrogs across the collection sites. Among males, statistically significant differences appeared within the medium-contaminated sites (Croshaw, Robson East and Robson West). Male body weight at Robson East was elevated 48.1% and 43.9% compared to both Croshaw and Robson West, respectively. However, when the data were collapsed by level of contamination (Figure 2.6), no significant differences in either sex appeared. Among male green frogs, body weight was significantly different between several sites (see Figure 2.7 for differences between sites and p values). There were no detectable differences among green frog females. When the data were collapsed by level of contamination, statistically significant differences remained

among green frog males (Figure 2.8). Body weights at low sites were 18.6% and 23.1% higher than at medium or high sites. Again, no significant differences were detectable among green frog females.

SVL. Similar to body weight, male bullfrog SVL measurements were significantly elevated 18.9% and 16.7% at Robson East compared to Croshaw and Robson West (Figure 2.9), with no differences between bullfrog females. Again, when the data were collapsed (Figure 2.10), no significant differences in either sex were detected. No apparent changes in SVL were determined in either sex of green frogs between individual sites (Figure 2.11) or between levels of contamination (Figure 2.12).

Tympanic membrane (eardrum) diameter. Figure 2.13 shows the tympanum membrane diameters of males by species across sites. Looking at the ear diameter across sites among bullfrog males, the pattern resembles that of body weight and SVL. Tympanum size was significantly elevated 30.9% and 25.0% at Robson East compared to Croshaw and Robson West. No difference was determined between populations of green frog males, though the distribution pattern for tympanum membrane size resembled that for SVL (Figure 2.11). When the data were collapsed by level of contamination (Figure 2.14), no significant differences existed

between eardrum diameter in either species. However, the means did decrease with increasing levels of contamination among bullfrog males.

HLL/SVL indices. Stages of growth are highly variable in the field. In order to compare the relative growth patterns of the frog populations across sites, hind limb length (HLL) and snout vent length (SVL) were measured and compared. Normally, these measurements are proportional at a 1:1 ratio (Gillilland 2000). If we look across sites in Figure 2.15, there were no significant differences in the HLL/SVL indices between the frog populations. Even when bullfrogs and green frogs are separated (Figure 2.16), this measurement uniformity for the most part remains and is comparable between the two species. There were significant differences between bullfrog and green frog HLL/SVL indices at Durr II. Figure 2.17 shows the HLL/SVL comparisons between females and males by site. Again, the indices were largely comparable with the exceptions of sites Durr II, Robson East and Robson West, which show significant differences.

Ear/eye indices (EEI). Figure 2.18 displays the EEIs between females and males for each species. Sex was determined by the presence of ovaries or testes. Though significant differences existed between both species, there was a greater separation

between males and females in green frogs (30.8%) compared to bullfrogs (20.9%). To analyze the differences further, average ear diameters were plotted against average eye diameters for each individual in order to compare males with females within each species. Figure 2.19 is a scatter plot of individuals from each species identified as either male or female (with r-square values and slopes of the regression lines listed). There was considerable overlap for the indices of the bullfrogs, where males did not become a distinct group until the eardrum reached about 14 mm. However, there is very little to no overlap in the green frogs.

When looking at the bullfrog sites individually (Figure 2.20), clear separation between the sexes was generally lacking. Similarly, when sites were collapsed by level of contamination, bullfrogs displayed an overlap of the indices that increased with contamination and the male and female regression slopes approached one another (Figure 2.21).

In contrast, when looking at sites individually, green frogs (Figure 2.22) always displayed good separation between the sexes and the sexes looked like distinct groups. Across sites collapsed for level of contamination (Figure 2.23), there was clear separation between green frog males and females. It is

important to note that while separation between female and male EEI was maintained among green frogs, that separation decreased as sites become more contaminated with agricultural chemicals.

In general, the slopes between the sexes were closer among bullfrogs than green frogs. In addition, the r-square was usually tighter for females, but varied among males in green frogs. Despite this distribution, the two sexes maintained distinct groupings for their respective EEIs. However, in bullfrogs the males generally displayed a tighter distribution than the females. Regardless, there was considerable overlap between the EEIs of the two bullfrog sexes.

Discussion

The differences in body weight, SVL and tympanum size among bullfrog males may reflect larger pesticide inputs to Robson West that subsequently flow into Croshaw. However, since body weight, SVL and ear diameter showed the same pattern, there is an indication that the bullfrog populations at Robson West and Croshaw may be younger than the populations at Robson East. Robson East may be the primary breeding site, where older fully mature adults are established, and from which juveniles disperse to Robson West and Croshaw. Although SVL and body weight differed across sites, they were similar by level of

contamination and should not influence analysis of indices in bullfrogs. Body weight among green frog males was variable across sites and by level of contamination. In contrast, no significant difference appeared for SVL either across sites or across level of contamination. This disparity between body weight and SVL distribution among green frog males indicates pathology whereby the frogs are the same size, but weigh less, potentially as a result of chemical contamination. Consequently, the change in body weight may influence ear and eye measurements in green frog males, though the primary indicator of reproductive maturity is SVL, not body weight. In addition, the high correlation between SVL and ear diameter among both species (data on females not shown) reflects the overall relatedness of length parameters in these animals.

Limb and snout-vent lengths are traits that display plasticity (i.e., the ability to produce alternative phenotypes in response to the surrounding environment) and may change under different environmental conditions (Relyea 2001c). Relyea (2001a) has shown that in response to predator-induced stress, the limbs of exposed tadpoles become longer in relation to body length as adults. Moreover, in response to competitor-induced stress, body girth became larger and body length shortened in exposed tadpoles (Relyea 2001b). Therefore, the HLL to SVL ratio is a

measure of developmental growth. With the growth (HLL/SVL) index being largely uniform in our captures (along with SVL and body weight), the populations at our sites are developmentally comparable across site, species and sex.

Studies have historically used tympanum size to sex bullfrogs in the field (Currie and Bellis 1969). In addition, this technique is still prevalent and appears on web sites (Casper and Hendricks 2006) and in government guides (Schwartz and Golden 2002). However, our analysis shows that the ear to eye ratio is highly variable among bullfrog males and is unreliable as a tool. Two possible explanations arise for this variability. First, it may be that the relatively long time to full sexual maturity (Rohr et al. 2006) also applies to the development of secondary sex traits. There appears to be a critical tympanum size that females cannot achieve, 12 - 14 mm. In this case, the males that plotted with the females were at various sub-stages of maturity. Another possibility is that the chemical contamination encountered by these animals interfered with the normal expression of secondary sexual characteristics. This possibility is suggested most clearly in Figure 2.21, where eye to ear diameters were plotted for individual bullfrogs based on the level of contamination they experienced in their habitat. A trend of increasing homogeneity (from Figure 21A-C) is shown

between males and females as contamination increases for bullfrogs and the slopes for both females and males converge. This trend may indicate a hindrance in males achieving a critical, sexually dimorphic tympanum size. Green frogs, however, maintained sex-related segregation of ratios regardless of contamination level (Figure 2.23). This difference in response between bullfrogs and green frogs may be due to the relative sensitivity to toxicants of the former species (Sparling et al. 2000), and the reported resistance of the latter to hormonal effects (Russell et al. 1997). In consequence, inappropriate field sexing may yield artificial numbers to a female-bias due to feminization of genetic males lacking masculine secondary sex characteristics (Lips 1998).

Sex steroids regulate the development of secondary sex characteristics with interaction from thyroid hormones (Hayes 1998). Changes in the expression of secondary sex traits may be indicative of changes in reproductive behavior (Kelley 1988). For these reasons, endocrine disruptors can have negative impacts on populations in the following ways. Impairment or inhibition of secondary sex trait development (demasculinization or feminization) or induction in the wrong sex (feminization or masculinization), can have dramatic effects on recruitment and reproductive output (Hayes and Menendez 1999). Males vocalize

to attract females and to protect territories from other males. Meanwhile, females receive vocalizations in order to identify males and to discriminate among them for mate selection (Kelley 1988). Therefore, it is necessary that studies are performed that tie reproductive output with physiological and behavioral endpoints, endpoints which differ depending on the species of interest.

One extreme result of sex reversal is possible when the sex-reversed individuals are reproductively functional. For example, ranid frogs are male heterogametic, XY, while the female is homogametic, XX (Richards and Nace 1978). Males treated with sufficient estradiol (E_2, see Figure 2.24) become XY females that are able to produce oocytes. When crossed back to normal males, only 25% of the progeny will be female while the rest will be male, with a third of the phenotypic males YY, instead of the expected 50:50 ratio (Ogata et al. 2003) without further estradiol input. Successful breeding of the YY males would produce 100% male progeny, again without further estradiol input. Thus, breeding between normal and sex-reversed animals only further skews sex ratios (Richards and Nace 1978).

Due to the influences of contaminants and other environmental stressors, reproductive determination and differentiation may

become altered, while the animals appear normal externally. Among other parameters, field contamination studies must account for animal life stages and sex. However, if external techniques cannot be used, due to the bias they introduce, then new techniques need to be developed so that sexing can be definitive and still non-lethal. For instance, genetic markers could be obtained from tissue samples (either clippings or biopsies) and used to sex male and female amphibians from the field or new metamorphs from the laboratory (Kratochv 2007). The difficulty lies in the fact that sex chromosomes in amphibians cannot be karyotyped because sex chromosomes have not been identified in these animals (Hayes 1998; Reeder et al. 1998). Very few species (<4%) have genetically-identifiable sex (Hayes 1998; Berset-Brändli et al. 2006).

Our data indicate that environmental exposures to agricultural pesticides can disrupt display of secondary sexual characteristics. Alterations in masculine secondary sex trait expression may be a subtle non-lethal effect of environmental endocrine disruptors, which have the potential to impact population stability.

Figure 2.1. Location of sites
A, map of New Jersey showing Burlington County highlighted in black and Jacobstown represented as a red star, with detail of Jacobstown and surrounding areas to the right. Site locations are indicated with icons. Marker = 0.5 km. Satellite picture was obtained from Google Earth (image ©2007 State of New Jersey, ©2007 Tele Atlas, ©2007 Europa Technologies). B, legend with sites listed by low, medium or high contamination in increasing order of overall chemical contamination.

B.

Low Contamination	Medium Contamination	High Contamination
○ Durr II	◊ Croshaw	□ Durr
● Bruch II	◊ Robson East	■ Hopkins
● Bruch I	◆ Robson West	■ Rahilly

Figure 2.2. Sites of low contamination
A, Durr II is located off of Route 527 (Monmouth Road) with access between Schoolhouse and Rahilly Roads. Durr II is approximately 23 x 46 yards^2. Marker = 0.02 km. B, sites Bruch I and II are located off of Matthew's Lane in Chesterfield, NJ. Bruch I is approximately 21 x 49 yards^2. Bruch II is approximately 22 x 44 yards^2. To the west of Bruch II is a small depression highlighted in a white box, which is approximately 11.5 x 24.5 yards^2. Marker = 0.01 km. All satellite pictures were obtained from Google Earth (image ©2007 State of New Jersey, ©2007 Tele Atlas).

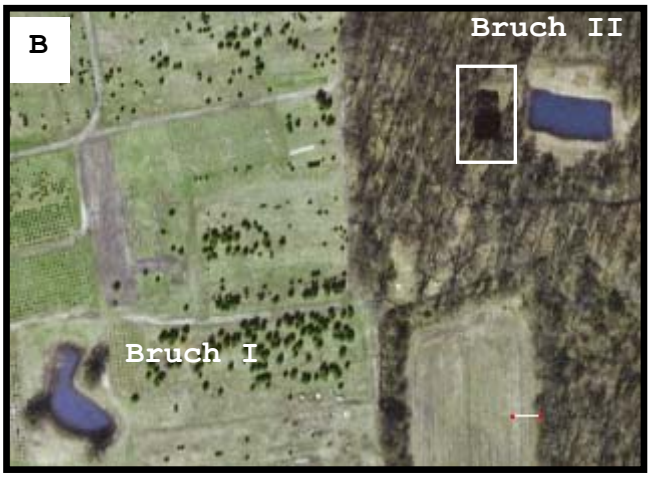
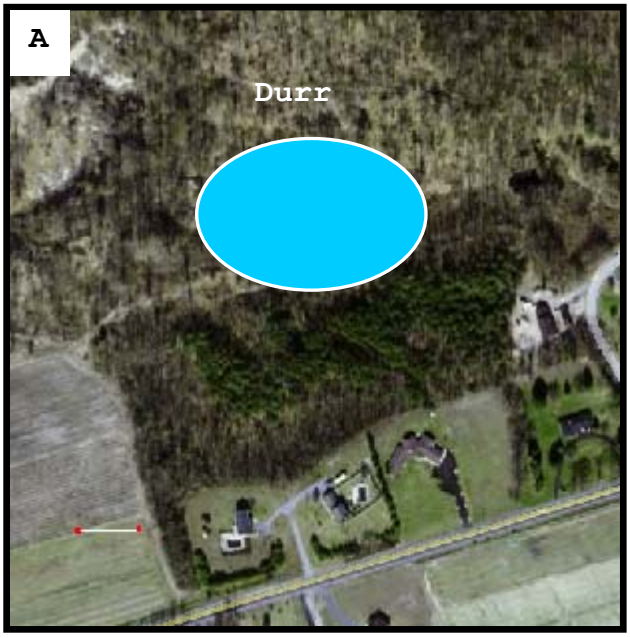


Figure 2.3. Sites of medium contamination
All three ponds are located off of Route 527 (Monmouth Road) with access between Schoolhouse and Jacobstown-Cookstown Roads. Approximate dimensions: Robson East = 36 x 110 yards^2; Robson West = 12 x 266.5 yards^2; Croshaw = 36.5 x 88 yards^2. Marker = 0.1 km. Satellite picture was obtained from Google Earth (image ©2007 State of New Jersey, ©2007 Tele Atlas).

Figure 2.4. Sites of high contamination
A, Durr is located off of Route 527 with access between Rahilly and Schoolhouse Roads. The pond is approximately 155 x 375 yards^2. Marker = 0.05 km. B, Hopkins is located off of Croshaw Road with access between Gander Way and Jacobstown-Cookstown Road. Hopkins is approximately 65.5 x 67 yards^2. Marker = 0.02 km. C, Rahilly is located off of Rahilly Road between Diane Lane and Croshaw Road. Rahilly is approximately 26 x 91 yards^2. Marker = 0.02 km. All satellite pictures were obtained from Google Earth (image ©2007 State of New Jersey, ©2007 Tele Atlas).


A
Durr


B
Hopkins


C
Rahilly

Table 2.1. Major crops and pesticides for each pond location Simazine and atrazine are selective triazine herbicides. Napropamide is a selective herbicide. Clomazone is a broad spectrum herbicide. Trifluralin is a dinitroaniline herbicide. Norflurazon is a preemergent pyridazinone herbicide. Alachlor and metolachlor are chloroacetanilide herbicides. Metribuzin is a trizinone herbicide. Acephate, chlorpyrifos and bensulide are organophosphorus insecticides. Methoxychlor is an organochlorine insecticide. Bifenthrin is a pyrethroid insecticide. Crops grown and pesticides applied at each site remained constant throughout all collection years.

Site	**Crops**	**Pesticides**
Durr II	NA	NA
Bruch II	NA	NA
Bruch I	Christmas trees	Simazine Acephate Chlorpyrifos
Croshaw	strawberries	Napropamide Clomazone
Robson East	peaches strawberries	Simazine Napropamide Chlorpyrifos
Robson West	asparagus cole crops chrysanthemums peaches strawberries	Trifluralin Norflurazon
Durr	flowers	Trifluralin Acephate Methoxychlor
Hopkins	sweet corn	Atrazine Alachlor Bifenthrin
Rahilly	cantaloupe eggplant tomatoes field corn	Atrazine Bensulide Metolachlor Devrinol Metribuzin

Table 2.2. Distribution of captures
List of captures by site, species and sex, and total caught per site. Total number captured per site per species is in parentheses. These numbers represent data used for the ear/eye indices analysis and does not reflect total frogs captured from the field.

	Durr II	**Bruch II**	**Bruch I**	**Croshaw**	**Robson East**	**Robson West**	**Durr**	**Hopkins**	**Rahilly**
Bullfrogs	(5)	(17)	(29)	(28)	(20)	(25)	(22)	(3)	(10)
Males	2	8	22	17	16	14	11	1	5
Females	3	9	7	11	4	11	11	2	5
Green Frogs	(12)	(18)	(2)	(15)	(8)	(10)	(37)	(8)	(1)
Males	10	17	1	11	7	7	31	7	0
Females	2	1	1	4	1	3	6	1	1
Total	**17**	**36**	**31**	**43**	**28**	**35**	**59**	**11**	**11**

Table 2.3. Technique accuracy, sex ratios and mortality rate A, error rate when using field sexing technique with bullfrogs and green frogs. B, resulting sex ratios in captures of both species (males were targeted). C, death rate in captures during transport.

A. Field technique accuracy

Species	% Accuracy	% Error
Bullfrog	85.2% (155/182)	14.8% (27/182)
Green Frog	100% (132/132)	0% (0/132)

B. Field capture sex ratios

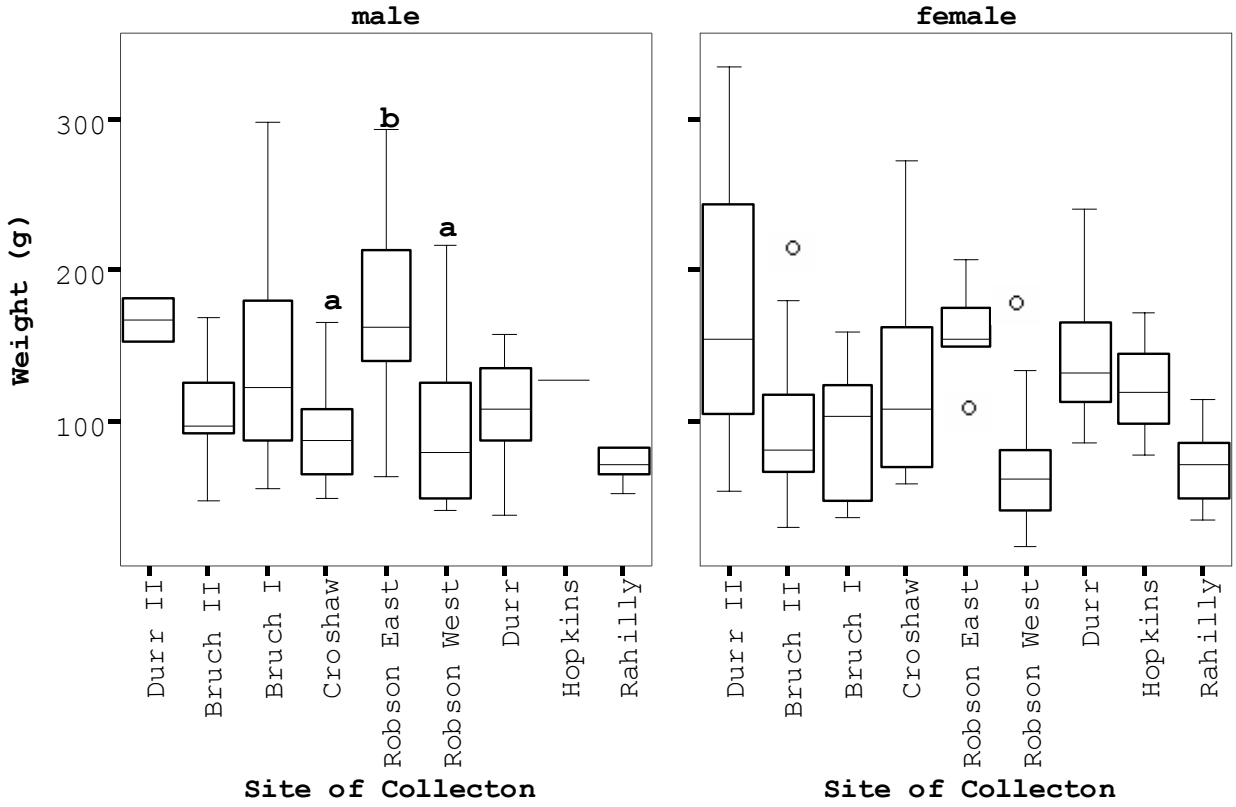
Species	Males	Females
Bullfrog	58.8% (107/182)	41.2% (75/182)
Green Frog	81.8% (108/132)	18.2% (24/132)

C. In route mortality rate

Species	Males	Females	Total†
Bullfrog	3.0% (4/133)	7.1% (7/99)	4.5% (11/242)
Green Frog	1.7% (2/120)	4.2% (1/24)	2.0% (3/149)
Total	2.4% (6/253)	6.5% (8/123)	3.6% (14/391)

†total includes unsexed juveniles

Figure 2.5. Bullfrog body weight across sites
A, body weight for bullfrogs across sites paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. Populations designated with different letters are significantly different ($p \leq 0.003$). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation, "--" indicates less than two samples and number of frogs included in each analysis is in parentheses.

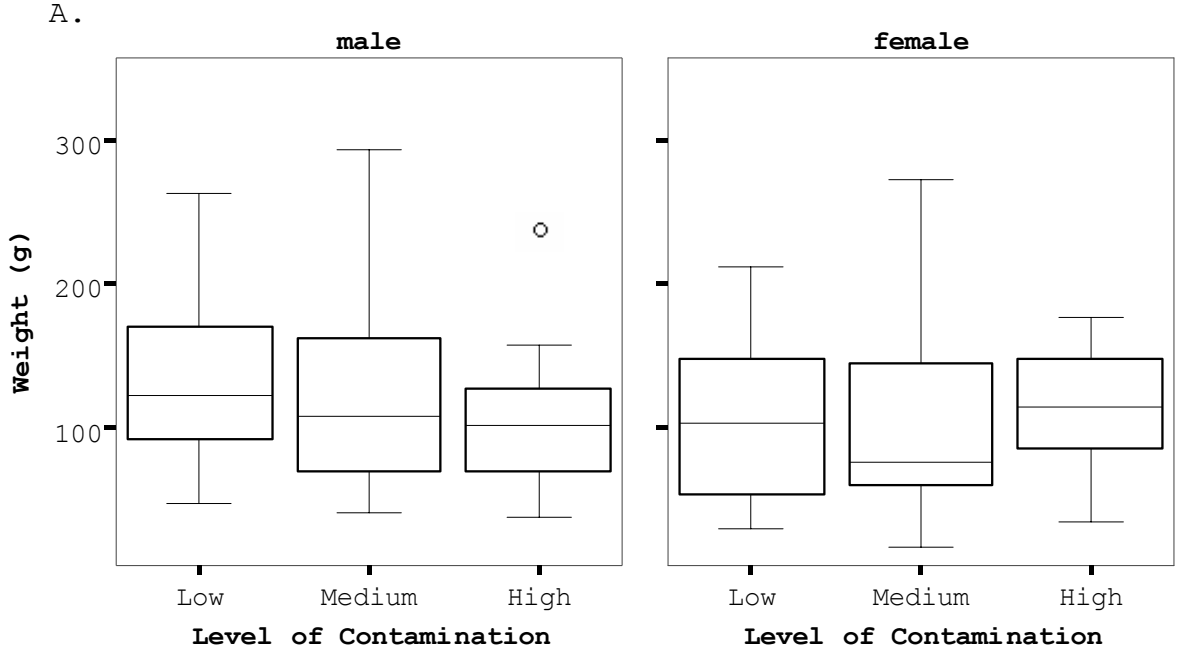


B.

Site	Males	SD/Range	Females	SD
Durr II	167.33 (2)	± 21.14	181.25 (3)	± 142.23
Bruch II	107.49 (9)	± 36.40	102.29 (9)	± 60.68
Bruch I	141.42 (22)	± 66.89	92.83 (8)	± 45.54
Croshaw	89.54 (17)	± 33.71	124.12 (11)	± 69.71
Robson East	172.65 (18)	± 59.56	158.06 (5)	± 37.23
Robson West	96.85 (20)	± 53.29	81.67 (19)	± 67.19
Durr	116.12 (10)	± 51.96	139.44 (10)	± 46.62
Hopkins	126.53 (1)	--	122.85 (3)	± 46.65
Rahilly	114.25 (5)	± 113.12	70.97 (5)	± 30.91

Figure 2.6. Bullfrog body weight by level of contamination A, body weight for bullfrogs grouped by site level of contamination and paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

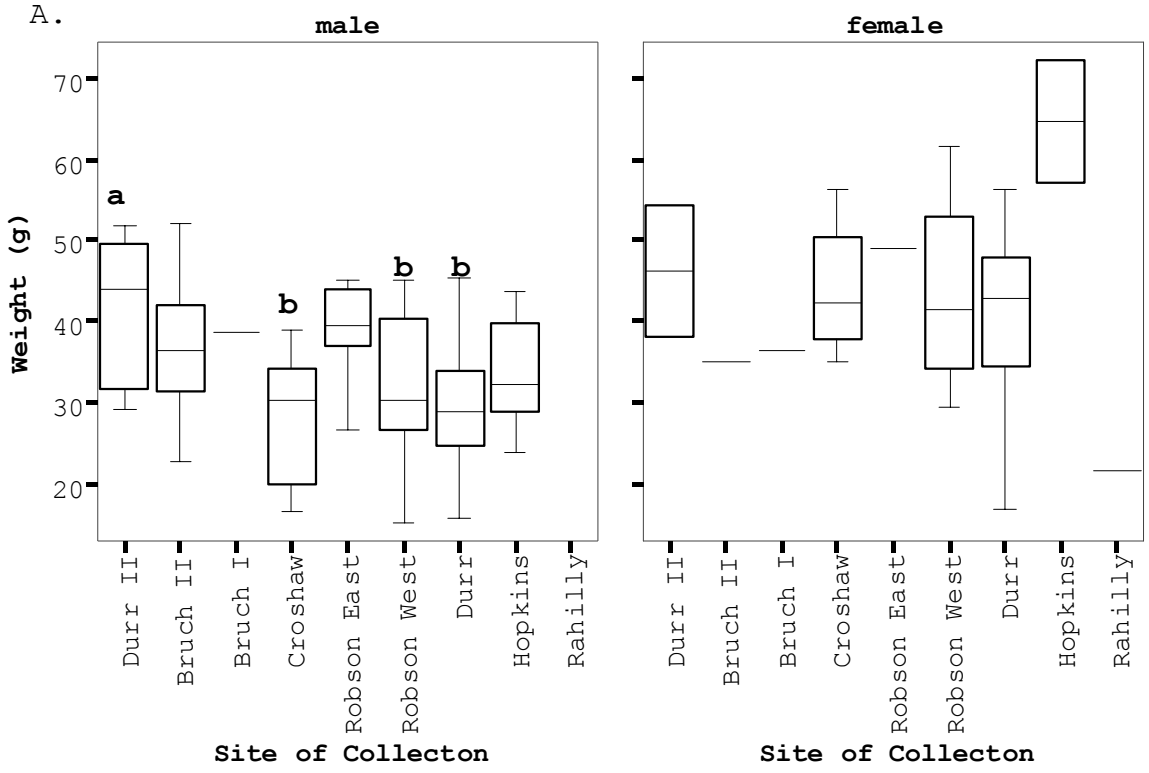


B.

Level of Contamination	Males	SD	Females	SD
Low	133.73 (32)	± 59.89	110.35 (19)	± 73.47
Medium	119.40 (55)	± 62.48	105.25 (35)	± 69.47
High	116.08 (17)	± 73.21	117.66 (17)	± 11.92

Figure 2.7. Green frog body weight across sites
A, body weight for green frogs across sites paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. Populations designated with different letters are significantly different ($p \leq 0.034$). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation, "--" indicates less than two samples and number of frogs included in each analysis is in parentheses.

A.

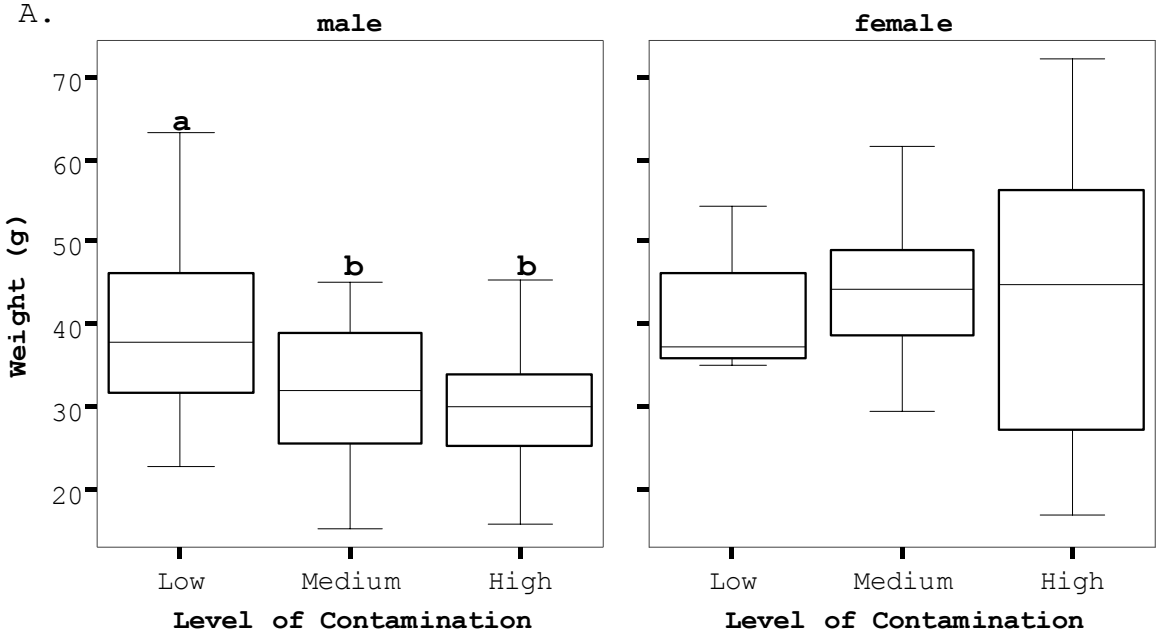


B.

Site	Males	SD	Females	SD/Range
Durr II	41.96 (10)	± 8.85	46.26 (2)	± 11.47
Bruch II	37.66 (16)	± 10.33	35.17 (1)	--
Bruch I	38.56 (1)	--	36.40 (1)	--
Croshaw	28.10 (14)	± 7.32	44.06 (4)	± 9.03
Robson East	39.08 (8)	± 5.97	48.96 (1)	--
Robson West	31.70 (18)	± 8.46	43.53 (4)	± 13.58
Durr	29.35 (32)	± 6.89	40.14 (7)	± 13.53
Hopkins	33.94 (7)	± 7.36	64.74 (2)	± 10.61
Rahilly	--	--	21.56 (1)	--

Figure 2.8. Green Frog body weight by level of contamination A, body weight for green frogs grouped by site level of contamination and paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. Populations designated with different letters are significantly different ($p \leq 0.002$). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

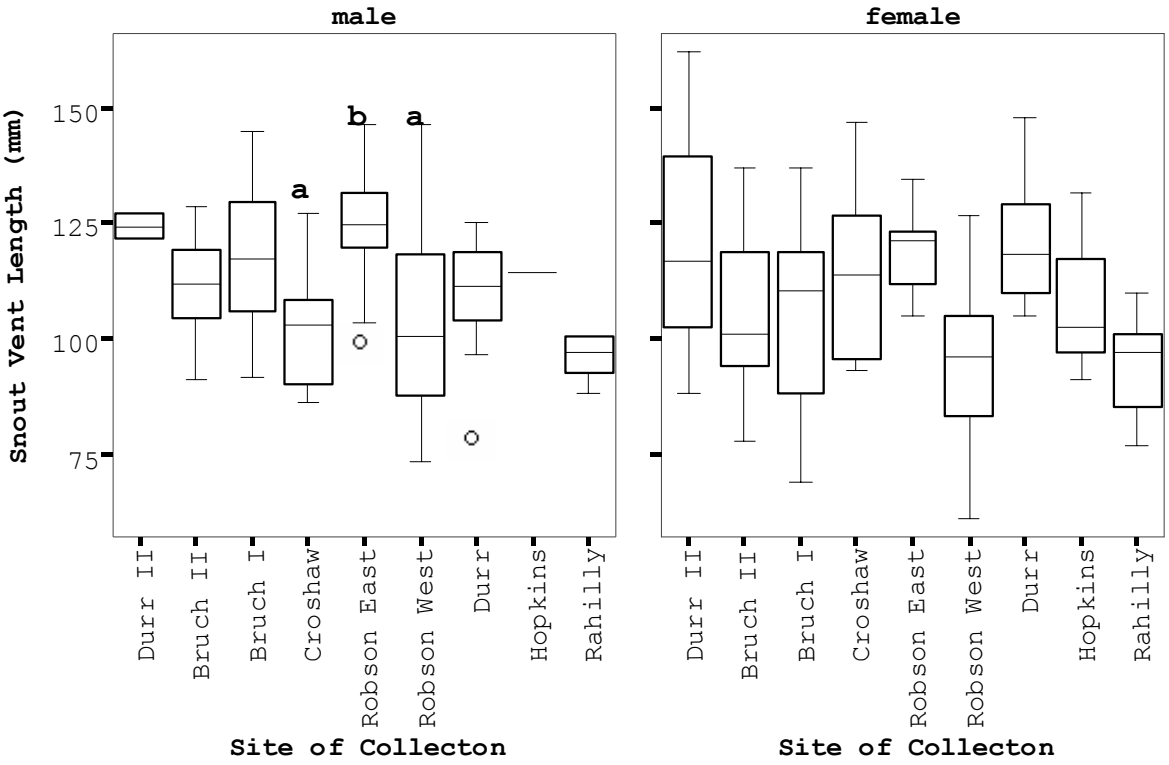


B.

Level of Contamination	Males	SD	Females	SD
Low	39.23 (28)	± 9.68	41.02 (4)	± 8.98
Medium	31.92 (40)	± 8.44	44.37 (9)	± 10.14
High	30.17 (39)	± 7.11	43.20 (10)	± 17.23

Figure 2.9. Bullfrog SVL across sites
A, SVL for bullfrogs across sites paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. Populations designated with the same letter are significantly different ($p \leq 0.003$). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation, "--" indicates less than two samples and number of frogs included in each analysis is in parentheses.

A.

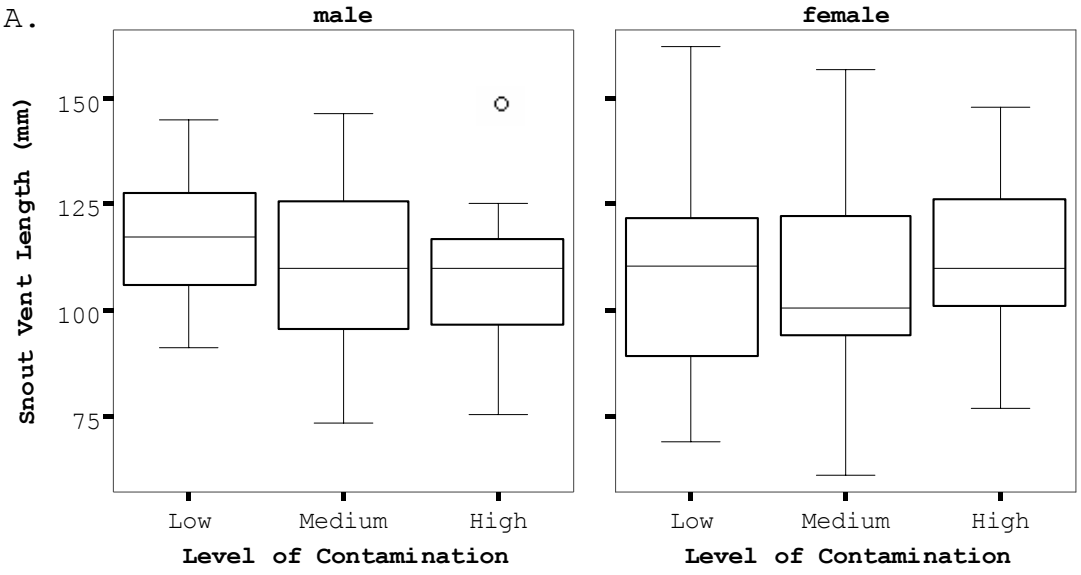


B.

Site	Males	SD/Range	Females	SD
Durr II	124.35 (2)	± 3.81	122.40 (3)	± 37.26
Bruch II	111.48 (8)	± 11.59	105.73 (9)	± 19.04
Bruch I	117.89 (22)	± 15.46	105.08 (8)	± 22.10
Croshaw	102.13 (17)	± 12.37	113.70 (11)	± 19.36
Robson East	126.00 (19)	± 12.61	119.18 (5)	± 11.29
Robson West	104.95 (20)	± 19.73	98.23 (19)	± 21.15
Durr	111.48 (10)	± 18.23	120.99 (10)	± 12.81
Hopkins	114.39 (1)	--	108.55 (3)	± 20.83
Rahilly	105.50 (5)	± 25.48	94.03 (5)	± 13.10

Figure 2.10. Bullfrog SVL by level of contamination
A, SVL for bullfrogs grouped by site level of contamination and separated by sex. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

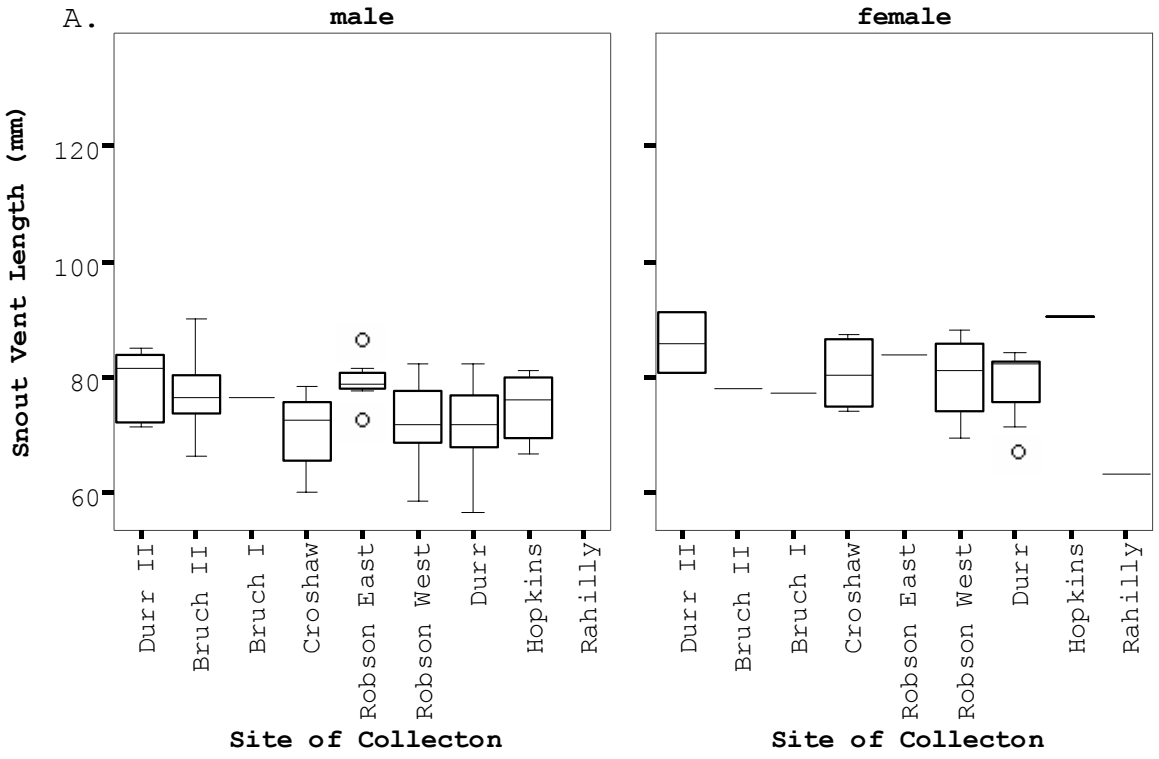


B.

Level of Contamination	Males	SD	Females	SD
Low	116.69 (32)	± 14.30	107.97 (20)	± 22.75
Medium	111.24 (56)	± 18.61	105.87 (36)	± 21.03
High	109.65 (17)	± 19.90	111.43 (18)	± 17.96

Figure 2.11. Green frog SVL across sites
A, SVL for green frogs across sites separated by sex. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation, "--" indicates less than two samples and number of frogs included in each analysis is in parentheses.

A.

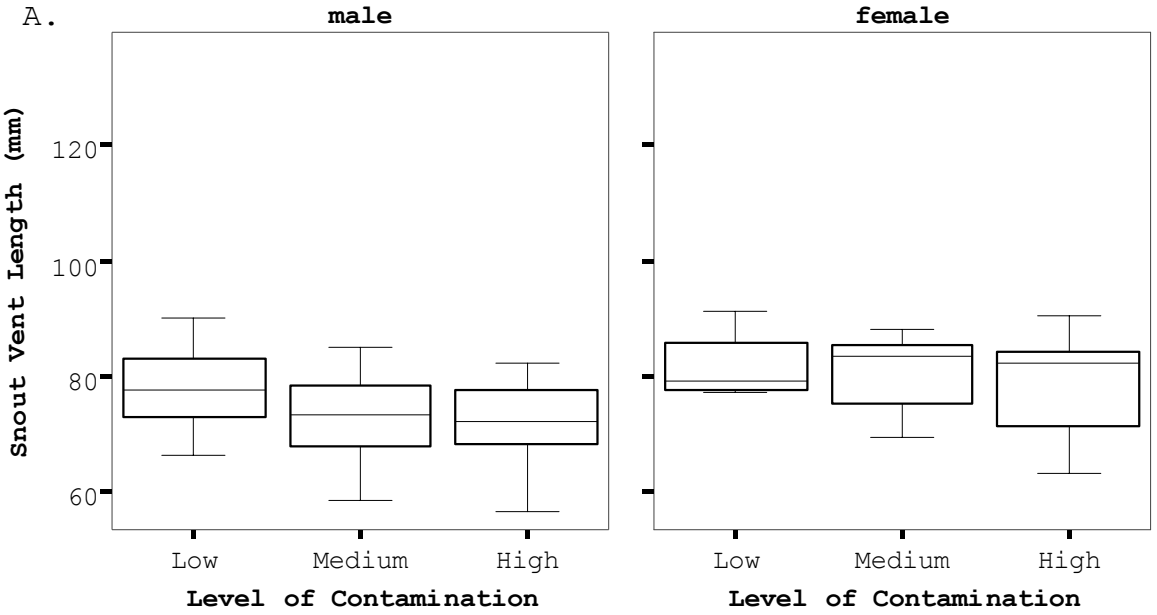


B.

Site	Males	SD	Females	SD/Range
Durr II	79.53 (10)	± 5.79	85.95 (2)	± 7.28
Bruch II	76.56 (17)	± 6.28	77.96 (1)	--
Bruch I	76.67 (1)	--	77.26 (1)	--
Croshaw	70.53 (14)	± 6.13	80.64 (4)	± 3.38
Robson East	78.94 (8)	± 3.75	83.83 (1)	--
Robson West	72.46 (18)	± 6.09	80.01 (4)	± 7.93
Durr	72.28 (31)	± 8.63	78.20 (7)	± 7.49
Hopkins	82.59 (6)	± 24.31	90.51 (2)	± 0.05
Rahilly	--	--	63.37 (1)	--

Figure 2.12. Green frog SVL by level of contamination
A, SVL for green frogs grouped by site level of contamination and paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

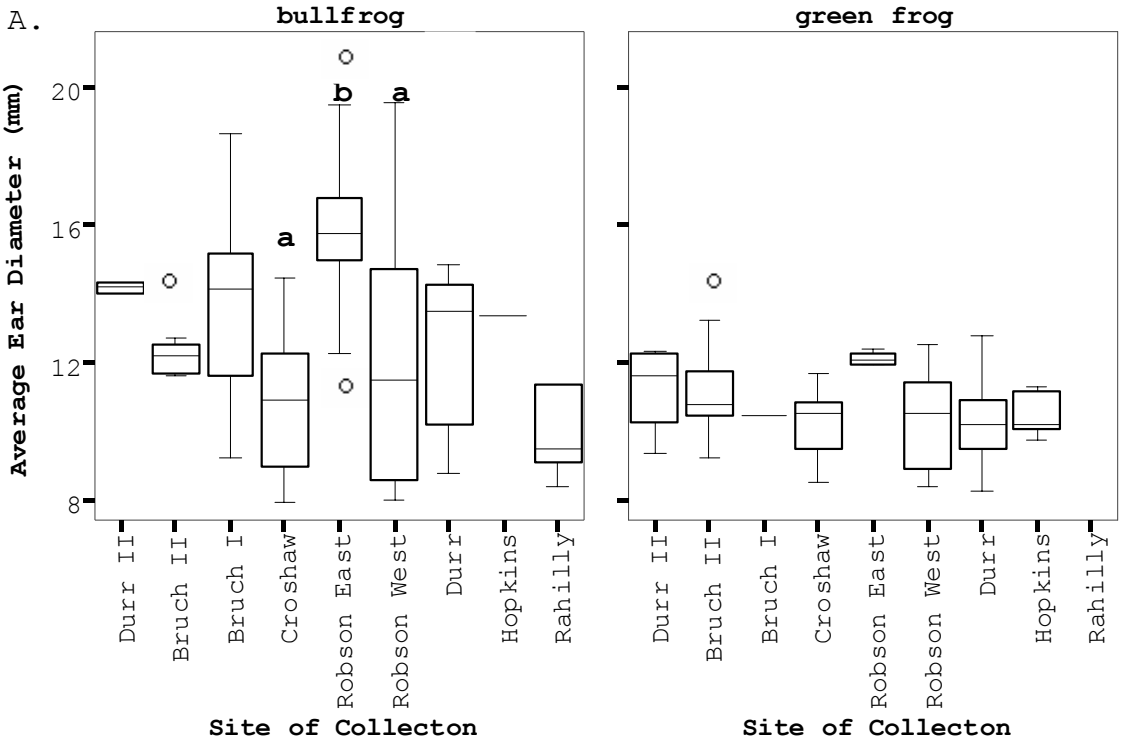


B.

Level of Contamination	Males	SD	Females	SD
Low	77.62 (28)	± 6.05	81.78 (4)	± 6.40
Medium	73.08 (40)	± 6.39	80.72 (9)	± 6.50
High	74.13 (37)	± 13.04	79.18 (10)	± 9.72

Figure 2.13. Male tympanum membrane size across sites A, tympanum membrane diameters for male frogs across sites and paneled by species. Lines in boxplots represent the 50th percentile, or median, of the data. Populations designated with the same letter are significantly different ($p \leq 0.002$). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation, "--" indicates less than two samples and number of frogs included in each analysis is in parentheses.

A.

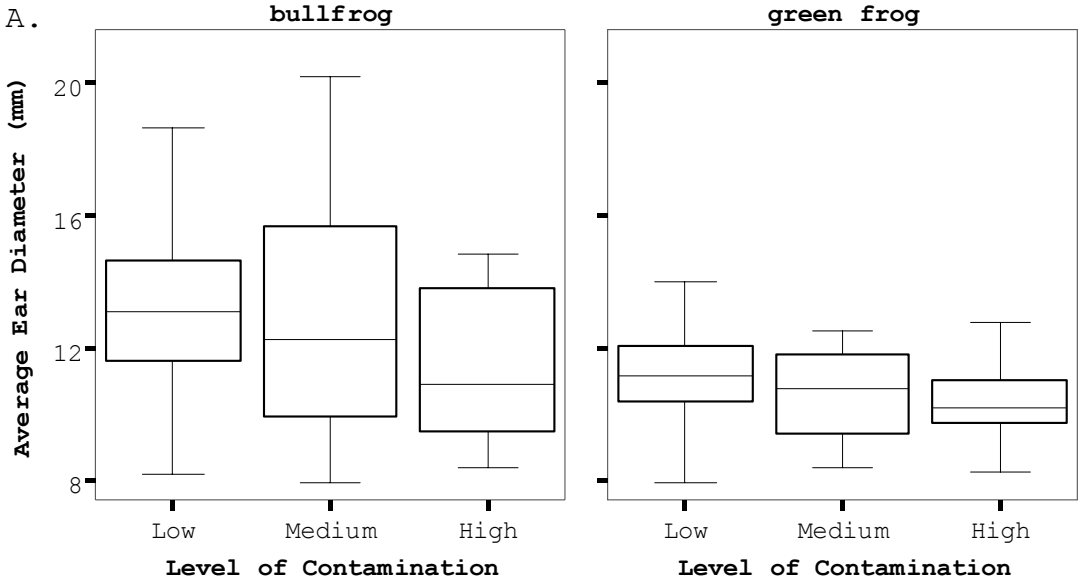


B.

Site	Bullfrogs	SD/Range	Green Frogs	SD
Durr II	14.19 (2)	± 0.22	11.28 (10)	± 1.11
Bruch II	11.90 (7)	± 1.74	11.02 (17)	± 1.42
Bruch I	13.62 (22)	± 2.72	10.47 (1)	--
Croshaw	10.91 (17)	± 1.96	10.23 (12)	± 1.04
Robson East	15.79 (17)	± 2.25	11.97 (6)	± 0.49
Robson West	11.84 (19)	± 3.54	10.25 (17)	± 1.40
Durr	12.79 (10)	± 3.55	10.48 (29)	± 1.76
Hopkins	13.35 (1)	--	11.51 (6)	± 3.03
Rahilly	11.40 (5)	± 4.65	--	--

Figure 2.14. Male tympanum membrane size by contamination level A, tympanum membrane diameters for male frogs by level of contamination and paneled by species. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

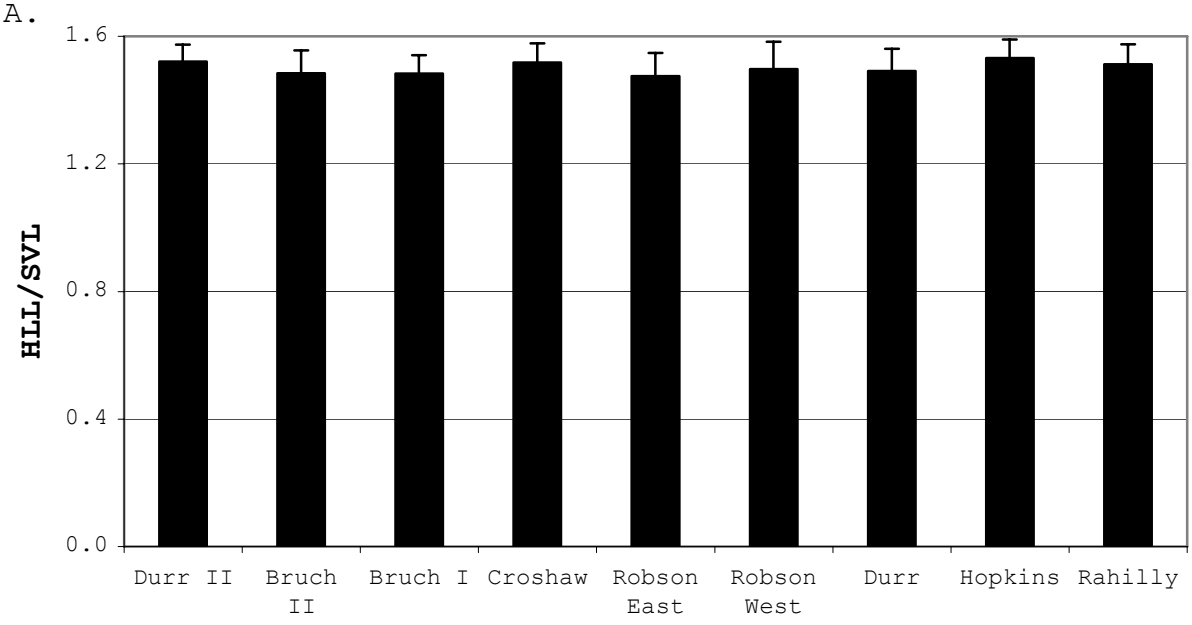


B.

Level of Contamination	Bullfrogs	SD	Green frogs	SD
Low	13.22 (32)	± 2.51	11.09 (28)	± 1.28
Medium	12.81 (53)	± 3.39	10.58 (36)	± 1.33
High	12.36 (16)	± 3.78	10.67 (35)	± 2.05

Figure 2.15. HLL/SVL across sites for all frogs
A, HLL/SVL indices for all frogs (both species, both sexes) across sites; error bars represent standard deviation. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation and the number of frogs included in each analysis is in parentheses.

A.

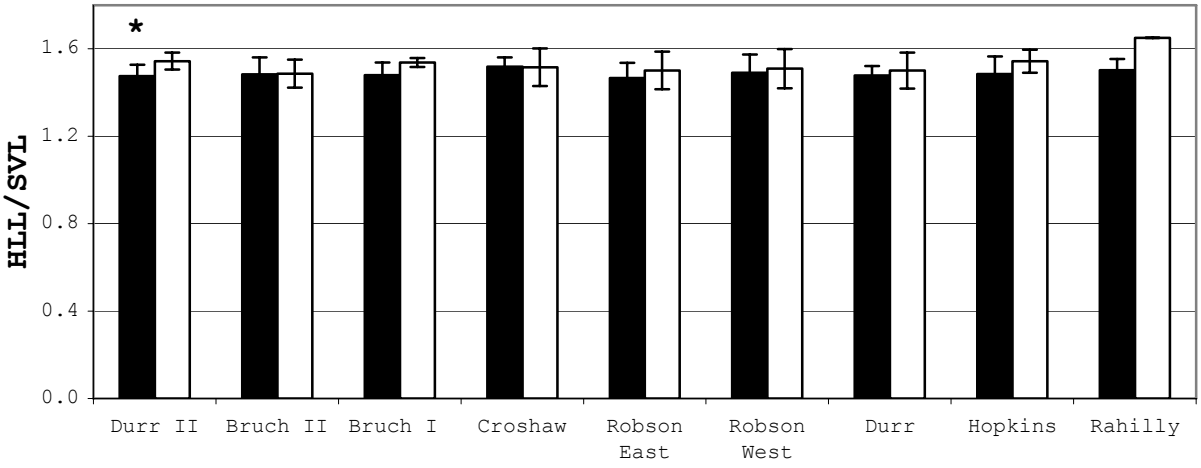


B.

Site	HLL/SVL Index	SD
Durr II (17)	1.5204	± 0.0531
Bruch II (37)	1.4849	± 0.0707
Bruch I (35)	1.4836	± 0.0575
Croshaw (49)	1.5176	± 0.0598
Robson East (46)	1.4751	± 0.0725
Robson West (70)	1.4976	± 0.0854
Durr (81)	1.4914	± 0.0690
Hopkins (10)	1.5315	± 0.0588
Rahilly (15)	1.5122	± 0.0630

Figure 2.16. Bullfrog and green frog HLL/SVL across sites A, HLL/SVL indices for all frogs across sites separated by species; error bars represent standard deviation or range. A significant difference ($p = 0.021$) between bullfrogs and green frogs within the Durr II site is indicated by "*****." Independent t-tests were used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is the standard deviation, "--" indicates less than two samples and number of frogs included in each analysis is in parentheses.

A.

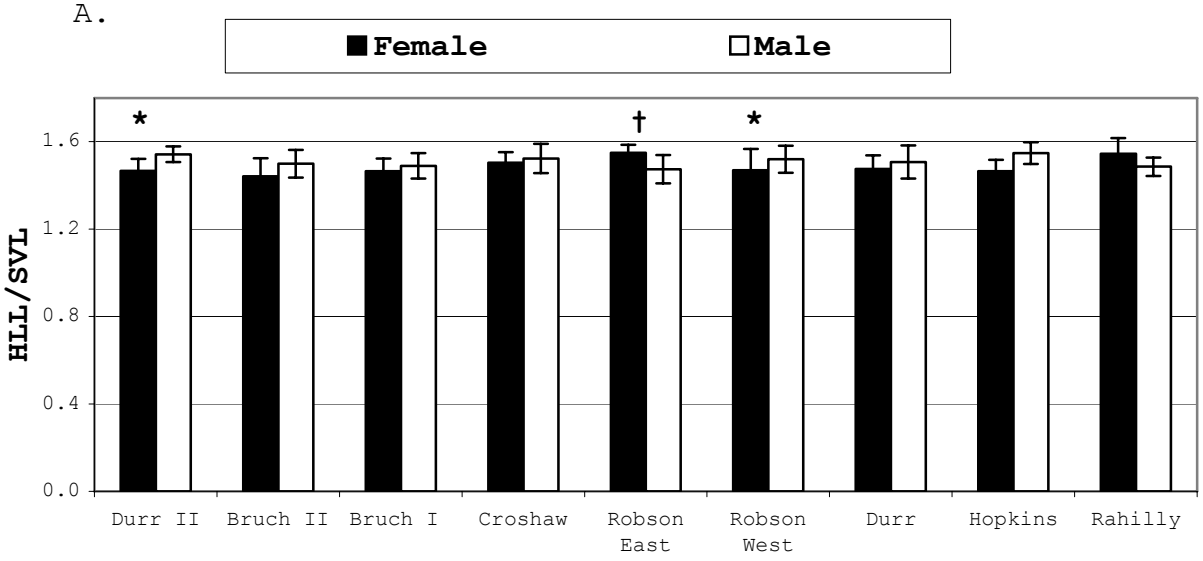


B.

Site	Bullfrogs	SD/Range	Green Frogs	SD/Range
Durr II	1.4768 (6)	± 0.0509	1.5442 (11)	± 0.0382
Bruch II	1.4830 (19)	± 0.0787	1.4870 (18)	± 0.0635
Bruch I	1.4804 (33)	± 0.0575	1.5374 (2)	± 0.0204
Croshaw	1.5185 (32)	± 0.0421	1.5160 (17)	± 0.0854
Robson East	1.4678 (36)	± 0.0681	1.5017 (10)	± 0.0853
Robson West	1.4909 (45)	± 0.0834	1.5096 (25)	± 0.0892
Durr	1.4786 (35)	± 0.0433	1.5012 (46)	± 0.0826
Hopkins	1.4854 (2)	± 0.0801	1.5431 (8)	± 0.0527
Rahilly	1.5024 (14)	± 0.0520	1.6504 (1)	--

Figure 2.17. Male and female HLL/SVL across sites
A, HLL/SVL indices for all frogs across sites separated by sex; error bars represent standard deviation or range. Significant differences (*****, p = 0.031; **†**, p **=** 0.006) between females and males within the Durr II, Robson East and Robson West sites are indicated by "*****." Independent t-tests were used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation and the number of frogs included in each analysis is in parentheses.

A.

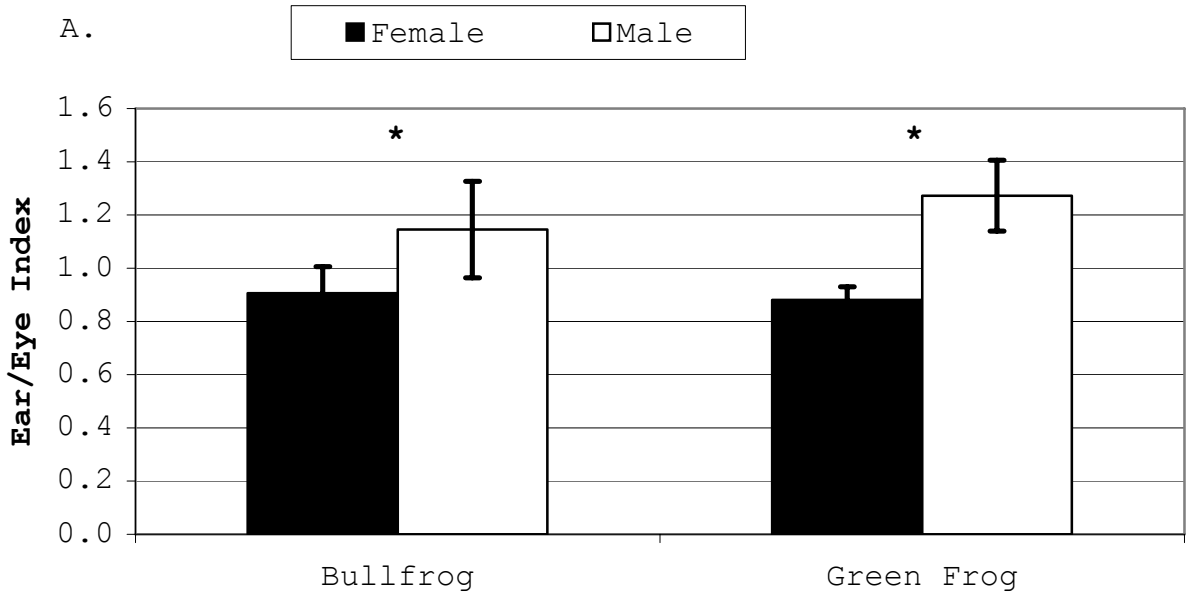


B.

Site	Females	SD/Range	Males	SD/Range
Durr II	1.4675 (5)	± 0.0534	1.5425 (12)	± 0.0356
Bruch II	1.4431 (10)	± 0.0811	1.4995 (25)	± 0.0634
Bruch I	1.4659 (9)	± 0.0569	1.4898 (26)	± 0.0575
Croshaw	1.5040 (15)	± 0.0483	1.5234 (30)	± 0.0667
Robson East	1.5494 (7)	± 0.0359	1.4745 (26)	± 0.0642
Robson West	1.4706 (24)	± 0.0967	1.5201 (39)	± 0.0611
Durr	1.4765 (17)	± 0.0612	1.5075 (44)	± 0.0749
Hopkins	1.4651 (2)	± 0.0514	1.5481 (8)	± 0.0500
Rahilly	1.5454 (6)	± 0.0719	1.4860 (5)	± 0.0417

Figure 2.18. Ear/eye index by species and sex
A, the ear/eye indices of all bullfrogs and green frogs separated by sex. The error bars represent standard deviation and "*****" indicates a significant difference ($p > 0.001$) between females and males of the same species. Independent t-tests were used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and the number of frogs in each analysis is in parentheses.

A.



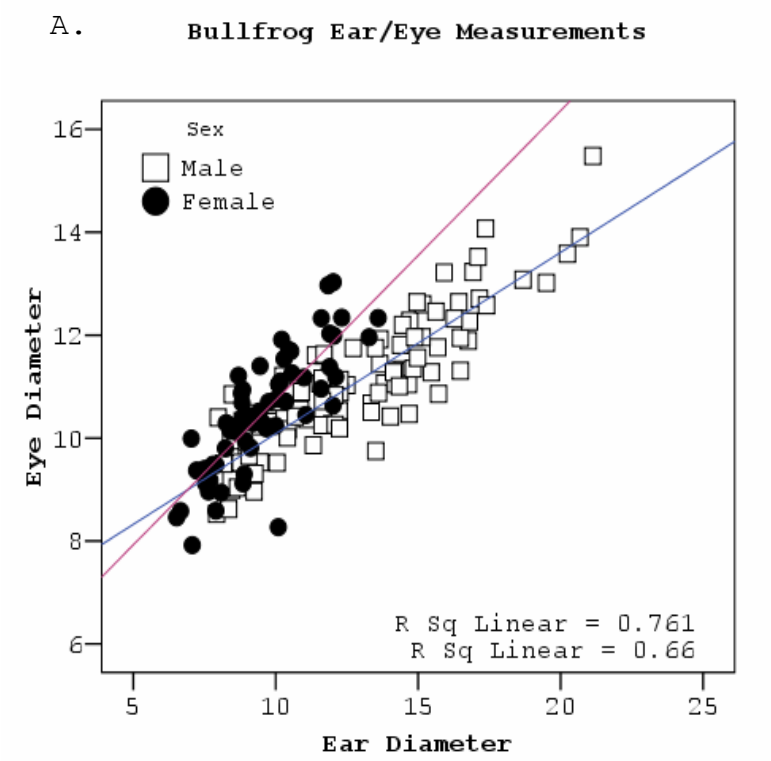
B.

Species	Females	SD	Males	SD
Bullfrogs	0.9062 (67)	± 0.0997	1.1454 (103)	± 0.1816
Green frogs	0.8811 (22)	± 0.0491	1.2727 (101)	± 0.1332

Figure 2.19. Ear/eye measurements by species
Scatter plots of average eye (y-axis) and ear (x-axis) diameters (mm) for individual (A) bullfrogs and (B) green frogs separated by sex. Lines (blue for males, pink for females) represent linear regression, whose slopes are listed below with the r-square values.

	Bullfrogs		**Green Frogs**	
	R-square	**Slope**	**R-square**	**Slope**
Male	0.761	6.56	0.460	4.93
Female	0.660	5.11	0.776	1.44

A.


Bullfrog Ear/Eye Measurements
Sex
Male
Female
Eye Diameter
Ear Diameter
R Sq Linear = 0.761
R Sq Linear = 0.66
16
14
12
10
8
6
5
10
15
20
25

B.

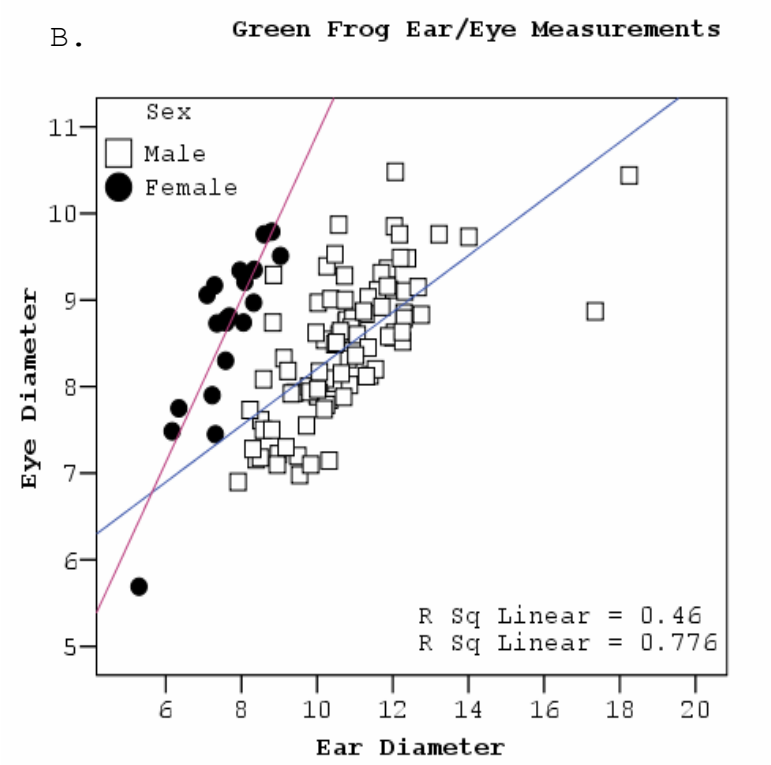
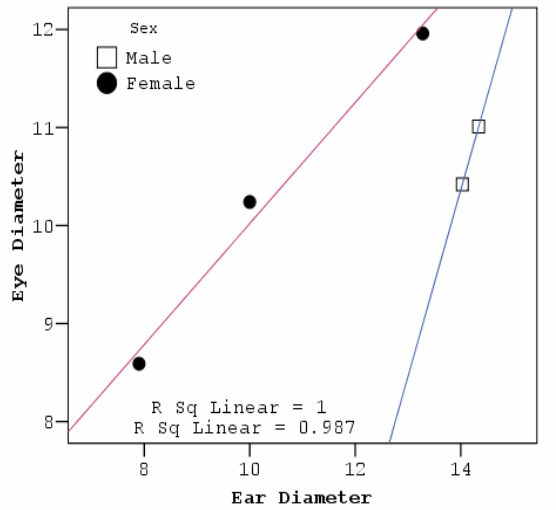

Green Frog Ear/Eye Measurements
Sex
Male
Female
Eye Diameter
Ear Diameter
R Sq Linear = 0.46
R Sq Linear = 0.776
11
10
9
8
7
6
5
6
8
10
12
14
16
18
20

Figure 2.20. Bullfrog EEI across sites
Scatter plots of average eye (y-axis) and ear (x-axis) diameters for individual bullfrogs separated by sex and paneled by sites with increasing contamination: low contamination, A - Durr II, B - Bruch II, C - Bruch I; medium contamination, D – Croshaw, E - Robson East, F - Robson West; high contamination, G – Durr, H – Hopkins, I - Rahilly. Lines (blue for males, pink for females) represent linear regression whose slopes are listed below with the r-square values. Regression lines do not appear in Hopkins due to fewer than two frogs among males. The slope and r-square values that are defined, are listed in the table below.

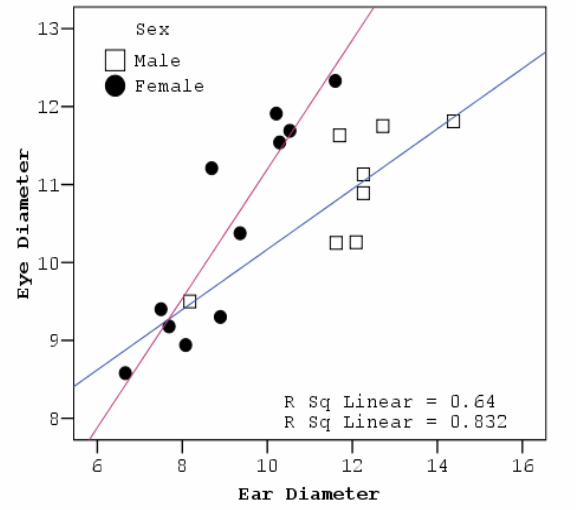
Site	**R-square**		**Slope**	
	Males	Females	Males	Females
Durr II	1.000	0.987	-16.28	3.85
Bruch II	0.640	0.832	6.30	2.91
Bruch I	0.853	0.698	5.60	5.96
Croshaw	0.588	0.845	6.78	5.82
Robson East	0.622	0.953	6.43	4.52
Robson West	0.775	0.499	7.21	7.03
Durr	0.844	0.550	5.11	3.52
Hopkins	--	1.000	--	2.54
Rahilly	0.980	0.940	5.54	3.82

"--" indicates an undefined r-square or slope where the N < 2.

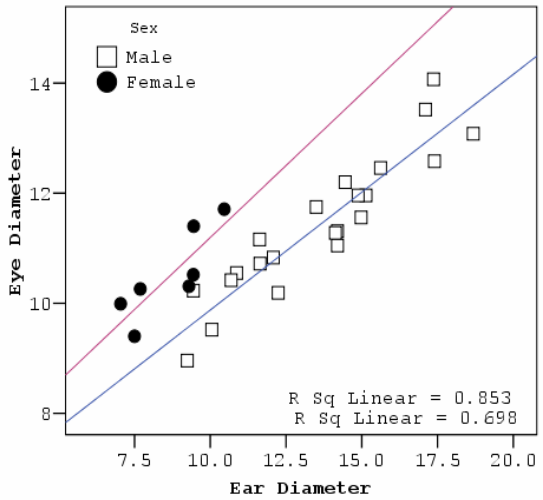
A. Bullfrog Ear/Eye Measurements at Durr II



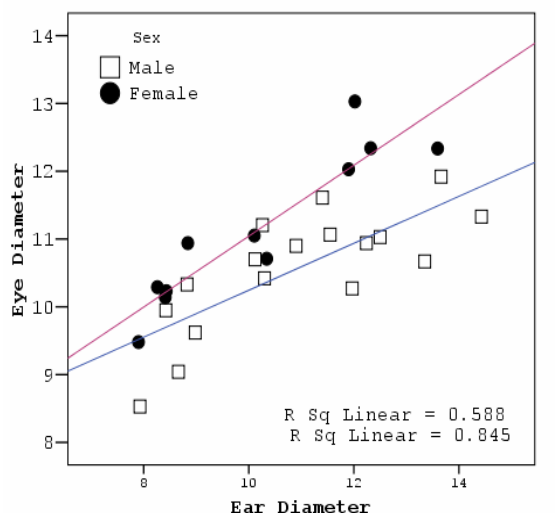
B. Bullfrog Ear/Eye Measurements at Bruch II



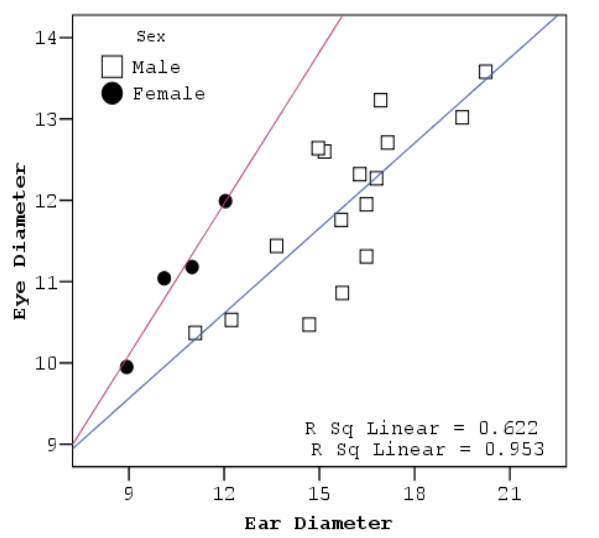
C. Bullfrog Ear/Eye Measurements at Bruch I



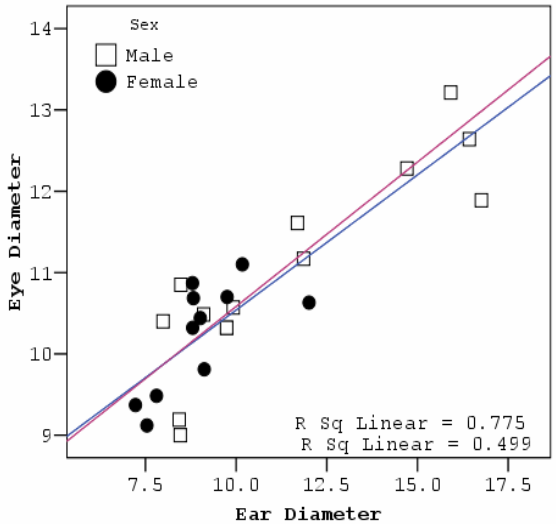
D. Bullfrog Ear/Eye Measurements at Croshaw



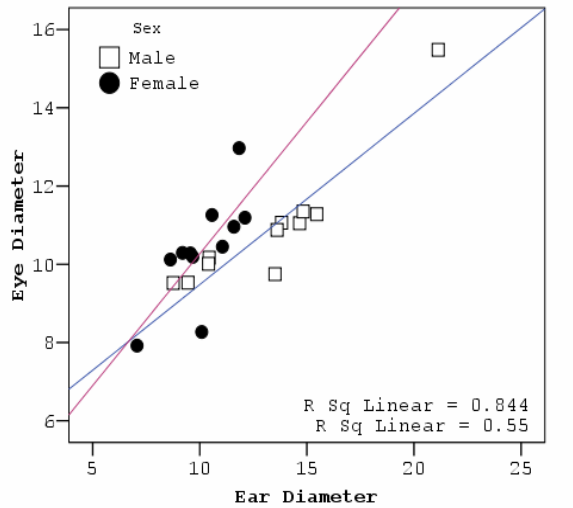
E. Bullfrog Ear/Eye Measurements at Robson East



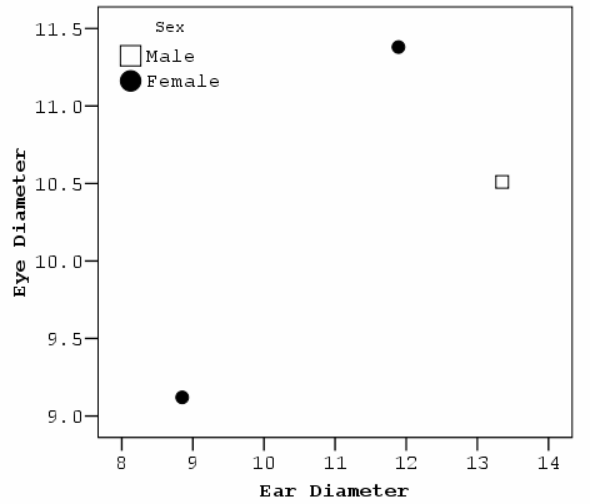
F. Bullfrog Ear/Eye Measurements at Robson West



G. Bullfrog Ear/Eye Measurements at Durr



H. Bullfrog Ear/Eye Measurements at Hopkins



I. Bullfrog Ear/Eye Measurements at Rahilly

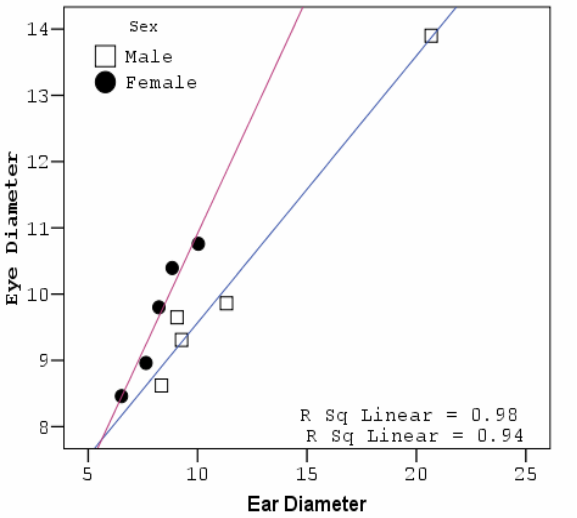
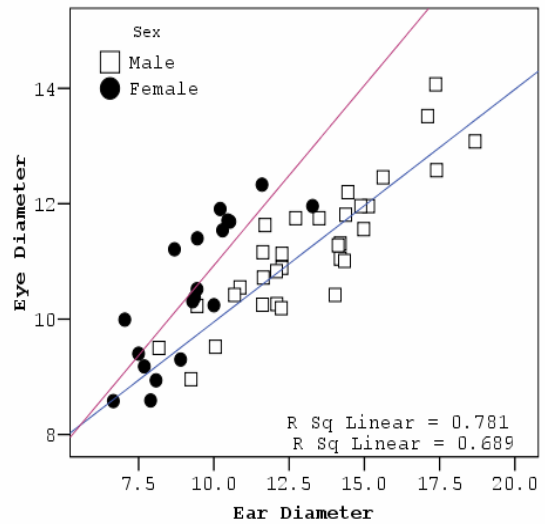


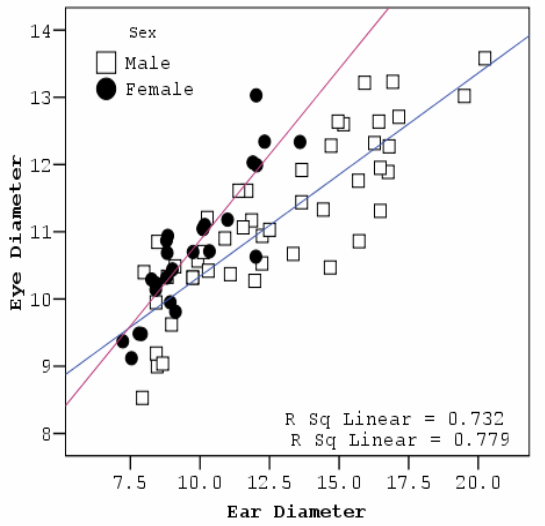
Figure 2.21. Bullfrog EEI by level of contamination
Scatter plots of average eye (y-axis) and ear (x-axis) diameters for individual bullfrogs separated by sex and paneled by level of contamination: A, low; B, medium; C, high. Lines (blue for males, pink for females) represent linear regression whose slopes are listed below with the r-square values.

Level of Contamination	**R-square**		**Slope**	
	Males	Females	Males	Females
Low	0.781	0.689	5.92	4.69
Medium	0.732	0.779	7.32	5.78
High	0.894	0.626	5.31	4.43

A. Bullfrog Ear/Eye Measurements at Low-Impacted Sites



B. Bullfrog Ear/Eye Measurements at Medium-Impacted Sites



C. Bullfrog Ear/Eye Measurements at High-Impacted Sites

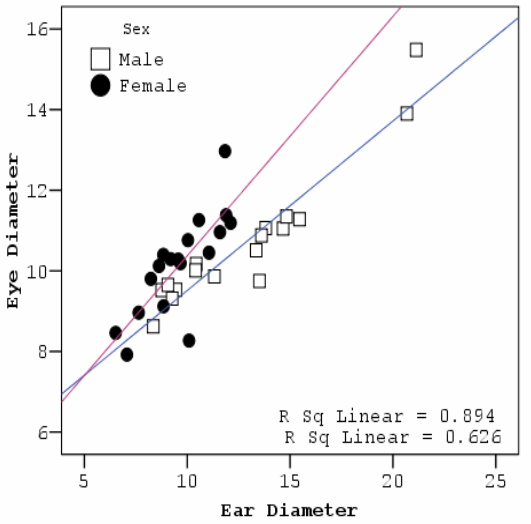
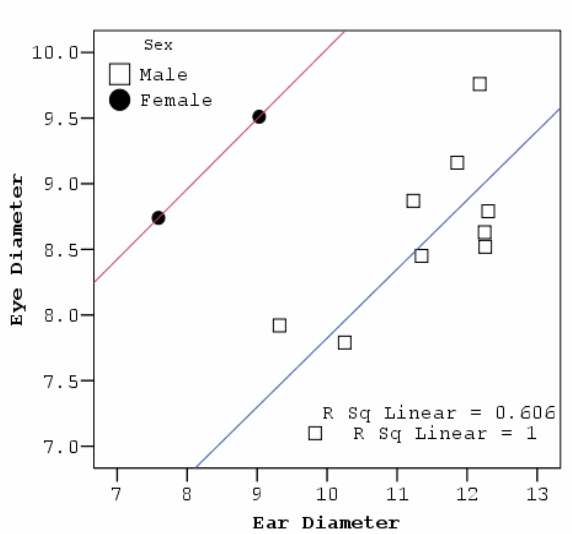


Figure 2.22. Green Frog EEI across sites
Scatter plots of average eye (y-axis) and ear (x-axis) diameters for individual green frogs separated by sex and paneled by sites with increasing contamination: low contamination, A - Durr II, B - Bruch II, C - Bruch I; medium contamination, D – Croshaw, E - Robson East, F - Robson West; high contamination, G – Durr, H – Hopkins, I - Rahilly. Lines (blue for males, pink for females) represent linear regression whose slopes are listed below with the r-square values. Regression lines do not appear in Bruch II, Bruch I, Robson East, Hopkins and Rahilly due to fewer than two frogs in males, females or both. If the slopes or r-square values are defined, they are listed in the table below.

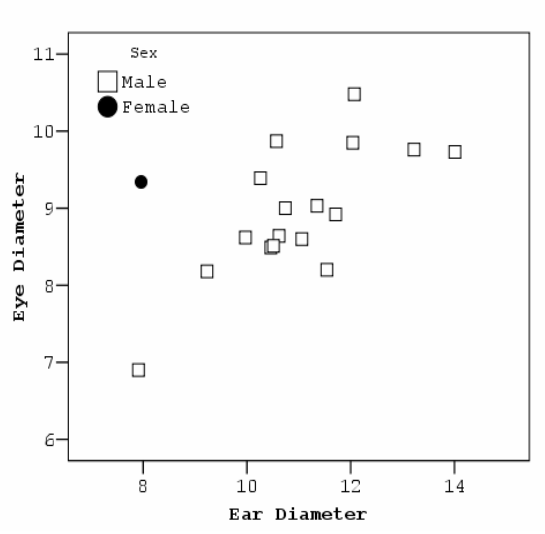
Site	**R-square**		**Slope**	
	Males	Females	Males	Females
Durr II	0.606	1.000	2.57	4.68
Bruch II	0.538	--	4.12	--
Bruch I	--	--	9.53	--
Croshaw	0.852	0.958	2.29	-2.79
Robson East	0.221	--	4.65	--
Robson West	0.466	0.897	4.22	2.49
Durr	0.308	0.872	5.87	-0.95
Hopkins	0.959	--	4.74	--
Rahilly	--	--	--	--

"--" indicates an undefined r-square or slope where the N < 2.

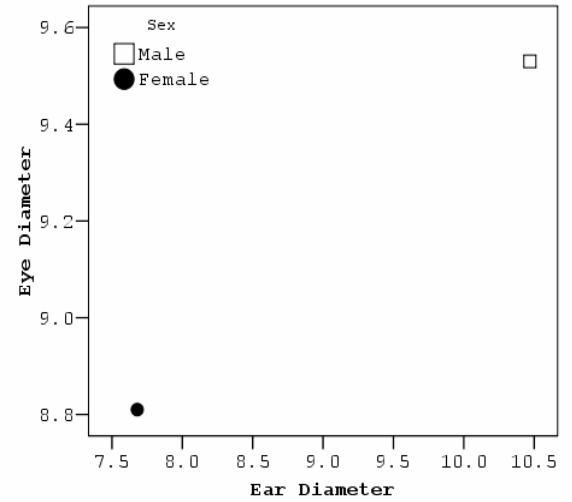
A. Green Frog Ear/Eye Measurements at Durr II



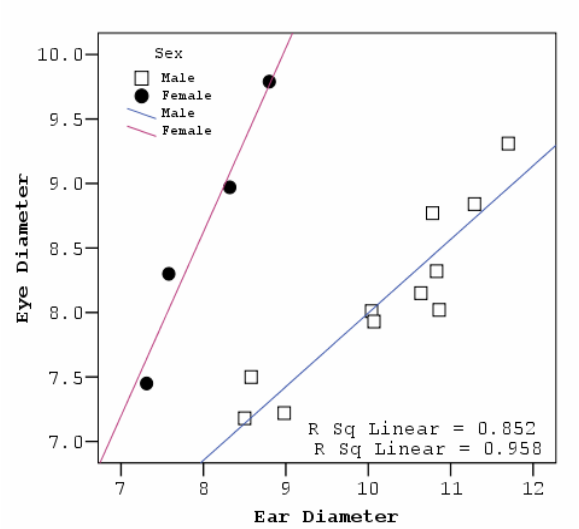
B. Green Frog Ear/Eye Measurements for Bruch II



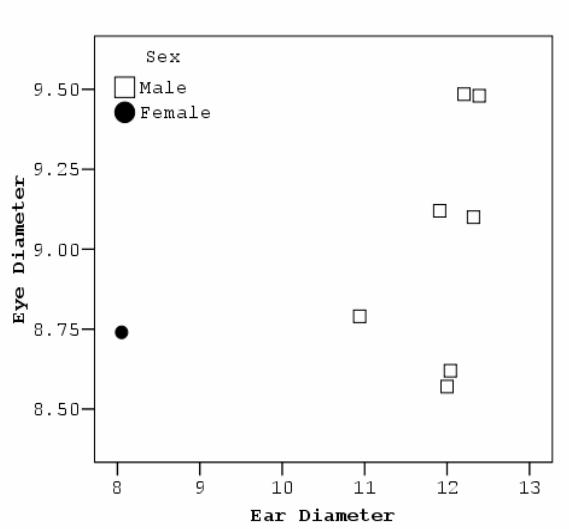
C. Green Frog Ear/Eye Measurements at Bruch I



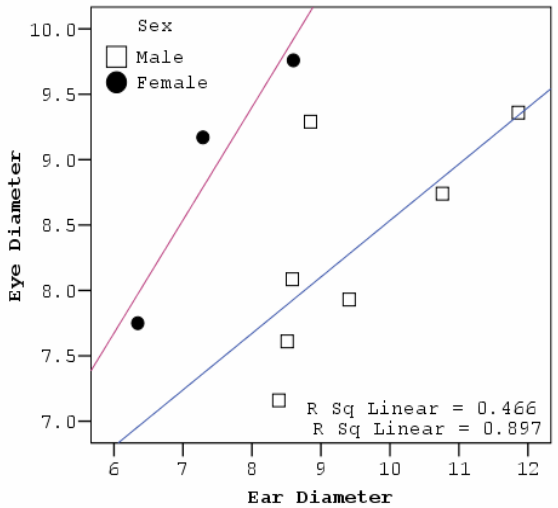
D. Green Frog Ear/Eye Measurements at Croshaw



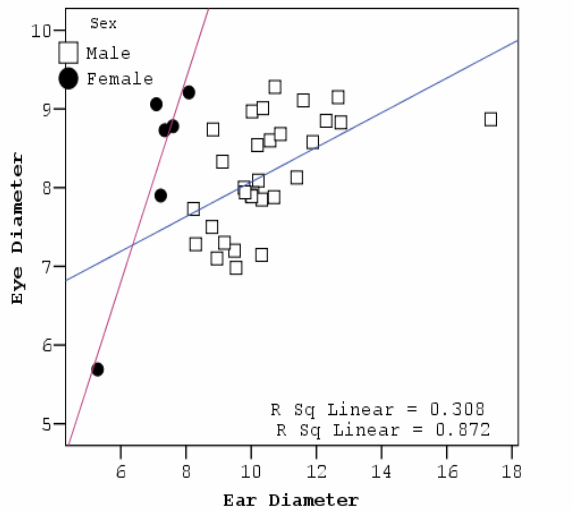
E. Green Frog Ear/Eye Measurements at Robson East



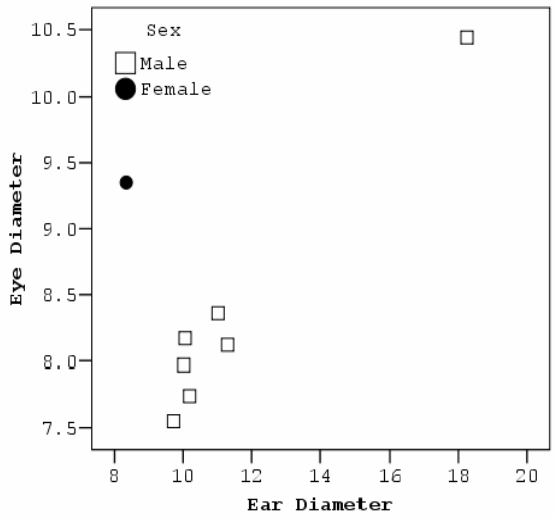
F. Green Frog Ear/Eye Measurements at Robson West



G. Green Frog Ear/Eye Measurements at Durr



H. Green Frog Ear/Eye Measurements at Hopkins



I. Green Frog Ear/Eye Measurements at Rahilly

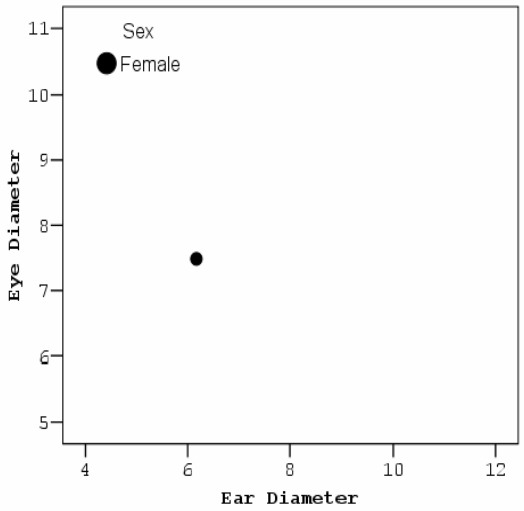
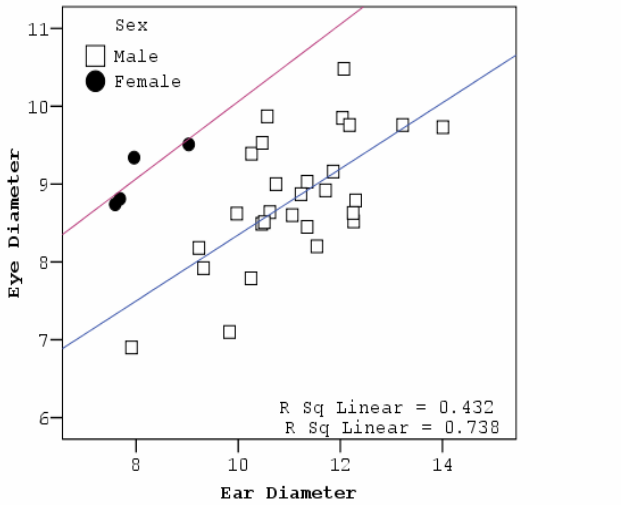


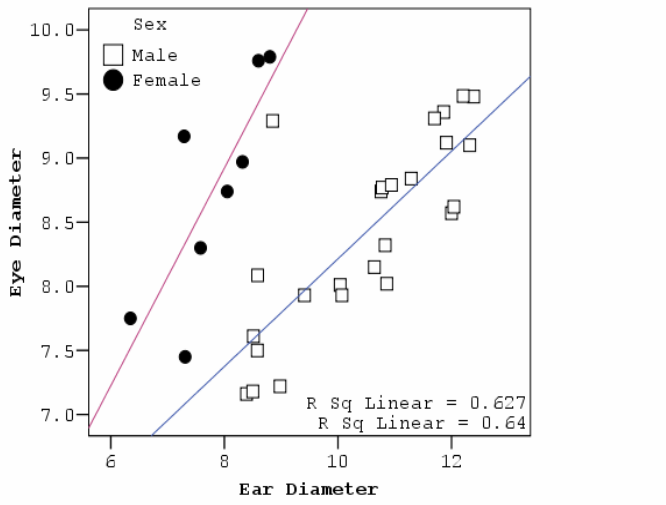
Figure 2.23. Green frog EEI by level of contamination
Scatter plots of average eye (y-axis) and ear (x-axis) diameters for individual green frogs separated by sex and paneled by level of contamination: A, low; B, medium; C, high. Lines (blue for males, pink for females) represent linear regression whose slopes are listed below with the r-square values.

Level of Contamination	**R-square**		**Slope**	
	Males	Females	Males	Females
Low	0.432	0.738	4.10	5.10
Medium	0.627	0.640	4.01	2.13
High	0.386	0.866	5.53	0.09

A. Green Frog Ear/Eye Measurements at Low-Impacted Sites



B. Green Frog Ear/Eye Measurements at Medium-Impacted Sites



C. Green Frog Ear/Eye Measurements of High-Impacted Sites

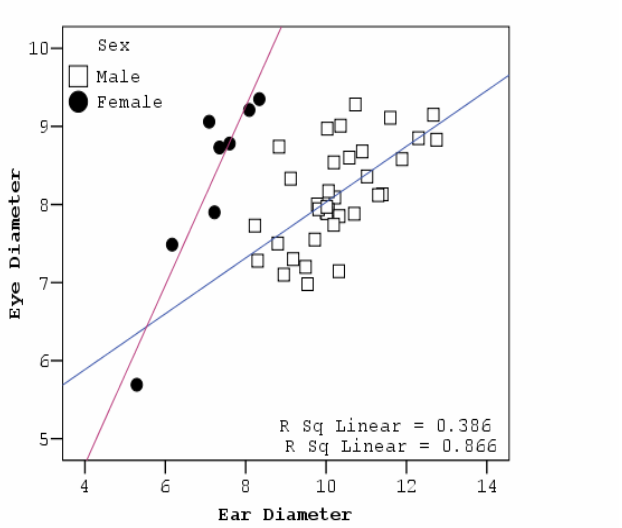
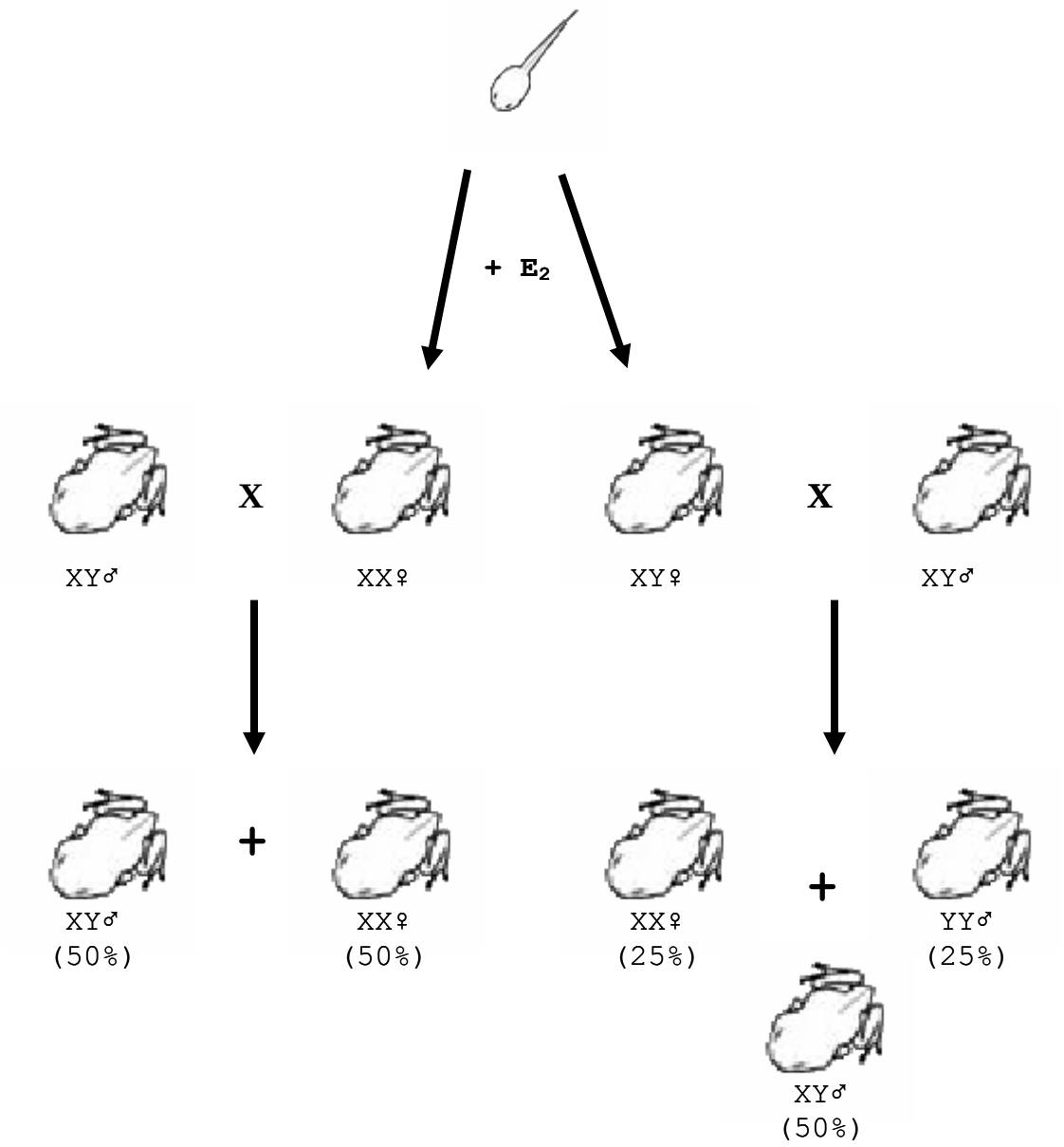


Figure 2.24. Effect of sex-reversed breeding
Example of the potential effect of breeding by sex-reversed animals. Exposure of tadpoles to estrogens results in 100% females at metamorphosis, half of which are genetic males with ovaries. Sex-reversed (XY) females are able to produce oocytes and when crossed with normal (XY) males will produce a disproportionate number of males. Adapted from (Richards and Nace 1978; Hayes 1998)



Role of atrazine in altered reproductive parameters in bullfrogs (*Rana catesbeiana*) and green frogs (*Rana clamitans melanota*) from New Jersey agricultural sites

Chapter 3

Abstract

Exposure to anthropogenic endocrine disruptors is listed as one potential cause of amphibian declines. Atrazine, an herbicide used worldwide, is reported to affect the reproductive systems of wild frogs, particularly males. Therefore, bullfrogs (*Rana catesbeiana*) and green frogs (*R. clamitans melanota*) were collected from agricultural sites in Burlington County, New Jersey to assess the effects of agrochemicals, which included atrazine, on the endocrine system, especially the reproductive tract. Various parameters were compared across sites and contamination levels to determine whether altered responses correlated with exposure. Testicular weights were decreased in bullfrogs at highly contaminated sites (21.2% compared to medium), whereas green frogs presented with reductions at medium-contaminated sites (21.6% compared to low). Reduced hematocrit was present among bullfrog females, with no differences among males of either species. Abnormal testicular

development (mainly in bullfrogs) included histologically delayed growth, hyperplasia, edema, dysplasia and calcification, and was 44.9-54.3% higher at medium-contaminated sites than low or high sites. Testicular oogenesis, the histological presence of oocytes within testicular tissue, was observed (in green frogs) with the highest incidence (16.7%) at a site impacted with atrazine. In addition, ten green frogs presented with spermatozoa within renal tissues. These two species were collected from the same sites, yet responded differently to contamination. The data raise concerns about the impact of atrazine and other endocrine disruptors on amphibian declines. This research also illustrates that a single species cannot act as a model for the whole class of amphibia because different life histories and strategies result in differential sensitivities.

Introduction

Amphibian declines have been documented in various parts of the world and reasons for the global declines are unclear (Pounds et al. 1997; Lips 1998; Alford and Richards 1999; Stokstad 2004; Stuart et al. 2004). However, chemical contamination has been implicated as one causal factor (Houlahan et al. 2000). A variety of agrochemicals disrupts endocrine function and may therefore contribute to amphibian declines by reducing

reproductive fitness. For example, Reeder et al. (2005) found that geographic distribution of endocrine disruption (as measured by percent intersexuality) were consistent with population declines in the cricket frog (*Acris crepitans*). Furthermore, drainage ditches and farm ponds, which serve as breeding sites for amphibians, receive major inputs from fertilizers and pesticides annually (Sparling et al. 2000). It is in these small volume systems where chemical exposures can become disproportionately large.

One such agrochemical that is heavily used worldwide is atrazine. The literature suggests that very low concentrations (>0.1 ppb) of atrazine in the environment have the ability to cause male frogs to develop into hermaphrodites (Reeder et al. 1998; Tavera-Mendoza et al. 2002a; Hayes et al. 2003), lower testosterone levels (by ten-fold) and develop smaller vocal organs (Hayes et al. 2002b). Reeder et al. (1998) found that the prevalence of intersexed cricket frogs approached significance ($p = 0.07$) when correlated with atrazine in their habitats. In addition, a field study conducted by Hayes et al. (2003) found that the lowest doses (0.1 ppb) of atrazine had the largest effects on frogs, including retarded gonadal development and testicular oogenesis (the presence of egg cells within testicular tissue) in native leopard frogs (*Rana pipiens*) across

the United States. Endocrine disruptors have been reported to cause more marked developmental effects at lower doses because these concentrations are within the range of maximal responsiveness in target tissues (Hayes et al. 2002a; Roberts 2006).

Based on this evidence of reproductive effects, some researchers contend that the widespread use of atrazine may contribute to the worldwide declines of amphibian populations (Howe et al. 1998; Diana et al. 2000; Hayes et al. 2002a; Hayes et al. 2002b; Tavera-Mendoza et al. 2002a, 2002b; Goulet and Hontela 2003; Sullivan and Spence 2003; Rohr et al. 2006). However, atrazine's effects have been evaluated in few species other than *Xenopus laevis* (Hayes et al. 2002b; Tavera-Mendoza et al. 2002a) and the leopard frog (Hayes et al. 2002a, 2003), and it is unknown whether these reproductive effects are widespread among amphibian populations or are confined to a few species.

To determine if environmental exposure to atrazine has the potential to adversely affect amphibian populations in New Jersey, a study was conducted over three breeding seasons (2003, 2004 and 2005). The objectives of this reconnaissance study were to survey the levels of atrazine and related compounds in New Jersey agricultural ponds, survey the resident frog

populations for gross morphogenic changes and evaluate potential endocrine-mediated adverse effects, mainly through histopathologic observations of the reproductive organs. Effects at the histological level were correlated with levels of contaminants in the habitats of bullfrogs and green frogs.

Nine ponds were identified on farms with histories of atrazine and other pesticide use and the presence of frog populations. Pesticide presence and level were determined for each pond, and the ponds were classified as low, medium or high pesticide exposure. These bodies of water were located near treated fields and received agricultural runoff, which would include atrazine if applied.

As a part of ongoing research investigating risk factors for endocrine burdens and their effects on public health, this study's goal was to determine whether native New Jersey frogs exposed to environmental atrazine concentrations had a higher incidence of gonadal anomalies than unexposed frogs. The purpose of this assessment was to determine if atrazine poses an environmental health concern in the state.

Materials and Methods

Study area. All collection sites were within the same vicinity of Jacobstown in Burlington County, New Jersey (see Chapter 2 for location details). The main crops and pesticides (as listed in Chapter 2) remained consistent throughout the collection years.

Test organisms. The target species (bullfrogs and green frogs) were chosen for several reasons. These species are abundant, not threatened in New Jersey, and likely to be the most numerous ranid species at our collection sites. The species were also chosen for their size: as the two largest frogs in the United States they are easy to spot in the field and straightforward to dissect. In addition, their aquatic nature keeps both species in continual contact with potentially contaminated waters as both larvae and adults {Roy, 2002 #584;Frogwatch, 2001 #1044}.

Water collection. Where possible, a 1 liter amber glass bottle was carried into the pond until the pond was at a depth of mid-thigh; otherwise, water was collected where accessible as one is not able to wade in all ponds. The bottle was rinsed with pond water and inserted into the water face down. About a foot deep, the bottle was turned up and allowed to fill. Once filled, the bottle was secured with its cap, labeled with site name and

date, transferred to an ice chest and transported to 4°C storage until analyzed.

Frog collection. Representative samples of frogs (three males of each target species) were collected from each site during three time periods of the breeding season: early, mid and late. The goal was to collect nine male frogs per site, per species, per year. Collections proceeded as previously described in Chapter 2, with the following additional details. Frogs were anesthetized one at a time with 1% tricaine methanesulfonate (MS-222). Once the frog was anesthetized and unresponsive to stimuli of toe pinch or eye touch (about five minutes), blood was collected on site via cardiac puncture. Cardiac puncture was performed as follows: using a 1 mL syringe with a 26 gauge ½" needle (with larger bullfrogs, a 5/8" needle, Becton Dickinson & Co., was used when necessary), the needle was positioned almost flat on the frog's ventral surface, beveled tip up, and just below the frog's xiphisternum while the animal laid on its back. The needle was inserted through the skin and the plunger pulled back a few millimeters to create a slight vacuum. The needle was slowly advanced toward the heart until blood flow into the syringe began. If this technique proved unsuccessful, the heart could also be accessed through the coracoids on either side of the heart. As the syringe filled

with blood, the plunger was gently pulled back. Once blood flow ceased or the syringe was full, the needle was removed. Contents of the syringe were transferred into a 2 mL vacutainer containing sodium heparin (Becton Dickinson & Co.). Vacutainers were labeled and stored in a small chest containing wet ice for transfer to the laboratory. Captures were held in half-gallon glass jars containing pond water from the frogs' habitats and allowed to recover from the anesthesia.

Water analysis. All chemical analyses were performed at the CORE Facility, under the supervision of Dr. Brian Buckley, in the Environmental and Occupational Health Sciences Institute (EOHSI), a research facility at Rutgers University and the Robert Wood Johnson School of Medicine. Pond water samples were prepared for continuous flow-solid phase extraction (CF-SPE) by addition of methanol and hydrochloric acid. Cartridges (6 mL Supelclean ENVI-18SPE tubes, Supelco, St. Louis, MO) were conditioned and samples eluted using the following solvent systems: ethyl acetate, methylene chloride, methylene chloride:ethyl acetate (1:1), acetone:hexane (3:2) and methanol. Concentrated, eluted samples were loaded onto a Varian 3400 CX gas chromatograph (GC) system with helium as the carrier gas. Data acquisition started at five minutes and the total GC program time was 60 minutes. The GC was coupled to a Saturn

2000 GC/MS ion trap mass spectrometer (ITMS, Varian Inc., Walnut Creek, CA). For screening of semi-volatile organic compounds in the water samples, the ITMS was operated in the electron ionization positive mode.

Animal dissection and tissue harvesting. In the laboratory, all measurements and observations were recorded on data recording sheets prepared for each animal. At the top of the sheet was each animal's unique accession number determined as follows: Collection#SiteDevelopmentalStageSpeciesSexCapture#; e.g., 16REABM3 was from the 16th collection event, from the Robson East site, was an adult bullfrog male and was the third (3) capture from that site.

Frogs were placed in 1% MS-222 as before (see frog collection). Once anesthetized, animals were patted dry with paper towels and weighed to the nearest hundredth gram. With digital calipers, length measurements were taken as per Chapter 2. Blood was collected again in the laboratory in order to test whether hormone levels changed after capture as previously described (see frog collection) with death intended by exsanguination. Frogs were laid on their stomachs and pithed at the base of the head. Frogs were then turned over onto their backs, a midline incision made and the skin and muscle covering underlying

tissues removed. The aortic arch of the heart was located and cut. Two capillary tubes were filled with blood from the heart, sealed at one end and spun for five minutes in a micro hematocrit centrifuge, measurements taken and recorded. The heart was removed to ensure death. The left liver lobe was removed and fixed in 10% buffered formalin. The right lobe was weighed, wrapped in labeled aluminum foil, frozen in dry ice then stored at -80°C for potential future chemical analyses. Once the liver was removed, gonads were visualized, identified and recorded. If the gonads were testes, each testis was removed and weighed individually before being placed in fixative. Kidneys were then removed from the body cavity, weighed in males, and placed in fixative. The larynx was then dissected out and cut along the anteroposterior axis. The right side was cut again along the dorsoventral axis, then fixed and decalcified in Cal-EX II (Fisher Scientific). The frog was decapitated and the upper portion of the head containing the brain was placed in 10% buffered formalin. After three days in fixative, tissues were preserved in 70% ethanol, while skulls were washed and decalcified in Cal-Ex overnight. Cal-Ex II was not used on brain tissue as subsequent histological observations revealed artifacts due to rapid hydropic change, that is cavity formation in the tissue. Once decalcified, brains were dissected out of their casings and cut on the anteroposterior

axis, and preserved in 70% ethanol until processing. Small juvenile brains were fixed, decalcified, processed and sliced within their skull casings.

Testosterone radioimmunoassay. In the laboratory, vacutainers from the field or the laboratory were spun for 10 minutes at ~3500 rpms. The plasma (upper clear portion) was transferred to labeled 1.5 mL microcentrifuge tubes at a maximum volume of 500 µl and stored at -20°C until utilized for testosterone analysis. Frozen plasma samples were thawed on ice. Samples (20 µL) were prepared in double-distilled water and spiked with ^{3}H-testosterone. The sample and tritiated hormone were allowed to reach equilibrium. Hormones were then extracted using ether, evaporated in a 37°C water bath under ultra-filtered air and reconstituted in phosphate-buffered saline with gelatin (PBSG) overnight. Approximately one-third of the reconstituted hormone, ^{3}H-testosterone and testosterone antibody were assayed for hormone-antibody binding in duplicate overnight to allow ratios of bound and unbound hormones to reach equilibrium. In addition, a standard curve was assayed in triplicate. The reactions were stopped using charcoal, then spun at 4°C at 2200 rpms for fifteen minutes. The bound fraction of testosterone was decanted into liquid scintillation vials and ^{3}H-testosterone was detected by a scintillation counter. Using the standard

curve, the program RIAMENU calculated the amount present (in pg) of each sample based on the sample's counts per minute (cpm). After adjustment calculations (for dilution factor, duplication, percent recoveries and sample volume), the concentration of plasma testosterone (ng/mL) was obtained. Detection limit: 2.4 pg (~0.46 ng/mL).

Testiculo-kidney index (TKI). The combined weight of the testes was divided by the weight of the kidney to obtain the testiculo-kidney index (TKI). The TKI is a measure of relative testes weight normalized to the kidney. Kidney weight was used as it is an organ that will not undergo much weight change, in contrast to body weight, which fluctuates with energy assimilation and utilization during the season.

Histology. Tissues preserved in 70% ethanol were processed overnight and dehydrated in graded alcohols (70% ethanol, 95% ethanol, 100% ethanol and finally xylene), then infiltrated with paraplast. Processed tissues were embedded in paraffin wax, and tissue sections were cut at 6 µm and mounted onto glass slides. Slides were stained with hematoxylin and eosin, coverslipped and viewed under a microscope. Observations were recorded on data sheets created for each frog and photographs taken. Photographs were taken using IPWIN32 software and a microscope with a

mounted digital camera. All observations and pictures were recorded on the animal's data sheet.

Statistics. Comparison of means were performed by one-way analysis of variance (ANOVA) with Bonferroni correction for hematocrit, hepatosomatic index (HSI), plasma testosterone between sites and TKI between sites; by independent t-test for TKI between species; and by paired t-test for plasma testosterone measurements in the field versus the laboratory. A Pearson's correlation test was performed for proportions of abnormalities found between populations. Significance for each test was set at $p < 0.05$. Data points that are 1.5 to 2 standard deviations from the mean are denoted by "o" in all graphs. Data points greater than two standard deviations from the mean were excluded from analyses.

Results

Site characterization. Detailed descriptions of the collection sites are provided in Chapter 2. The 2003 and 2004 collecting seasons experienced a great deal of rain, while the 2005 season was a relatively dry one. In addition, the 2005 season began and ended late (late May into mid-October rather than mid-April to mid-September), as warmer spring weather began later than usual and cooler conditions were also delayed.

Levels of contaminants. Table 3.1 lists the contaminants found at each pond at their highest recorded concentration. Pond sites are listed in increasing order of contamination and grouped by contamination level. Also listed are two specific analyses for the site Rahilly. These recordings illustrate the fleeting nature of the sites' contaminants; in less than a month, concentrations went from near maximal to below detection (detection limit: 1.7 ng/L). Figure 3.1 shows the structures of all the contaminants detected at sites by our analyses.

Captures. In thirty-one collection events involving nine sites, 391 bullfrog and green frog adults and juveniles were captured. Table 3.2 contains the distribution of captures across sites, species and sex for each year of collection. Further details appear below by year.

2003. Over three collection events involving five sites, a total of 41 frogs were captured. 35 of the captured animals were subsequently analyzed. The captures consisted of ten male, three female and nine unsexed juvenile green frogs, and five male, nine female and five unsexed juvenile bullfrogs. Collection events occurred on: August 27th and September 9 and 30th at Bruch I, Croshaw, Robson East, Robson West and Durr

ponds. The Rahilly site was visited twice, few frogs were spotted and no animals were captured. Four of the green frog juveniles collected from Croshaw were too small to efficiently dissect and were therefore not analyzed. In addition, two juvenile bullfrogs captured from Bruch I were consumed by a female bullfrog en route from the collection site to the laboratory and were therefore not analyzed.

2004. In nine collection events involving nine sites, a total of 208 frogs were captured. All but one of these animals were subsequently analyzed. The captures consisted of 77 male and fifteen female green frogs, 72 male and 44 female bullfrogs. Frogs were collected on the following dates: May 6th, June 7th, July 1, 4, 10, 19 and 21st, and September 19 and 21st. The only frog that was not analyzed was a male green frog from Robson East, which was consumed by a male bullfrog from the same site. The green frog was in amplexus with the bullfrog and could not be separated (trying to lift off the green frog only lifted the bullfrog as well). The green frog was later missing and subsequently found in the stomach of a bullfrog upon dissection. It is important to note that the Hopkins site did not yield any frogs when visited during the day; only during night collections were frogs found. It is possible that frogs at this site hide

in the tall grasses surrounding the pond to avoid predation and only venture to the uncovered pond at night.

2005. In nineteen collection events involving seven sites, 142 frogs were caught. All animals were subsequently analyzed for gross and histological effects. The captures consisted of 29 male and six female green frogs, and 56 male, 46 female and five unsexed juvenile bullfrogs. Frogs were collected on the following dates: June 17, 22 and 28th, July 13, 19, 22 and 26th, August 2 and 3rd, September 2, 4, 6, 9, 12, 13, 28 and 30th, and October 2 and 6th. Durr II was not visited during this season as it became inaccessible by the owner. In addition, Hopkins was only surveyed once and no frogs were observed.

Hematocrit. Average hematocrit was determined for each frog, then compared across sites and levels of contamination. There were no significant differences among males of either species; however, a significant difference was revealed among female bullfrogs (numbers for female green frog hematocrit were too small for statistical analysis). Figure 3.2 shows that percent hematocrit decreased as level of contamination increased, and was significantly reduced among female bullfrogs from high contaminated sites (16.4%) compared to other female bullfrogs from low (21.1%) or medium (19.7%) contaminated sites.

Hepatosomatic index (HSI). Liver weight was divided by body weight of each frog to obtain its HSI. Changes in HSI would indicate an effect on liver function as induction of metabolizing enzymes in response to xenobiotic exposure increases liver weight (Peakall 1976). There were no significant differences found across sites in either bullfrogs (Figure 3.3) or green frogs (Figure 3.4). When data was collapsed by site level of contamination, no significant differences were seen among bullfrogs in either males or females (Figure 3.5). However, HSI decreased among female bullfrogs with greater site contamination. In green frogs, no statistical differences in HSI appeared when levels of site contamination were compared in either sex (Figure 3.6).

Testosterone levels. Blood was obtained both on-site shortly after capture and again upon arrival at the laboratory in order to detect any changes in testosterone levels due to capture. The differences between these two measurements were analyzed with a paired t-test. Figure 3.7 shows that the plasma concentrations of testosterone were significantly reduced by the time captures were transported to the laboratory for dissection compared to at the time of capture. This observation confirms that hormone concentrations can change dramatically just two

hours following removal from the environment (Licht et al. 1983). Accordingly, samples from the field were used to compare sites by level of contamination. Figure 3.8 illustrates the high variability within each contamination level, reflecting the high variability within field populations. As a consequence, no significant differences resulted.

Testiculo-kidney index (TKI). Testiculo-kidney indices were calculated for each frog. Figure 3.9 reveals the different strategies to reproductive output of the two species; green frog males putting forth a significantly greater percentage of energy toward testicular function than bullfrog males with 68.6% greater TKI. Therefore, the two species were analyzed separately. Figure 3.10 shows bullfrog TKI across individual sites. Although the average TKI at Rahilly was lower than the other sites, the difference was not significant. However, when the sites were nested by level of contamination (Figure 3.11), a significant difference appeared between the medium- and high-contaminated sites, showing a 21.2% reduction at high-contaminated sites compared to medium (there was no significant difference between low- and high-contaminated sites). For green frogs, the Robson West site displayed a TKI that was significantly different from all other sites, except those classified within its contamination level (Figure 3.12). When

the sites were nested by level of contamination (Figure 3.13), the TKI at the medium-contaminated sites was significantly reduced 21.6% compared to low contaminated sites.

Testicular dysgenesis. Testicular dysgenesis refers to the abnormal development of the gonads. Figure 3.14A shows the normal histology of a testis from a green frog. In normal spermatogenesis, as spermatozoa develop, they move into the lumen of the tubule. Early spermatogonia lie next to the basement membrane of the tubule. As these cells undergo mitotic divisions, they move away from the basement membrane toward the lumen. After a period of growth, DNA replicates and the cells become primary spermatocytes. After meiotic divisions, the primary spermatocytes become secondary spermatocytes, which divide immediately to form spermatids. Spermatids lie close to the lumen of the tubule and together with sustenacular cells, develop into spermatozoa, which lie in the lumen of the tubule (Aughey and Frye 2001). As Figure 3.14B illustrates, although the testis of a juvenile frog is immature and poorly organized, there is evidence of spermatogenesis with cells at different stages, including spermatozoa. Because spermatogenesis is evident in juveniles, albeit in the early stages, it would be expected that older males, even if not at full sexual maturity, would possess more developed testes. As Figure 3.14C shows,

however, male bullfrogs were found that had testes almost devoid of sperm. The tubules of the testis as shown in Figure 3.14C were full of spermatogonia, the early stage cell that is normally limited to the basement membrane of the tubule. In addition, enlarged cells appear at the edge of the tubules (see close up in Figure 3.14D). These cells, often termed sperm granulomas, are a result of cell growth with failure of kinesis for cytoplasmic division. In addition to retarded testicular development, hyperplasia (Figure 3.15A), edema (Figure 3.15B), dysplasia (Figure 3.15C) and calcification (Figure 3.15D) were also observed in testicular tissues. When sites were nested, the highest incidence of testicular dysgenesis was among the medium-contaminated sites, which was significantly elevated 44.5-54.3% compared to the low- and high-contaminated sites (Figure 3.16). These lesions appeared primarily in bullfrogs (over 70% of all examples were from bullfrogs).

Testicular oogenesis. While testicular dysgenesis was seen most often in bullfrogs, testicular oogenesis was found solely in green frogs. Testicular oogenesis is the phenomenon where oocyte formation occurs in testicular tissue. Figure 3.17A shows a single egg in a tubule, which is surrounded by normal testicular tissue. Figure 3.17B shows several oocytes from a single testis, which contained seven oocytes altogether in a

single section (one oocyte was found in the other testis of the same frog). Figures 3.17C-D display the periodic acid schiff's (PAS) stains of the tissues in Figures 3.17A-B, highlighting the basement membrane of the oocytes and confirming that these eosinophilic masses are indeed physiological structures and not aggregates of protein material.

Renal sperm. In addition to testicular oogenesis, ten green frog males also presented with spermatozoa within their kidneys (Figure 3.18) with only a single observance in bullfrogs. Such a phenomenon has not been reported previously and may arise from a failure of complete separation of the intermediate mesoderm adjacent to the developing kidney from the testes' rudiments.

Discussion

The abiotic components of a habitat may affect a species individually or collectively (Freda et al. 1991). When an agricultural habitat receives abundant rainfall, as in years 2003 and 2004, this increases the amount of chemical input to surrounding bodies of water, especially when pesticide applications coincide with spring rains. Conversely, in years where there is little rain, as in 2005, less contamination results from runoff; however, as a pond dries up, stress hormones increase in its inhabitants introducing another

stressor. Since it is nearly impossible to separate all variables in field experiments, several times of year were utilized over multiple years in order to minimize the impact of potential annual and/or seasonal variation of abiotic and atrazine effects (Rohr et al. 2006). In addition, because agricultural sites in the same area were used as reference sites, habitats at these sites should be generally similar, thus reducing the number of confounding variables, especially since collections were performed under the same temporal, seasonal and weather conditions using the same techniques.

Pesticide presence. Our water analyses show that atrazine concentrations in some of the collection sites exceeded the effective concentration of atrazine as reported by Hayes et al. (0.1 ppb, 2002b) for hemaphroditism and retarded gonadal development. In addition, with the rapid disappearance of contaminants (as shown with Rahilly) it was shown that the agrochemical exposure at these sites might be pulsatile in nature, rather than continuous. Therefore, concentrations will vary from year to year and during the season depending on application rates and timing, which may or may not correspond to runoff events and sensitive developmental periods. This observation indicates that the half-life of surface contaminants at these sites is on the order of a few weeks. The sites with

multiple chemical inputs present a more complicated challenge as interactions become possible. Indeed, Howe et al. (1998) found that atrazine and alachlor had synergistic effects on acute toxicity testing in amphibians.

It is important to note that methoxychlor, detected at the Durr site and now used as a replacement for DDT (Anway et al. 2005), is metabolized into active compounds, like 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). These compounds have ERα agonist, ERβ antagonist and anti-androgenic activities (Akingbemi and Hardy 2001; Anway et al. 2005; Kaiser 2005). HPTE may exert its effects through the estrogen receptor (ER), which mediates inhibition of the cholesterol side-chain cleavage enzyme ($P450_{SCC}$), that in turn prevents the conversion of cholesterol into testosterone (Akingbemi and Hardy 2001). This chemical has also demonstrated effects on the central nervous system, epididymal sperm numbers, accessory sex glands and delays in mating without any effect on luetinizing hormone, prolactin, or testosterone secretion (Ostby et al. 1999), though it has been shown to affect hypothalamic and pituitary function (Akingbemi and Hardy 2001). Consequently, methoxychlor may be playing a role in effects seen in our field analyses, particularly since ovotestes were observed at a site heavily impacted with this insecticide. In addition, some of our sites

contained metolachlor and its presence may have had an influence in reduced TKI among green frogs residing at medium-contaminated sites.

Hematocrit. Some contaminants directly affect energy metabolism pathways and since atrazine's action in plants is on the electron transport chain, it could be expected for atrazine to have a direct toxic effect on cells (Sparling et al. 2000). For example, Allran & Karasov (2001) found that atrazine exposure in the leopard frog decreased oxygen consumption with increased formation of methemoglobin. This effect has also been seen in fish (Prasad et al. 1991). These observations indicate that the oxygen-carrying components of the blood may be affected by atrazine exposure (Allran and Karasov 2001). As among female bullfrogs in this study, the number of red blood cells decreased in other species following atrazine exposure, including mice (Mencoboni et al. 1992) and fish (Prasad et al. 1991; Hussein et al. 1996). It is important to remember that temperature plays a critical role in oxygen consumption, which increases almost exponentially with increased temperatures (Casper and Hendricks 2006). However, the sites of this study would be expected to be similar in temperature; therefore, there is evidence of atrazine's action as a respiratory distressor that may be sex-specific.

Hepatic function. Although one would expect increased liver function due to upregulation of xenobiotic metabolizing enzymes to elevate liver weights (Peakall 1976), our field observations did not yield such an effect. Rather, a trend was seen whereby liver weight decreased with pesticide exposures (bullfrog females, Figure 3.5). This observation may indicate an effect on tissue growth.

Testosterone levels. Because previous studies have observed feminization of male frogs, it would be expected that testosterone levels of atrazine-exposed frogs would be lower than unexposed frogs, as found by Hayes et al (2002b). Hecker et al. (2004) also found that atrazine depressed testosterone levels in *Xenopus laevis* under certain exposure regimes, as well as increased the estradiol to testosterone ratios in the same exposure paradigms. Although a decrease in plasma testosterone levels in exposed male frogs was expected, the different degrees of sexual maturity among frogs at our sites may have caused the wide distribution and have been a confounding variable in the analysis.

Testicular effects. Reduction in testicular weight is a measure of gonadal growth. The observation of reduced testicular weight

was found in both species and indicates gonadal dysfunction. However, the two species did differ in which of their populations displayed reduced testicular weight. The two species also differed in their response to chemical contamination at the histological level. Bullfrog testes presented with effects consistent with demasculinization and the lack of testosterone influence (retarded gonadal development). In contrast, green frogs presented with effects consistent with feminization and tissue responsiveness to estradiol (testicular oogenesis). This study extends findings of both gonadal dysgenesis and oogenesis to new species, the bullfrog and the green frog respectively. In addition, this is the first report of renal sperm aggregation. This phenomenon may arise from abnormal development of the testes and/or kidney and is consistent with an estrogenic influence as estradiol has a role in organogenesis. In order to determine whether these physical changes translate into functional impairments, reproductive output studies need to be performed.

Bullfrogs are one of the most sensitive species to atrazine exposure, the channel catfish (*Ictalurus punctatus*) being the only other studied species that is more sensitive (Sparling et al. 2000). Steroids and their alterations have dramatic effects on sex differentiation in anurans (Hayes 1998). For the

bullfrog, the major effect was retarded testicular development, which arises from loss of proper cues from testosterone for spermatogenesis.

Though genetically determined, sex differentiation can be influenced by many factors, including temperature, pH and chemical contamination as the undifferentiated gonad is very sensitive to steroidal compounds (Reeder et al. 1998). Sexual development in anurans displays more plasticity than terrestrial vertebrates as it is more readily influenced by environmental factors. For example, gonadal reversal displays a thermosensitive window, whereby temperature may influence the phenotypic sex of the animal regardless of its genetic sex (Chardard et al. 1995). However, temperatures necessary to induce these sex reversals also induce high mortalities and are not considered environmentally relevant (Chardard et al. 1995). Because sexual development is sensitive to changes in the environment, like temperature, it may also be perturbed by chemical contamination (Hsu et al. 1971; Reeder et al. 1998). For cricket frogs, baseline intersex incidence is about 2%, though it has fluctuated with periods of chemical contamination in the environment (Reeder et al. 2005).

Reproductive strategies including sex reversal are very rare. For example, the reed frog (*Hyperolius viridiflavus*) can reverse sex under extreme environmental stress and skewed sex ratios (Grafe and Linsenmair 1989). In addition, some *Bufo* males maintain embryonic tissue that can develop into ovarian tissue when the testes become nonfunctional (Sullivan et al. 1996). There are no reports that bullfrogs or green frogs employ sex reversal as a normal part of any life stage or as part of their reproductive strategy. Therefore, the presence of ovotestes in the green frog is not considered normal, but an anomaly. The presence of ovotetes is not true hermaphroditism, as the tissue is still testicular and no separate ovarian tissue has formed. This is more accurately a feminized state of the male reproductive tract. The presence of either masculinized or feminized tissue does not necessarily impact mating; however, reproductive function may be adversely influenced by the changes in hormonal environment and impede reproductive output (Reeder et al. 1998). In addition, other reproductive parameters are regulated by the sex steroids and are under androgen and/or estrogen control. Androgen regulated parameters include thumb-pad development (Varriale and Serino 1994), gular pouch and larynx development (Sassoon et al. 1987; Cohen and Kelley 1996; Robertson and Kelley 1996; Hayes and Menendez 1999) in males. In addition, estrogen directed measures involve induction of

vitellogenin (Herman and Kincaid 1988; Palmer et al. 1998), regulation of gonaduct growth (Norris et al. 1997) and cloacal gland development (Norris and Moore 1975) in females. Changes is steroidal concentrations will also affect these secondary sex traits.

Possible mechanisms of effects. Since the observed effects of atrazine exposure on amphibians have been primarily feminization of males, a mode of action involving the estrogen receptor (ER) was suspected. However, investigations into atrazine's estrogenic effects failed to yield evidence for its interaction with the ER (Eldridge et al. 1994b; Tennant et al. 1994; Danzo 1997; Reeder et al. 1998; Eldridge et al. 1999a; Sanderson et al. 2000; Sanderson et al. 2001). Many endocrine disruptors act through mechanisms other than receptor mediation (Connor et al. 1996; Crain et al. 1997; Pickford and Morris 1999; Welshons et al. 1999) and it has been shown that chemicals alter male sex differentiation via mechanisms other than receptor interference (Wolf et al. 1999).

For the above reasons, researchers began to look at enzymes in the steroid system. It has been proposed that the normal function of the enzyme aromatase, cytochrome $P_{450}19$ (CYP19), may be disrupted by atrazine through induction. Aromatase is the

rate-limiting enzyme in the conversion of androgens to estrogens in vertebrates, including humans (Roberts 2006 Chem Heritage 24:39) (Crain et al. 1997; Sheehan et al. 1999; Sanderson et al. 2000; Roberts 2006). In addition, it is a key component in the ovarian-determining pathway in amphibians (Miyata and Kubo 2000). Mills et al. (2006) reported induction of brain aromatase in the fish cunner (*Tautogolabrus adsperus*) following atrazine treatment. Atrazine may cause endocrine disruption through alteration of this steroidogenic enzyme's activity (Crain et al. 1997), leading to the observed feminization of male frogs exposed to atrazine (Reeder et al. 1998; Hayes et al. 2002b; Hayes et al. 2003). Since this increase in estrogen is at the expense of androgrens, there is a double effect: increased estrogens promote ovary development within the testes, while lowered androgens disrupt larynx development and spermatogenesis in males (Sanders 2002).

Normally, gonadotropin-releasing hormone from the hypothalamus (Figure 3.19) stimulates the pituitary to release luetinizing hormone (LH) and follicle-stimulating hormone (FSH, Trentacoste et al. 2001). In turn, LH and FSH act on the testes to convert testosterone to dihydrotestosterone (DHT), which activates the androgen receptor (AR). Under these circumstances, a small amount of testosterone may be converted to estradiol (E_2) by

aromatase. However, in the presence of atrazine, aromatase is induced and a larger amount of testosterone is aromatized to E_2, resulting in depressed levels of testosterone and DHT (Trentacoste et al. 2001). Consequently, AR action is reduced and male-specific traits are not expressed.

Aromatase is also an important enzyme involved in the crosstalk between the developing gonads and the brain (Kwon et al. 2001). Endocrine disruptors often target the hypothalamic-pituitary-gonad axis due its abundance of ERs and ARs (Akingbemi and Hardy 2001). Aromatase has a key role in sexual differentiation of the central nervous system by controlling the ratios of hormones in the developing brain (Fox 1975; Renner 2000), which is thought to be bipotential and undifferentiated prior to sexual differentiation (Matuszczyk 2003). Androgens in the brain masculinize certain neuroendocrine and behavioral responses (Matuszczyk 2003). Estradiol is produced within brain cells by the aromatization of testosterone (Fox 1975; Montano et al. 1995); because the brain converts testosterone to estradiol, effects on the brain by androgens may be mediated by the estrogen receptor (Fox 1975). In this manner, reduced androgens in the brain hinder its masculinization and differentiation (Matuszczyk 2003). Indeed, ERs are high in brain regions associated with male sexual behavior, like the amygdala and the

preoptic area of the hypothalamus (Akingbemi and Hardy 2001). Interference at this level may lead to the development of inappropriate sexual traits. This phenomenon, coupled with atrazine's effects on the gonads, further supports the theory that atrazine's sublethal toxicities are aromatase-mediated. As an endocrine-disruptor, atrazine may be able to alter cellular differentiation and proliferation, leading to permanent changes in structure and function of the brain and reproductive tract (Mackenzie et al. 2003). Indeed, Mills et al. (2005) found that following atrazine exposure, male fish cunner (*Tautogolabrus adsperus*) brain aromatase was increased at all but one concentration tested. In addition, they found no change in vitellogenin production, a feminization marker, but reproductive effects were observed (Mills et al. 2006). It may be the case that atrazine's reproductive effects are primarily brain-aromatase mediated.

In a study conducted by Danzo (1997), atrazine was able to block 5α-dihydrotestosterone (5α-DHT) binding to the androgen binding protein (ABP) by 45%. Atrazine may bind ABP, preventing 5α-DHT delivery to sites of spermatogenesis and leading to the gonadal dysgenesis observed in male frogs treated with atrazine (Hayes et al. 2003) and in the bullfrogs from our field collections. In addition, ABP stimulates adenylyl cyclase, which generates

cAMP (Danzo 1997; Akingbemi and Hardy 2001). The primary target for cAMP is protein kinase A (PKA), the activation of which is the proposed mechanism whereby aromatase is induced to convert androgens to estrogens (Sanderson et al. 2001). (See Figure 3.20 for illustration.) A recent study has also shown that atrazine acts as a competitive inhibitor of phosphodiesterase, the enzyme that hydrolyzes cAMP (Roberge et al. 2004), leading to sustained elevated levels of cAMP and prolonged activation of the PKA pathway. Accordingly, the actions of atrazine ***in vivo*** may be quite complex, resulting in unexpected and varied responses.

Though suspected, there is no evidence for this mechanistic pathway in amphibians (Hayes et al. 2002b). Evidence does exist for alligators (Crain et al. 1997), rats (Babic-Gojmerac et al. 1989; Kniewald et al. 1995), the human adrenocortical carcinoma cell line H295R (Sanderson et al. 2000; Sanderson et al. 2001) and fish (Mills et al. 2005; Mills et al. 2006). The induction of aromatase in H295R cells was concentration dependent (Sanderson et al. 2000; Sanderson et al. 2001), showing the necessity of atrazine presence. Coady et al. (2004) observed a metamorphosis pattern in green frog larvae exposed to 25 ppb atrazine similar to larvae exposed to estradiol. In addition, the sex ratio among frogs exposed to 25 ppb atrazine was skewed

toward females by the same proportion as those exposed to estradiol. Further, Murphy et al. (2004) found a positive correlation between atrazine exposure in the field and juvenile male estradiol concentrations. These studies indicate an upregulation of E_2 following atrazine exposure that could be aromatase-mediated.

Consequently, there is concern about wildlife and human exposure to atrazine because errant aromatase induction during irreversible developmental periods may result in inappropriate feminization or altered physiological states of certain tissues (White et al. 1994; Sanderson et al. 2000). In fact, aromatase is known to be active in the undifferentiated larval gonad of *X. laevis* and altering its activity can affect gonadal differentiation (Miyata and Kubo 2000), despite former genetic sexual determination. Steroidogenesis may be actively ongoing in the tadpole gonads (Hsu, et al. 1985 Gen Comp Endocrinol 57:393), or even contribute to estrogen-mediated toxicities. It has been shown that tadpoles can synthesize and metabolize gonodal steroids (Gray and Janssens 1990); and therefore, ratios would be affected by exogenous hormones. According to the literature, moderate or even low concentrations of estrogenic compounds are sufficient to induce sex reversal in genetic male tadpoles (Mackenzie et al. 2003) and potentially alter the

morphology and physiology of animal, including human, reproductive systems permanently (Guillette et al. 1994; Honma et al. 2002; Timms et al. 2002). Indeed, ER agonists tend to be more active at low levels (Akingbemi and Hardy 2001). Furthermore, Gupta (2000a) found via ***in vitro*** studies that the effects of estrogenic compounds are independent of testosterone levels. An increase in free estradiol as low as 0.1 pg/ml in serum is sufficient to elicit a biological response in tissues with estrogen receptors (vom Saal et al. 1997). This finding indicates that tissues are extraordinarily sensitive to estradiol exposure (Takai et al. 2001; Welshons et al. 2003). Of course, it is important to point out that changes in gonadal development do not always translate into effects on reproductive output. However, these early changes leave an animal vulnerable to reproductive dysfunction.

Aromatase induction may also occur in adipose tissue (Miller and O'Neill 1987) and the adrenal cortex (Sanderson et al. 2000), and be most deleterious in those tissues where androstenedione is relatively higher than testosterone (Sanderson et al. 2001). Indeed, males may be more adversely affected than females because androstenedione is a better substrate for aromatase than testosterone (Gonzalez and Piferrer 2002). In addition, contaminant concentrations tend to be higher in males than

females (Sparling et al. 2000). It is important to look at more tissues than the gonads because atrazine's impact may be tissue dependent. For again, Mills et al. (2006) observed elevated aromatase activity in male and female brains following atrazine exposure that correlated with alterations in reproduction, but there were no significant changes in gonadal aromatase activity.

In numerous studies, bullfrogs were found to be more sensitive to lethal effects from pesticide exposures compared to green frogs (the most tolerant, Freda and Dunson 1984), or leopard frogs (Berrill et al. 1993; Berrill et al. 1994; Berrill et al. 1995). This sensitivity includes exposure to atrazine (Detenbeck et al. 1996), with an LC_{50} of 0.41 mg/L for larval stage bullfrogs compared to 7.7 mg/L for larval ***Rana pipiens*** (Sparling et al. 2000). This difference in susceptibility among species may lead to changes in the structure of amphibian communities, due to differential mortality. As the relative fitness of one species falls compared to another, a shift in dominance may occur and lead to the decline of the former species in that community, altering overall amphibian diversity (Sparling et al. 2000).

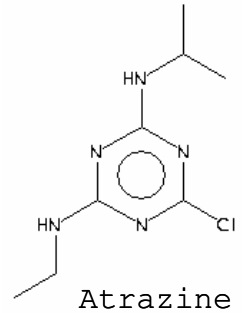
Conclusion. Overall, more attention needs to be given to sublethal impacts on fitness parameters, as endocrine-disrupting

chemicals may have effects at concentrations below detectable levels (Sparling et al. 2000). These effects include changes in growth, development and behavior of larvae, juveniles and adults (Carey and Bryant 1995). Our field observations support the hypothesis that native New Jersey frogs are under endocrine disruptive influences due to agrochemical contamination of their habitat. These influences are likely not due to the action of a single chemical, but of multiple, with methoxychlor and metolachlor as potential drivers in the sites investigated in this study.

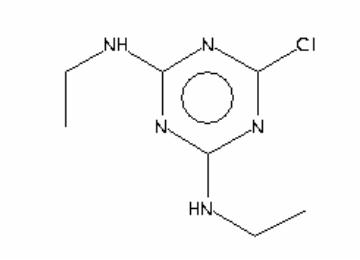
Table 3.1. Levels of pond contaminants
Concentrations are in ppb; ND = non detectable; maximum detection limit (MDL) = 1.7 ng/L; numbers represent highest concentration detected per contaminant per site.

Site	**Atrazine**	**Simazine**	**Alaclor**	**Metolaclor**	**Methoxychlor**	**Oxamyl**
Durr II	ND	ND	ND	ND	ND	ND
Bruch II	ND	ND	ND	ND	ND	ND
Bruch I	ND	**0.3**	ND	ND	ND	ND
Croshaw	ND	ND	ND	**10.4**	ND	ND
Robson East	ND	ND	ND	**12.8**	ND	ND
Robson West	ND	ND	ND	**17.4**	ND	ND
Durr	ND	ND	ND	ND	**54.1**	ND
Hopkins	**22.3**	ND	ND	ND	ND	ND
Rahilly	**45.4**	ND	**24.5**	**92.1**	ND	**1.8**
Rahilly (7/24/03)	**25**	**ND**	**24.5**	**92.1**	**ND**	**1.8**
Rahilly (8/9/03)	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**

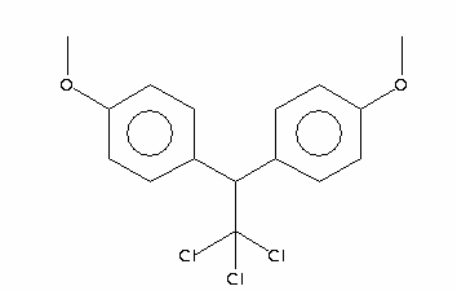
Figure 3.1. Structures of identified pond contaminants All structures were obtained using ChemSketch (Stein et al. 2002).



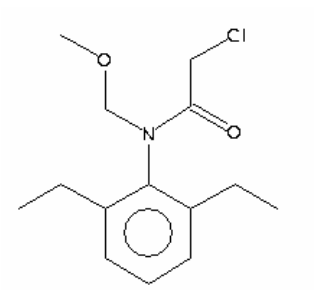
Atrazine



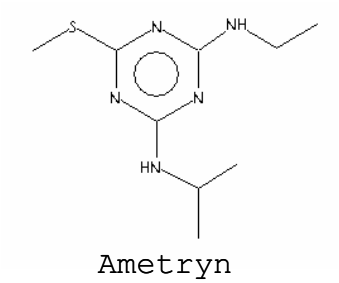
Simazine



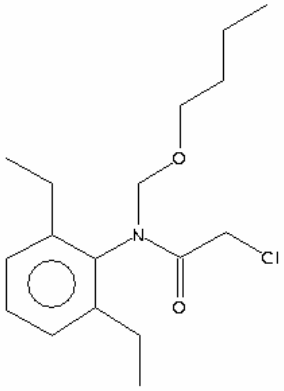
Methoxychlor



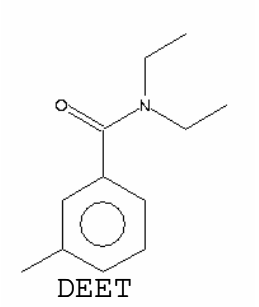
Alachlor



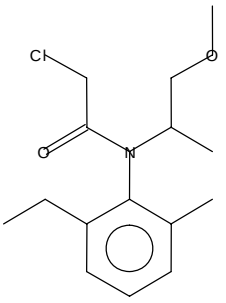
Ametryn



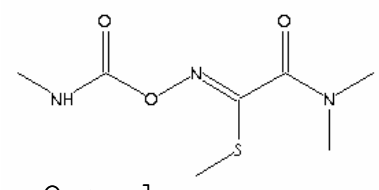
Butachlor



DEET



Metolachlor



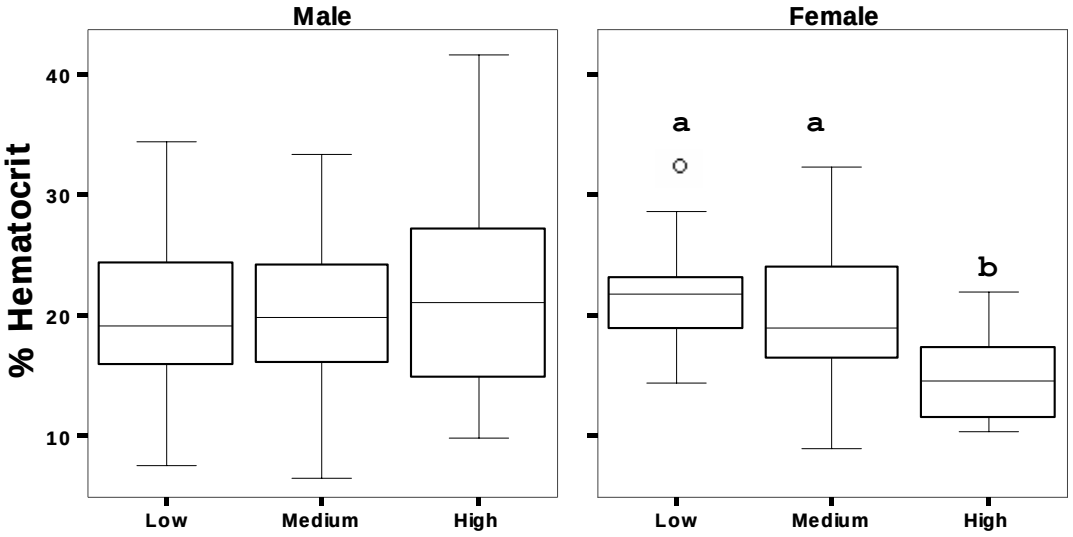
Oxamyl

Table 3.2. Collection summary
List of captures by site, species, sex and year of collection. Total caught per site per species is in parentheses. Totals include unsexed juveniles.

	Durr II	Bruch II	Bruch I	Croshaw	Robson East	Robson West	Durr	Hopkins	Rahilly
2003									
Bullfrogs			(8)		(6)	(5)			
Males	--	--	1	--	3	1	--	--	--
Females			3		2	4			
Green Frogs			(1)	(7)	(1)	(3)	(10)		
Males	--	--	--	2	1	1	6	--	--
Females			--	--	--	--	3		
2003 Total	**0**	**0**	**9**	**7**	**7**	**8**	**10**	**0**	**0**
2004									
Bullfrogs	(5)	(11)	(24)	(12)	(20)	(15)	(17)	(5)	(7)
Males	2	6	18	8	15	8	10	1	4
Females	3	5	6	4	5	7	7	4	3
Green Frogs	(12)	(14)	(1)	(10)	(7)	(9)	(31)	(8)	
Males	10	13	1	6	6	6	28	7	--
Females	2	1	--	4	1	3	3	1	
2004 Total	**17**	**25**	**25**	**22**	**27**	**24**	**48**	**13**	**7**
2005									
Bullfrogs		(9)	(10)	(20)	(17)	(25)	(19)		(7)
Males	--	3	7	11	11	14	6	--	4
Females		5	3	9	5	11	10		3
Green Frogs		(4)	(1)	(7)	(2)	(14)	(6)		(1)
Males	--	4	--	7	2	12	4	--	--
Females		--	1	--	--	2	2		1
2005 Total	**0**	**13**	**11**	**27**	**19**	**39**	**25**	**0**	**8**
Total	**17**	**38**	**45**	**56**	**53**	**71**	**83**	**13**	**15**

Figure 3.2. Hematocrit
A, hematocrit by level of contamination and paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. Populations designated with the different letters are significantly different ($p \leq 0.034$). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

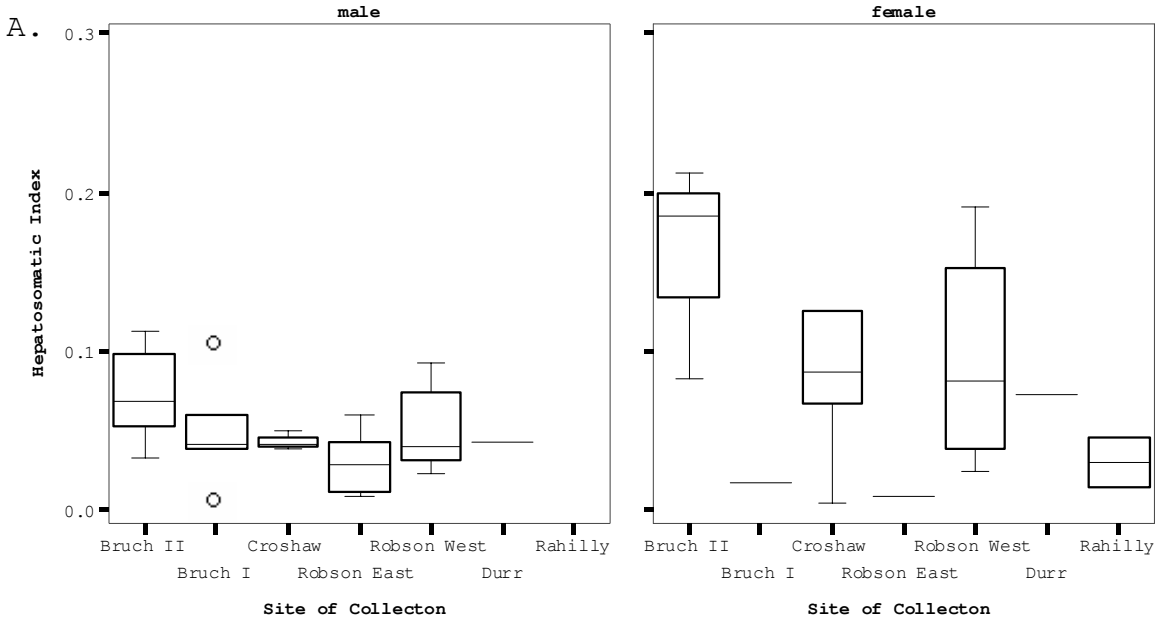


B.

Level of Contamination	Males	SD	Females	SD
Low	20.63 (46)	± 7.42	21.07 (19)	± 3.36
Medium	20.89 (42)	± 7.65	19.68 (30)	± 4.76
High	21.72 (46)	± 7.95	16.38 (16)	± 3.91

Figure 3.3. Bullfrog HSI across sites
A, bullfrog HSI across sites and paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation, "--" indicates less than two samples and number of frogs included in each analysis is in parentheses.

A.

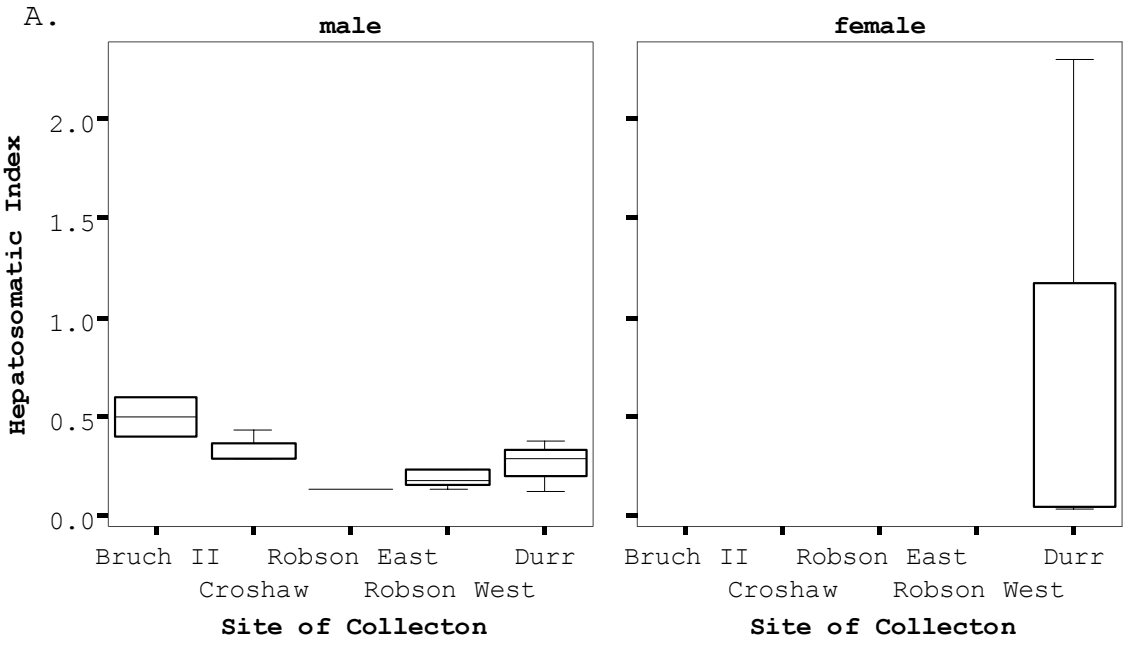


B.

Site	Male	SD/Range	Female	SD/Range
Bruch II	0.0861 (6)	± 0.0564	0.1604 (3)	± 0.0680
Bruch I	0.0429 (6)	± 0.0429	0.0180 (1)	--
Croshaw	0.0928 (9)	± 0.0810	0.1111 (5)	± 0.0983
Robson East	0.0307 (6)	± 0.0201	0.0087 (1)	--
Robson West	0.0830 (11)	± 0.0187	0.0969 (9)	± 0.0621
Durr	0.0577 (2)	± 0.0210	0.0725 (1)	--
Rahilly	--	--	0.0300 (2)	± 0.0218

Figure 3.4. Green frog HSI across sites
A, green frog HSI across sites and paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation, "--" indicates less than two samples and number of frogs included in each analysis is in parentheses.

A.

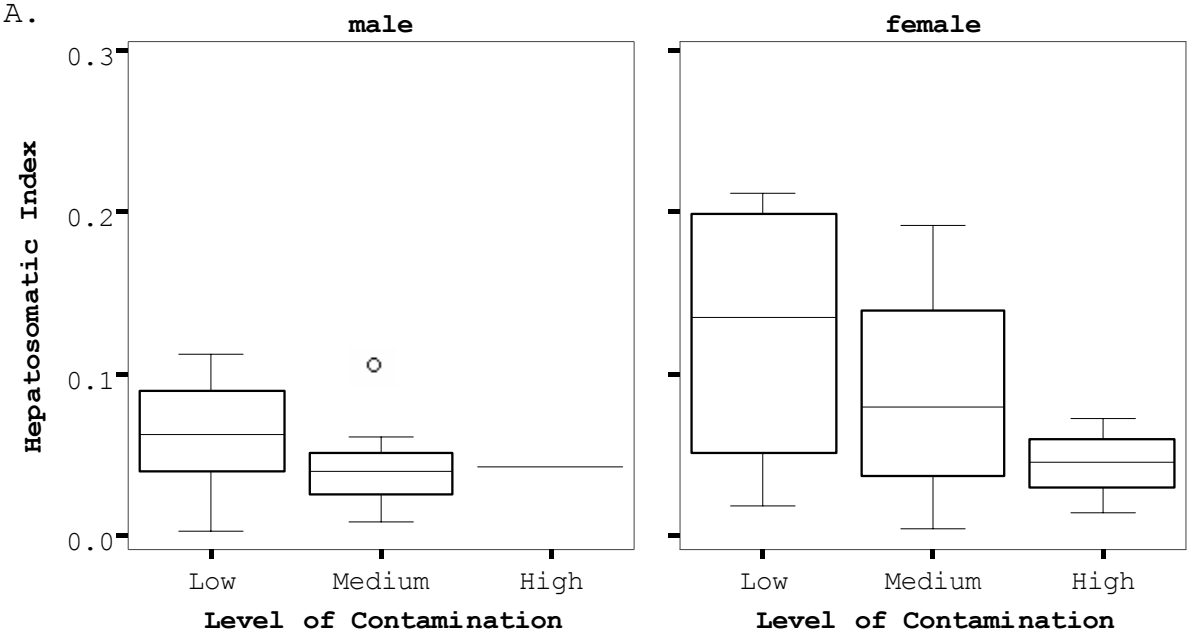


B.

Site	Males	SD/Range	Females	SD
Bruch II	0.4995 (2)	± 0.1466	--	--
Croshaw	0.3364 (3)	± 0.3364	--	--
Robson East	0.1301 (1)	--	--	--
Robson West	0.2225 (6)	± 0.1165	--	--
Durr	0.2695 (4)	± 0.2695	0.7937 (3)	± 1.3031

Figure 3.5. Bullfrog HSI by level of contamination
A, bullfrog HSI by level of contamination and paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

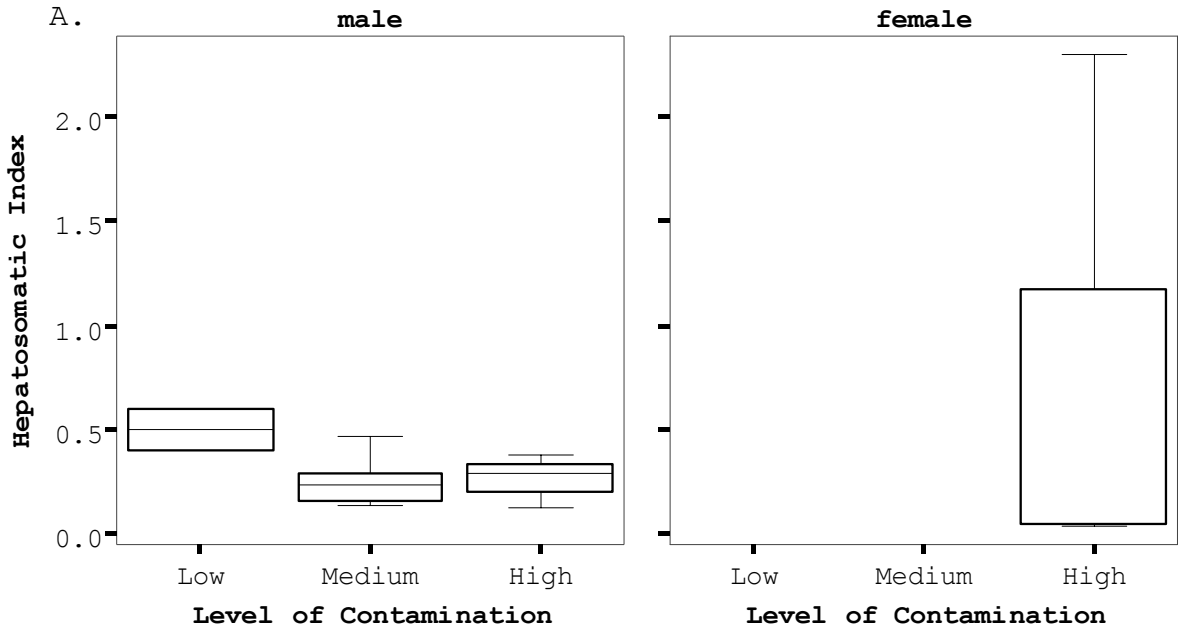


B.

Level of Contamination	Males	SD/Range	Females	SD
Low	0.0662 (12)	± 0.0503	0.1248 (4)	± 0.0903
Medium	0.0747 (26)	± 0.0668	0.0967 (15)	± 0.0767
High	0.0577 (2)	± 0.0210	0.0442 (3)	± 0.0158

Figure 3.6. Green frog HSI by level of contamination
A, green frog HSI by level of contamination and paneled by sex. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation, "--" indicates less than two samples and number of frogs included in each analysis is in parentheses.

A.

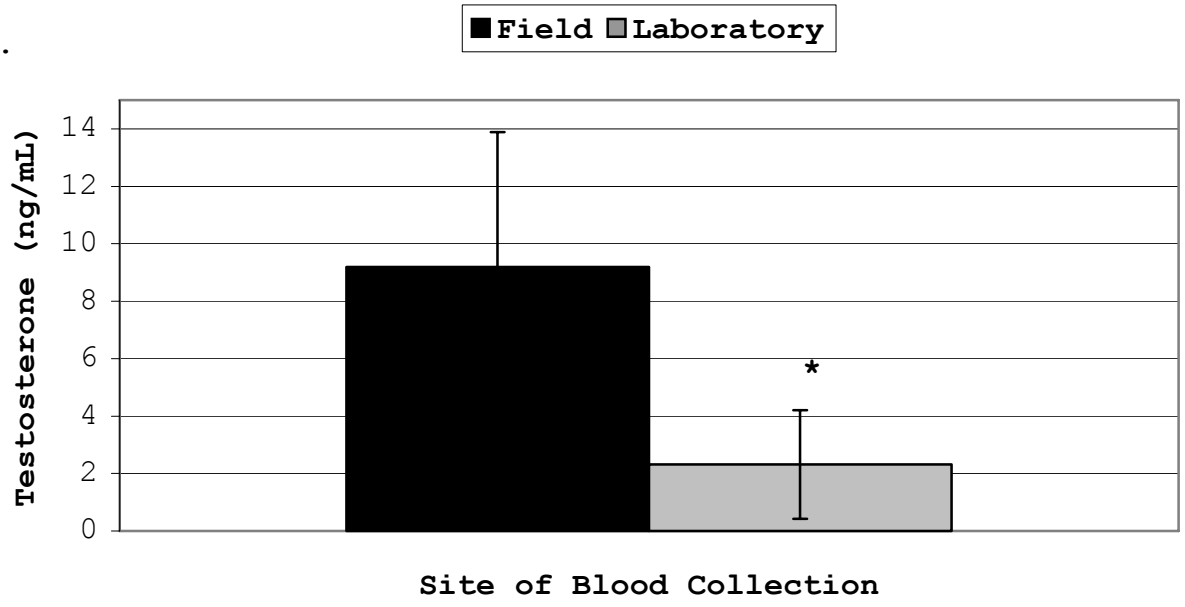


B.

Level of Contamination	Males	SD/Range	Females	SD
Low	0.4995 (2)	± 0.1466	--	--
Medium	0.2452 (11)	± 0.1170	--	--
High	0.2695 (4)	± 0.1055	0.7937 (3)	± 1.3031

Figure 3.7. Effect of time on plasma testosterone
A, testosterone levels for all frogs measured from the field or laboratory; error bars represent standard deviation. A significant difference ($p = 0.015$) between field and laboratory measures is indicated by "*****." A paired t-test was used to test for significant difference between means. B, raw numerical data where SD is the standard deviation and number of frogs included in each analysis is in parentheses.

A.

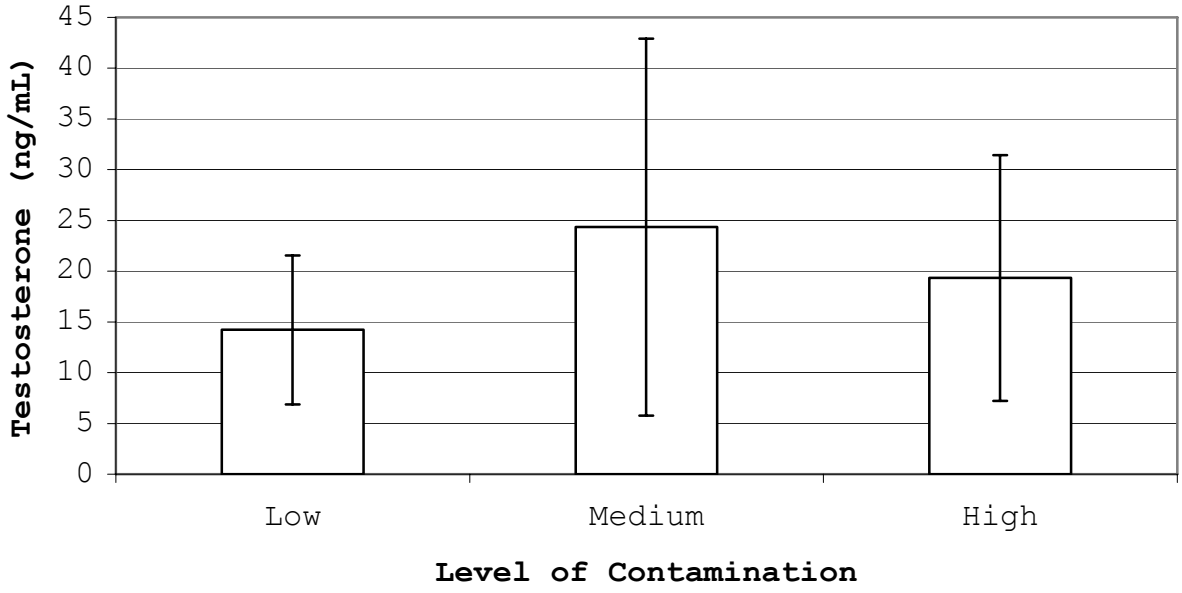


B.

Site of Collection	Testosterone (ng/mL)	SD
Field	9.167 (102)	± 4.977
Laboratory	2.223 (102)	± 2.015

Figure 3.8. Male plasma testosterone by level of contamination A, male plasma testosterone by level of contamination; error bars represent standard deviation. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

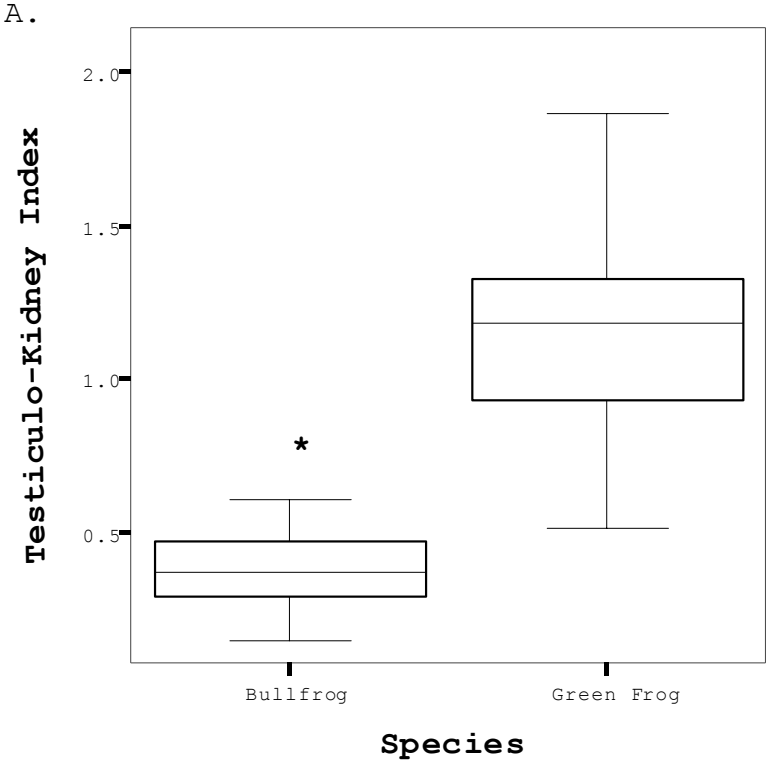


B.

Level of Contamination	Testosterone (ng/mL)	SD
Low	14.217 (34)	± 7.341
Medium	24.350 (41)	± 18.576
High	19.924 (27)	± 12.093

Figure 3.9. TKI between the species
A, TKI of all male bullfrogs versus all male green frogs. Lines in boxplots represent the 50th percentile, or median, of the data. A significant difference ($p < 0.001$) between bullfrog and green frog TKI is indicated by "*****." An independent t-test was used to test for significant difference between means. B, raw numerical data where SD is the standard deviation and number of frogs included in each analysis is in parentheses.

A.

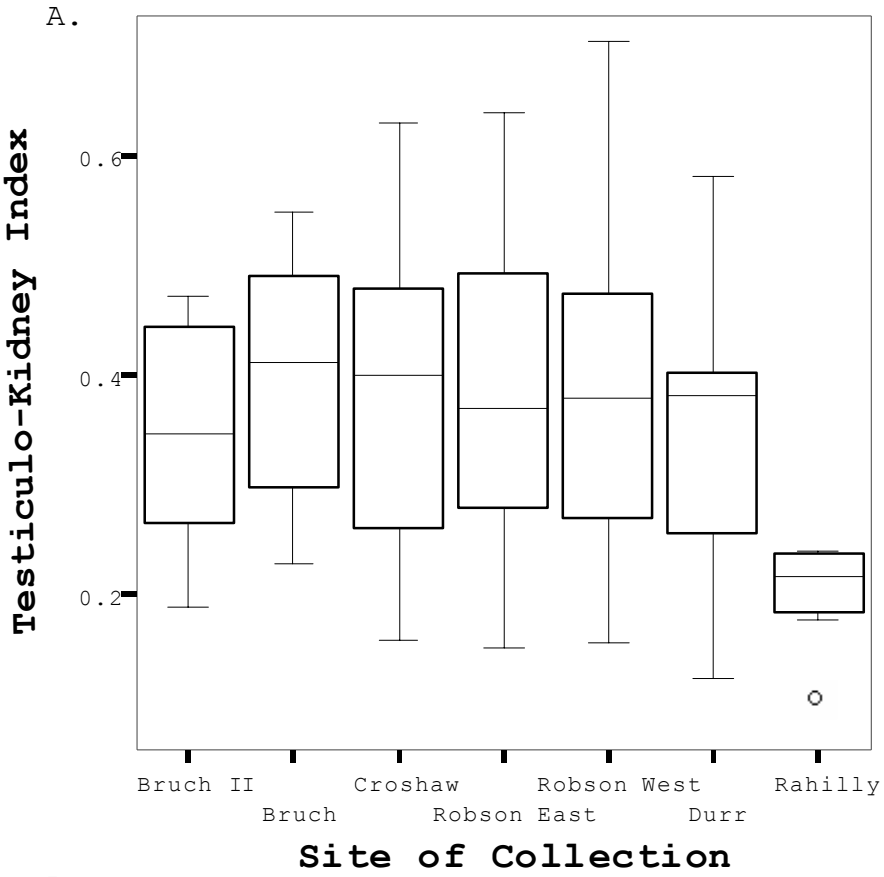


B.

Species	TKI	SD
Bullfrogs	0.3682 (51)	± 0.1108
Green Frogs	1.1721 (54)	± 0.3071

Figure 3.10. Bullfrog TKI by site
A, bullfrog TKI across sites. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation, "--" indicates less than two samples and number of frogs included in each analysis is in parentheses.

A.

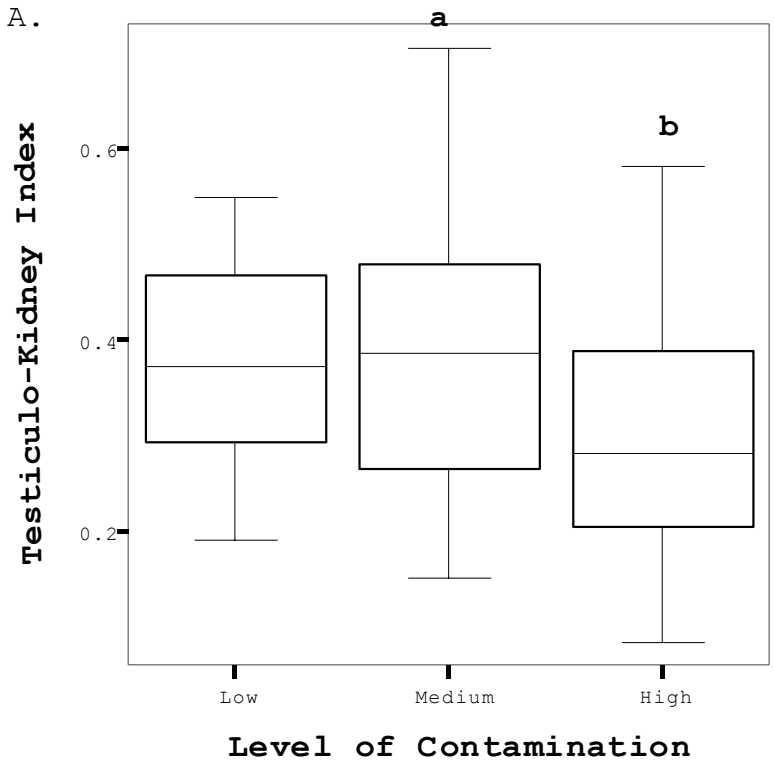


B.

Site	TKI	SD/Range
Durr II	0.3327 (2)	± 0.0488
Bruch II	0.3436 (6)	± 0.1073
Bruch I	0.3937 (19)	± 0.1056
Croshaw	0.3863 (19)	± 0.1329
Robson East	0.3819 (17)	± 0.1457
Robson West	0.3876 (18)	± 0.1555
Durr	0.3433 (13)	± 0.1270
Hopkins	0.3061 (1)	--
Rahilly	0.2386 (7)	± 0.1350

Figure 3.11. Bullfrog TKI by level of contamination
A, bullfrog TKI by level of contamination
Lines in boxplots represent the 50th percentile, or median, of the data. Populations designated with different letters are significantly different (p = 0.046). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

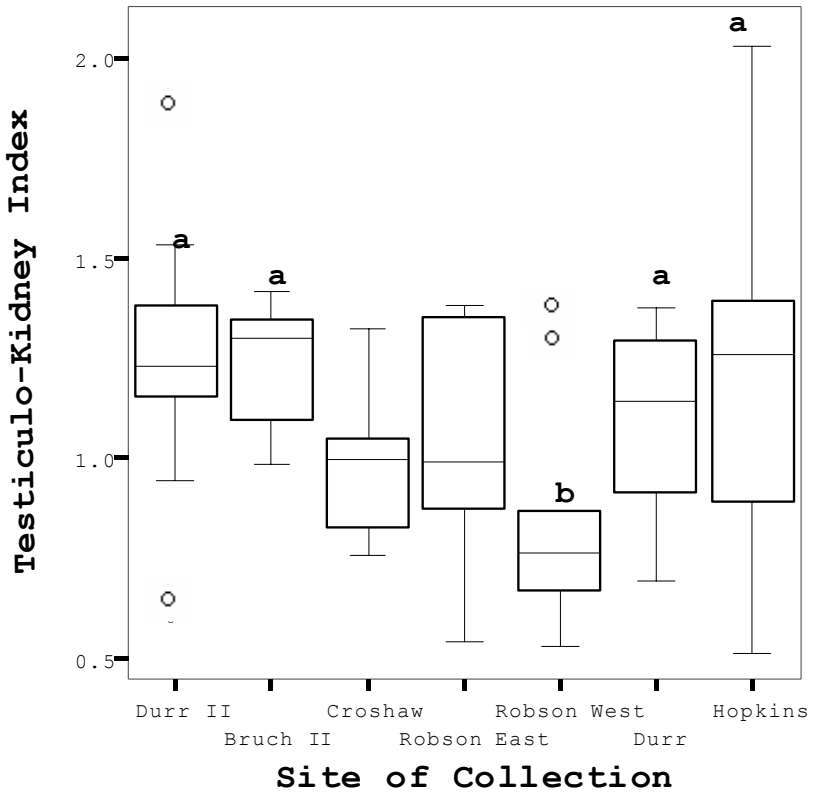


B.

Level of Contamination	TKI	SD
Low	0.3780 (27)	± 0.1032
Medium	0.3853 (54)	± 0.1420
High	0.3035 (22)	± 0.1337

Figure 3.12. Green Frog TKI by site
A, green frog TKI across sites. Lines in boxplots represent the 50th percentile, or median, of the data. Populations designated with different letters are significantly different ($p \leq 0.040$). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. Sites are in order of increasing contamination. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

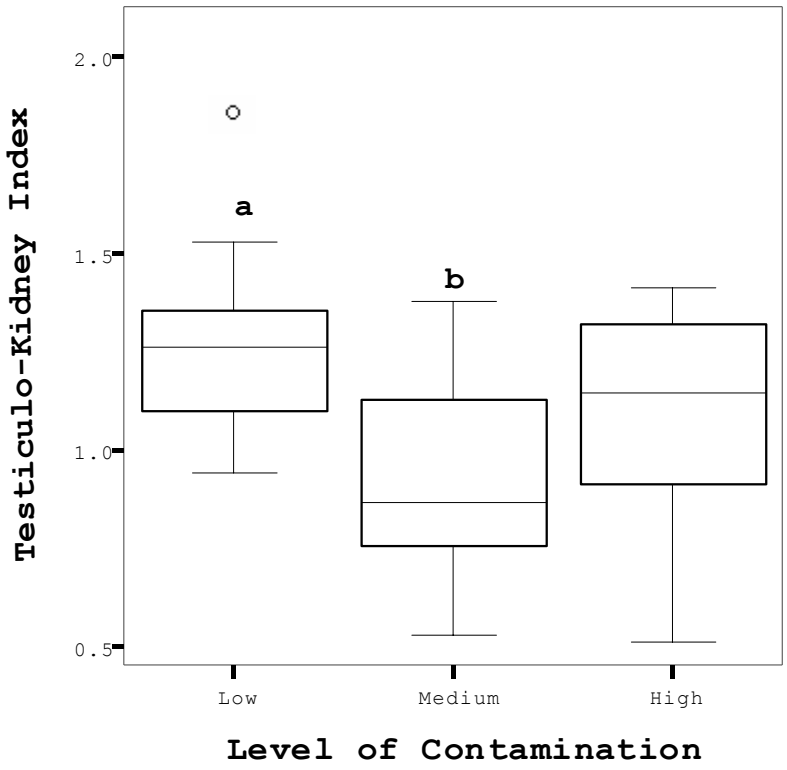


B.

Site	TKI	SD
Durr II	1.2371 (9)	± 0.3574
Bruch II	1.2333 (9)	± 0.1601
Croshaw	0.9764 (11)	± 0.1761
Robson East	1.1507 (6)	± 0.4943
Robson West	0.8714 (13)	± 0.3388
Durr	1.0965 (27)	± 0.2071
Hopkins	1.1944 (7)	± 0.4870

Figure 3.13. Green frog TKI by contamination level
A, green frog TKI by level of contamination. Lines in boxplots represent the 50th percentile, or median, of the data. Populations designated with different letters are significantly different ($p = 0.011$). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of TKI means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.



B.

Level of contamination	TKI	SD
Low	1.2352 (17)	± 0.2687
Medium	0.9686 (30)	± 0.3423
High	1.0857 (33)	± 0.2802

Figure 3.14. Testicular retardation
A, normal testicular structure of a green frog male (100x). As spermatozoa develop, they move into the lumen of each tubule. B, juvenile spermatogenesis. The presence of spermatozoa (arrow head) in a juvenile bullfrog testis indicates that juveniles undergo spermatogenesis. C, testicular dysgenesis in a male bullfrog (100x). Except for a small patch of spermatids (arrow), there is no evidence of active spermatogenesis within this testis. The tissue is instead completely filled with spermatogonia, some of which are enlarged (a few are highlighted in circles). D, spermgranuloma. Close-up (400x) of an enlarged spermatogonia presumably arrested in its growth phase.

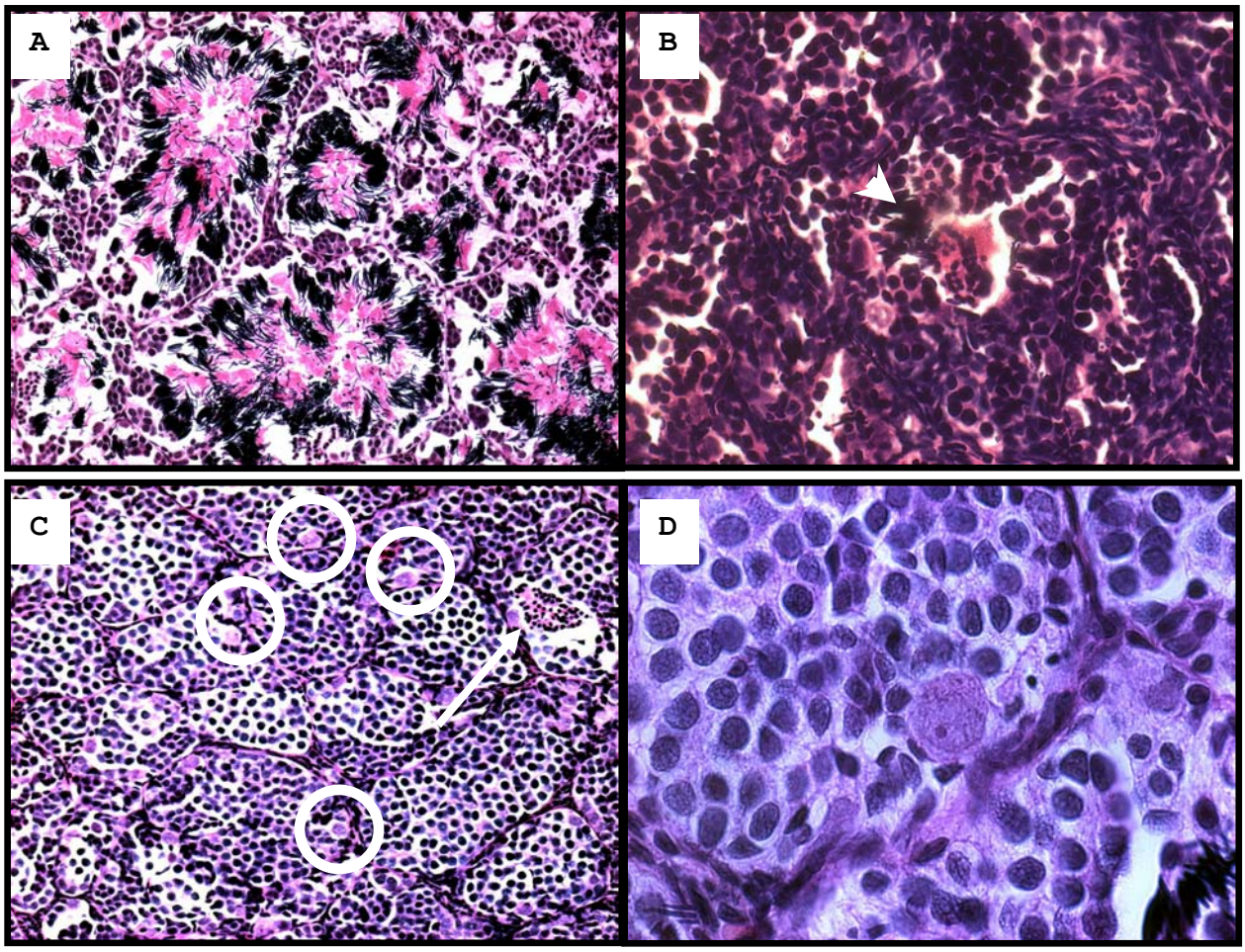


Figure 3.15. Testicular dysgenesis
A, testicular hyperplasia in a green frog male captured at the Durr site (200x). Notice that the interstitial cells are tightly packed. B, edema within a tubule from a mall bullfrog collected from Robson East (200x). C, testicular dysgenesis in a green frog collected from Croshaw (100x). D, calcification from a green frog from the Durr site (200x).

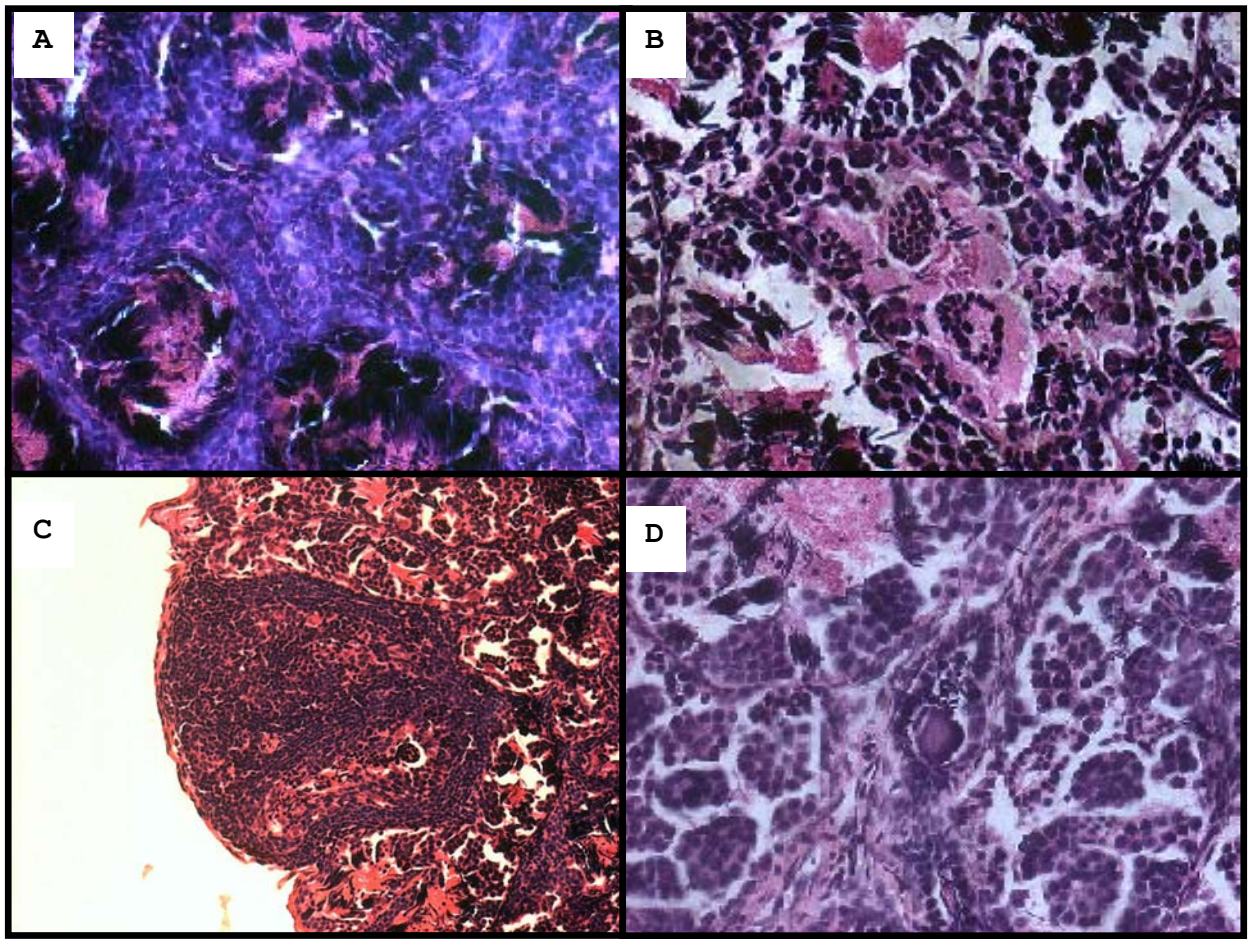
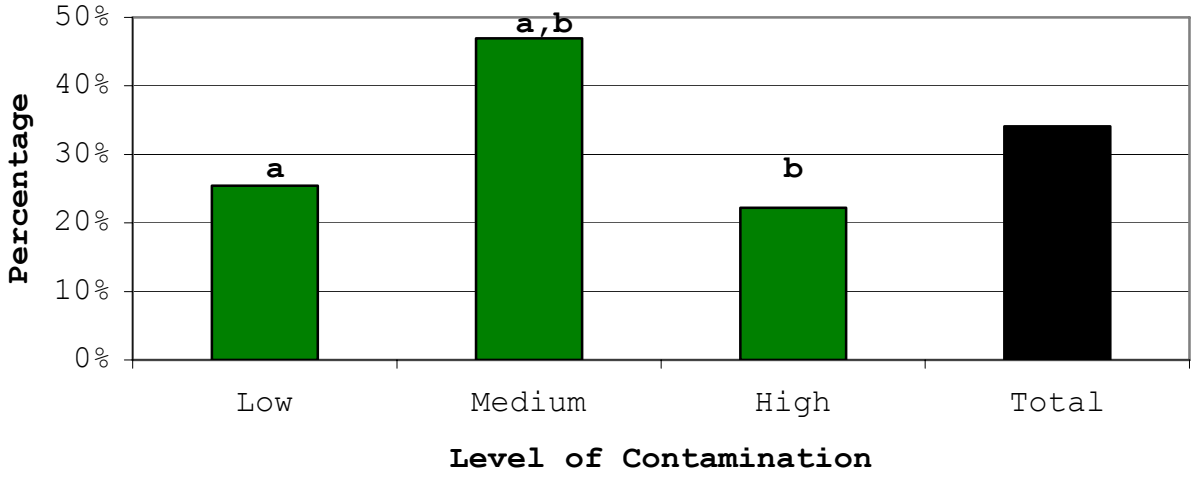


Figure 3.16. Testicular dysgenesis prevalence
A, percent testicular dysgenesis by level of contamination. Populations designated with the same letter are significantly different (a, p = 0.002; b, p = 0.001). Pearson's chi-square analysis was used to test for significant differences between proportions. B, percent prevalence of testicular dysgenesis with proportions shown in parentheses.

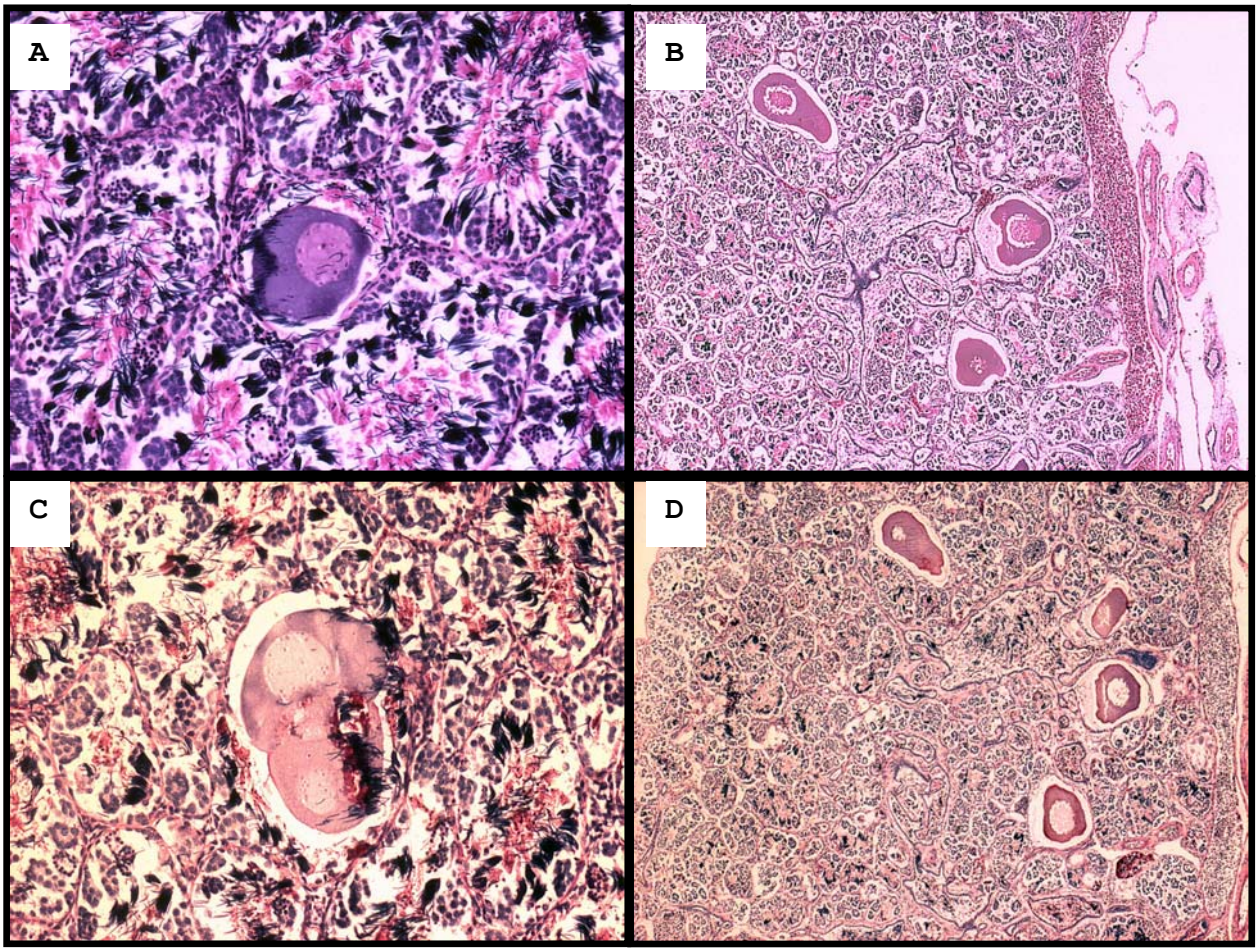
A.



B.

Low	Medium	High	Total
26.3% (15/57)	47.7% (42/88)	21.8% (12/55)	34.5% (69/200)

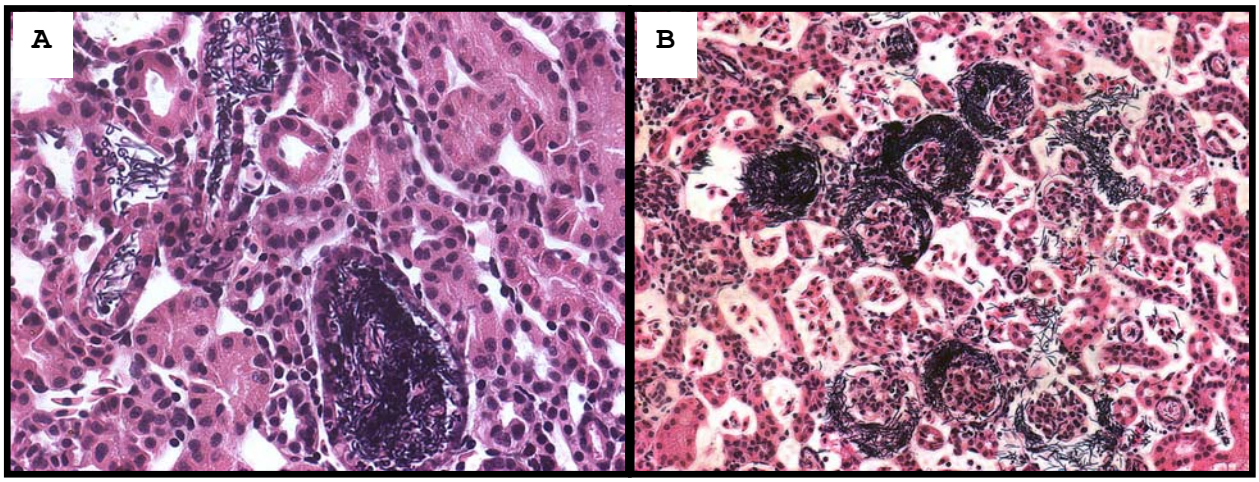
Figure 3.17. Testicular oogenesis
A, ovotestes from a green frog (100x). B, multiple oocytes within testicular tissue from a green frog male (25x). C, PAS stain of another egg in the tissues of A. D, PAS stain of eggs present in B. E, percent prevalence of testicular oogenesis in the three sites where it was observed with proportions shown in parentheses.



E.

Bruch II	Durr	Hopkins
9.1% (1/11)	10.3% (3/29)	16.7% (1/6)

Figure 3.18. Renal sperm
A, spermatozoa in the distal convoluted tubules of a green frog kidney (100x). B, spermatozoa in the Bowman's capsules of a green frog kidney (50x). C, percent prevalence in each species and overall with proportions shown in parentheses.



C.

Bullfrog	Green Frog	Total
0.5% (1/218)	7.5% (10/133)	3.1% (11/351)

Figure 3.19. Atrazine effect on steroid hormone
Consequences of atrazine action. GnRh = gonadotropin-releasing hormone; LH = luetinizing hormone; FSH = follicle-stimulating hormone; T = testosterone; DHT = dihydrotestosterone; ATR = atrazine; AROM = aromatase; E_2 = estradiol; AR = androgen receptor; modified from (Trentacoste et al. 2001).

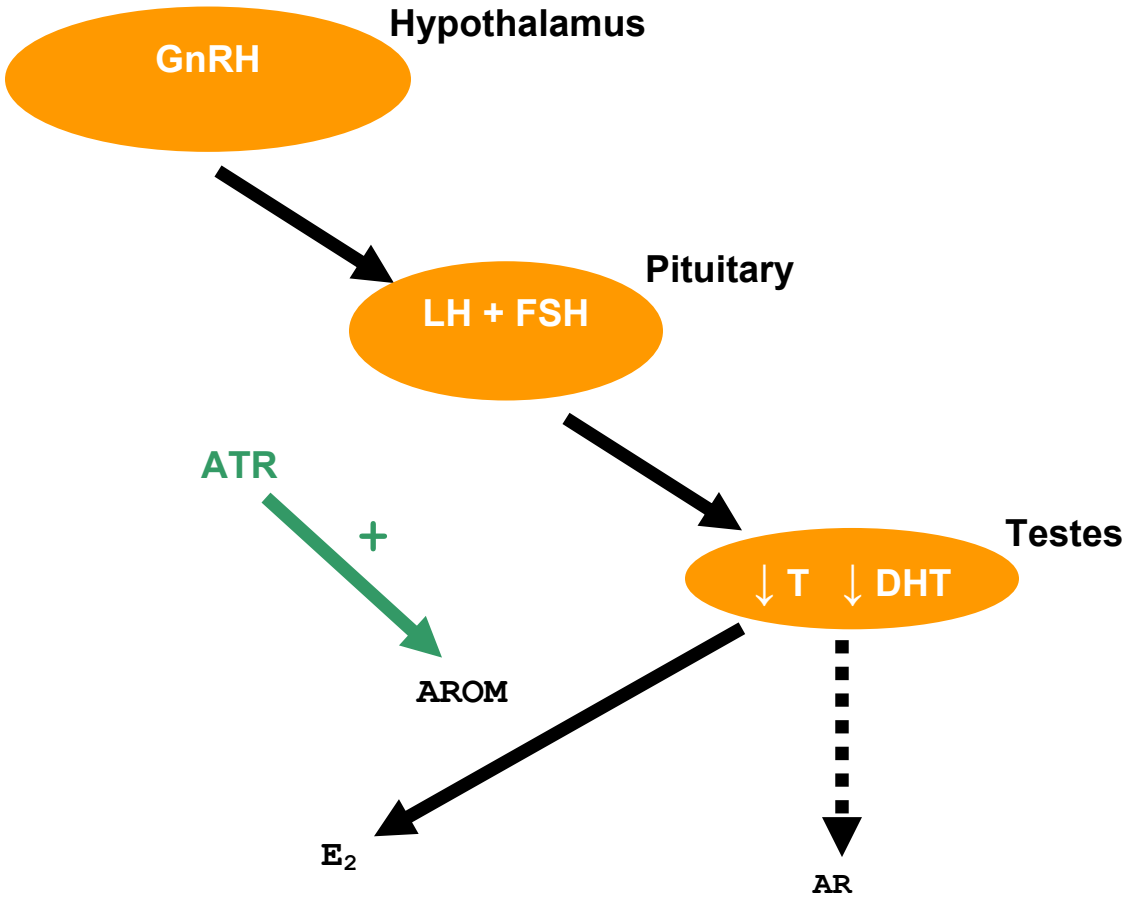
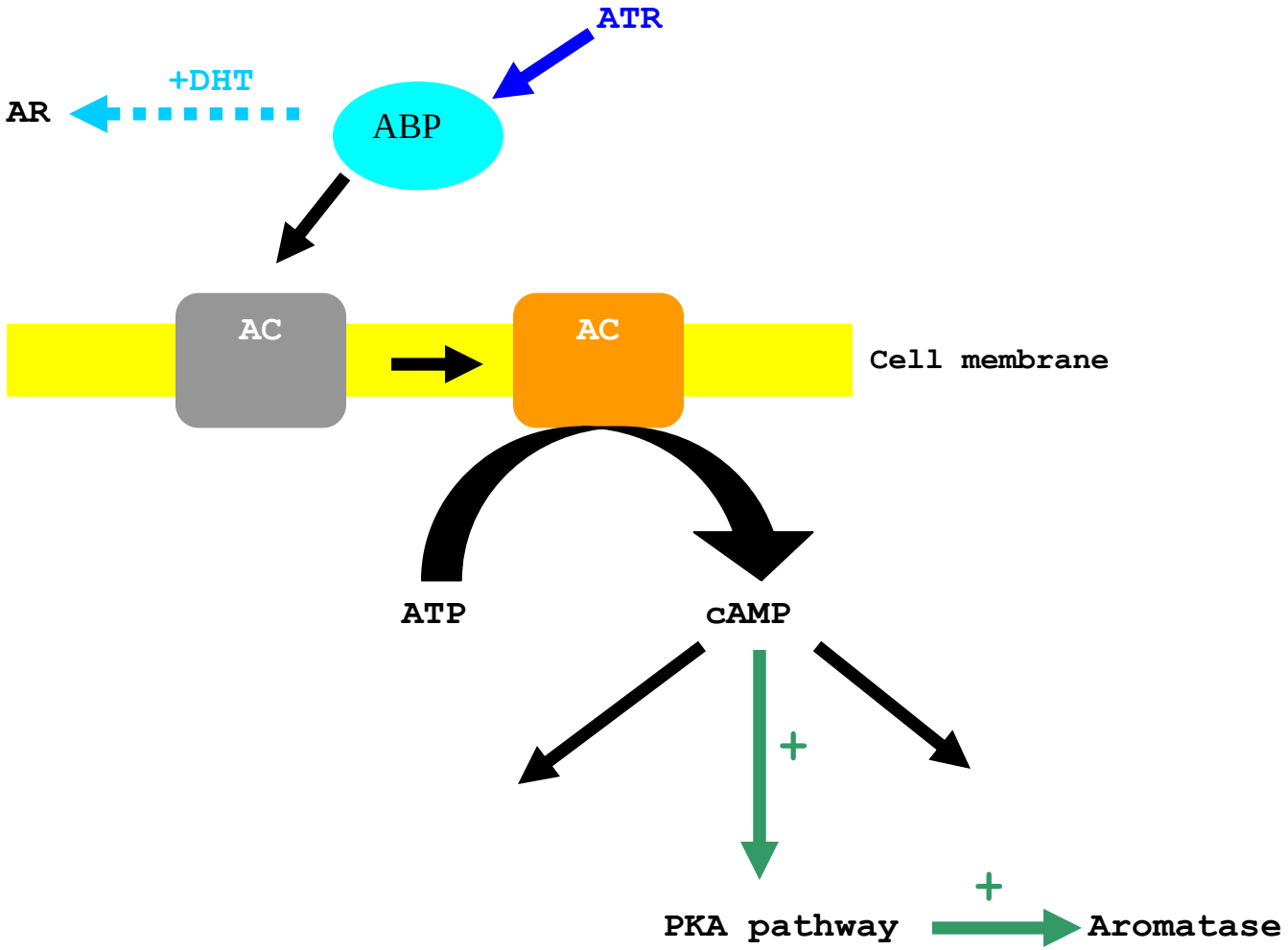


Figure 3.20. Atrazine action within the cell
Atrazine proposed mode of action for aromatase induction. DHT = dihydrotestosterone; ATR = atrazine; ABP = androgen binding protein; AC= adenylyl cyclase; modified from (Sanderson et al. 2001).



Prevalence of parasites and deformities in bullfrog (*Rana catesbeiana*) and green frog (*Rana clamitans melanota*) populations from sites contaminated with agricultural chemicals

Chapter 4

Abstract

Pathogens have been implicated as one cause in worldwide amphibian declines. In addition, increases in amphibian deformities have raised concerns about the etiology and role of gross malformations in amphibian losses. We evaluated the prevalence of parasitism and signs of disease and deformities in bullfrog (*Rana catesbeiana*) and green frog (*Rana clamitans melanota*) populations residing on agricultural sites in New Jersey. These sites incur various degrees of contamination from several pesticides, including atrazine. Incidences of parasites and deformities (both gross and histological) are shown by site and tissue. The frequencies of renal parasites and metacercarial cysts were 48% higher and 60% lower, respectively, at sites characterized as high compared to low or medium. Prevalence of hepatic inflammation was 44% higher in captures from medium-contaminated sites, while renal inflammation was 14% higher at high contamination compared to low. Incidence of inflammation in both tissues decreased over the collection

years. In addition, renal dysgenesis was 37.5% more prevalent in frogs from medium contaminated sites than low or high. White blood cell content is also presented with a discussion on the possible interactions between chemical contamination and other environmental factors, with parasitism, malformations and immune function. Our analyses show trends relating chemical contamination and prevalence of parasites, inflammation and renal dysgenesis. However, environmental interactions are quite complex and the extent of influence of each parameter remains ambiguous.

Introduction

Frog declines have been associated with signs of disease, where potential causative agents include viruses, bacteria, fungi and even parasites (Lips 1998; Carey 2000). Since the mid-1970s, outbreaks of disease have increased, coinciding with amphibian declines (Lips 1998; Carey 2000). It is unclear if the reported increases in amphibian mortalities resulting from disease are due to novel pathogens entering naïve populations. Novel agents are suspected with the patterns of progressive decline as witnessed in Queensland, Australia and other areas (Laurance et al. 1996; Johnson and Speare 2005).

Alternatively, increased disease rates could be a consequence of an increased virulence in the infectious agents, possibly stemming from their interactions with environmental factors, like contamination. Chemicals present in the environment could cause mutations that transform nonpathogenic agents into pathogenic ones for amphibians. For example, the chytrid fungus, which causes thickening and sloughing of the skin and essentially suffocates its host without eliciting an immune response, was not always pathogenic toward frogs as it now appears to be (Berger et al. 1998). In a study exposing leopard frogs (*Rana pipiens*) to a pesticide mixture, lungworm (*Rhabdias ranae*) infection rate was unchanged compared to controls (Gendron et al. 2003). However, migration of the parasite was accelerated and twice as many adult worms were established in the lungs of the exposed frogs versus unexposed (Gendron et al. 2003).

The disease-related declines could result from depressed immune systems, weakened by stressors of the natural environment and of anthropogenic sources (Carey 2000). For example, Kiesecker (2002) found increased trematode infections in wood frogs (*Rana sylvatica*) exposed to the herbicide atrazine and the insecticides malathion and esfenvalerate. Furthermore, these increased infections were associated with reduced

immunocompetency as indicated by reduced eosinophil numbers (Kiesecker 2002). Similarly, Kiesecker and Blaustein (1995) observed that ultraviolet-B (UVB) radiation increased mortality in the western toad (*Bufo boreas*) only in the presence of the *Saprolegnia ferax* fungus. This observation suggests that UVB, like contaminants, decreased immune function, which allowed the fungus to lethally infect the animal. UVB may also act synergistically with contaminants to lower immune function that leads to mortality from disease. The complex and multifactorial deterioration of habitat may compromise the immune systems of resident amphibians, increasing their susceptibility to disease and infections.

Accompanying reports of unprecedented mortality associated with disease, are reports documenting increased incidences of developmental deformities. Proposed causes of these deformities fall into two categories: chemical contamination and parasitic infections (Kiesecker 2002). Ouellet et al. (1997) recorded a high incidence of hindlimb deformities in *Rana catesbeiana*, *R. clamitans*, *R. pipens* and *Bufo americanus* from agricultural sites contaminated by pesticide runoff from the St. Lawrence River Valley in Québec, Canada. Severe degrees of ectromelia (absence of all or part of limb) and ectrodactyly (absence of digit), as well as kidney and liver degeneration or general systemic

illness, were found primarily in juveniles (Ouellet et al. 1997).

An increase in limb deformities has also been found across the central United States where agricultural contaminants are most prevalent (Sparling et al. 2000). These deformities are mainly present in species with aquatic eggs and larvae, implying that the causative agent and/or its driving factors are in the water (Johnson et al. 2004). Furthermore, species with longer larval stages seem to be more susceptible than other species (Sparling et al. 2000). Experimentally, Allran and Karasov (2001) observed a dose-dependent increase in deformities in ***Rana pipiens***, ***R. sylvatica*** and ***Bufo americanus*** larvae following exposure (0-20 ppm) to atrazine.

Though amphibians are host to a wide array of metazoan and protozoan parasites, resultant disease is rare (Sparling et al. 2000). Even so, such parasites may lead to deformities, particularly of the limbs. Sessions and Ruth (1990) found trematode cysts localized in the limb regions of pacific treefrogs (***Hyla regilla***) that resulted in multiple limb abnormalities ranging from extra digits (polydactyly) to several whole limbs (polymely). It has been proposed that interactions of exogenous agents, such as pesticides and nutrient runoff,

with parasites elevate infection levels (Johnson and Sutherland 2003). Parasite-induced malformations are not a new phenomenon, rather they are a historic cause of limb deformities; however, their levels have recently increased (Johnson et al. 2003) and are associated with declines in tadpole survivorship (Johnson et al. 1999). What is unknown is whether there is an underlying factor leaving animals susceptible to such diseases and abnormalities. Contaminants could be the ultimate cause of both by compromising the immune system (Carey 1993).

In a field study conducted over three breeding seasons (2003, 2004, 2005), bullfrogs (*Rana catesbeiana*) and green frogs (*Rana clamitans melanota*) were collected from agricultural sites in Burlington County, NJ. The captures were examined grossly and histopathologically for parasites, as well as for any deformities to test if increased pesticide exposure increases parasitic load and/or deformity ratios. Presented in this chapter are the prevalence of parasites and observed deformities across different sites and in different tissues. In addition, blood smears were performed for each frog and percent white blood cells calculated.

Materials and Methods

Study area. All collection sites (in increasing order of contamination: Durr II, Bruch II, Bruch I, Croshaw, Robson East, Robson West, Durr, Hopkins and Rahilly) were within the same vicinity of Jacobstown in Burlington County, New Jersey (see Chapter 2 for location details). For analyses sites were nested by level of contamination.

Frog collection. Animals were collected by hand and net capture, transported to the laboratory and examined. For a detailed explanation, see Chapter 3.

Animal dissection, tissue harvesting and histology. Brain, gonads, larynx, liver and kidney were removed and preserved by procedures described in Chapter 3. Observations of gross or histological parasites and deformities were recorded on data record sheets created for each frog, photographs were taken and observations logged as described in Chapter 3. If a tissue (brain, liver or kidney) was not examined histologically, it was not included in subsequent analyses.

Blood smears. A drop of blood, obtained from cardiac puncture (see Chapter 4 for description), was placed at one end of a labeled glass slide. A second "spreader" slide was used to create a smear on the slide. The smear was dried under a hood,

fixed with methanol, and stored in a slide box until stained with Wright-Geisma (Richard-Allan Scientific). Under a microscope, percentage of white blood cells to total cell count was calculated as the average of four field views under 40x magnification.

Statistics. Pearson's correlation tests were performed for parasite and deformity incidences. For many analyses, the number of observances was too low for comparisons between sites; therefore, sites were compared by their level of contamination. Comparison of means was performed by one-way analysis of variance (ANOVA) with Bonferroni correction for white blood cell content. Significance for each test was set at $p < 0.05$. For each analysis, p values are provided in the results section.

Results

Site characterization. The pH at our sites was measured yearly and ranged from 4.0 to 5.0. Low pH can interact with other abiotic and biotic factors to lethally or sublethally affect amphibians (Sparling et al. 2000). Embryos are most sensitive to low pH and tolerance increases throughout the larval stage (Pierce et al. 1984). Acidic conditions modify sodium balance in that as pH declines sodium efflux increases. After a loss of about half of the initial body sodium, death ensues (Freda and

Dunson 1984). Of the species tested, the green frog (*R. clamitans*), one of our target species, was the most tolerant to changes in pH.

In low pH (4.0-5.0), time to hatching is significantly increased compared to neutral rearing media (Andrén et al. 1989). Prolonged time in the embryonic period, reduced hatchling size and modified hatchling behaviors increase susceptibility to egg predation and predation by gape-limited predators (Sparling et al. 2000). Acidic conditions is also associated with decreased body size; after eight months of exposure, growth rates were reduced by 45 to 60% in the salamander, *Plethodon cinereus* (Wyman and Hawksley-Lescault 1987).

Body size is an important variable in determining outcome of competitive and/or predatory interactions (Wilbur 1972). Metamorph size has fitness consequences to the amphibian's terrestrial life as larger metamorphs have greater fitness than smaller ones (Semlitsch and Gibbons 1988). One of the ways pH affects body size is by decreasing the algal and invertebrate food sources available in a given habitat (Sparling et al. 2000). For these reasons, the low pH at our sites is a confounding variable.

Based on pesticide use histories and water analyses (see Chapter 4 for details), study sites were classified as low (Durr II, Bruch II and Bruch I), medium (Croshaw, Robson East and Robson West) or high (Durr, Hopkins and Rahilly) in regards to level of chemical contamination.

Captures. Over three breeding seasons, 391 adult and juvenile bullfrogs and green frogs were captured. See Chapter 3 for a complete distribution of captures by site, species, sex and year.

Parasites. Figure 4.1 displays a variety of parasites found in frog tissues. Round worms are quite common and Figure 4.1A shows an example of the parasite's anchorage to intestinal mucosa. Acanthocephalans are thorny-headed parasites that are fairly rare (Sparling et al. 2000), but illustrated in Figure 4.1B. Amphibian tuberculosis has been attributed to *Mycobacterium marinum*, *M. xenopi* and *M. fortuitum*. This is a chronic disease localized in the skin in cysts, ulcers and nodules, or systemically with granulomas in the liver, spleen and other viscera (Hoff et al. 1984). Figure 4.1C demonstrates a possible tuberculosis infection in the liver. Blood protozoa, *Trypanosoma* and *Lankesterella*, are prevalent in wild amphibians but their consequence is unknown (Barta and Desser 1984).

Figure 4.1D is a picture of a collection of this type of parasite. Figure 4.1E is of a parasite traveling through a blood vessel of the kidney, while Figure 4.1F is a photograph of a parasite within testicular tissue, a rare observation. Figure 4.2 demonstrates the percent prevalence of parasitism by level of contamination. A majority of the frogs was parasitized and there were no significant differences between site groupings. In addition, when looking across the collection years (Figure 4.3), incidence of parasitism remained comparable.

The most commonly parasitized organs were the liver and kidney. Percent incidence in liver is displayed in Figure 4.4 with representative microphotographs of parasites observed. There were no significant differences in levels of parasitism of the liver by site level of contamination, nor across the collection years (Figure 1.5). In contrast, parasitism of the kidney did demonstrate significant elevation in the high contamination sites compared to both the low and medium sites (Figure 4.6). Parasites of the kidney, as with the liver, were varied and consisted most commonly of round worms (Figure 4.6A) and nematodes (Figure 4.6B). There were no significant changes in renal parasitic incidence over the collection years (Figure 4.7).

In addition to tissue-specific analyses, we analyzed the prevalence and incidence of specific parasites. Figure 4.8 illustrates the prevalence of infection with metacercariae, encysted forms of trematodes, in the brain. The frequency of infection decreased with increasing levels of contamination and the high contamination sites incurred 56.9-67.4% significantly less metacercarial infections than the other sites. This decrease in infection level may be due to a direct toxic effect by the contaminants on the parasites or a secondary effect due to amphibian population size. Compared to the other sites, the high contamination sites yielded fewer frogs from collections and abundant populations will have more parasites than scarce populations because a critical host density is necessary for effective transmission of parasites (Lafferty and Kuris 2005).

Trematode parasites use amphibians as intermediate hosts in their life cycle. The presence of the cysts may interfere with brain function and leave the infected frog easy prey for a definitive host. A typical life cycle for trematodes begins with the release of eggs into the water through the feces of a definitive host, like a heron. Aquatic snails become infected via ingestion of the eggs during grazing. Daughter sporocysts then shed free-swimming cercaria in the water. These cercaria access the internal tissues of a tadpole via penetration through

the skin or through openings like the cloaca (Sparling et al. 2000). Trematodes may infect their anuran host in ways that will increase the parasite's transmission to a definitive host. For example, ***Ribeiroia*** sp. often encyst near developing limb buds and cause malformation of the limbs. Additionally, ***E. trivolvis*** encyst the kidney and have been shown to reduce body length and weight in metamorphs (Fried et al. 1997). These infections are designed to increase the likelihood of predation by a paratenic or definitive host of the parasite by causing a handicap that prevents the amphibian from avoiding these predators. Encysted trematodes assume their adult and sexual form in the definitive host and reproduce. The eggs shed from reproduction are returned to the aquatic system in the feces of the definitive host and the cycle continues (Sparling et al. 2000). Incidence of metacercarial cysts did not change over collection years (Figure 4.9).

Figure 4.10 displays the prevalence of protozoan cysts, which were only found in the liver. These encysted clusters of the parasite are used to prevent eliciting an immune response from the host, or escape the effects of an immune response (Purner et al. 1996). In time, as immune activation diminishes, the cysts will release the protozoa to multiply (Figure 4.10B shows a cyst hatching). No incidences of protozoan infection were found in

amphibian tissues collected from low contamination sites. Furthermore, the frequency of this infection was so low, no significant differences were observed between levels of contamination or across years of collection (Figure 4.11).

Deformities. Several gross and histological abnormalities were found in our captures. Figure 4.12 displays a spectrum of lesions observed from our animals. This includes dysplasia within the testes (Figure 4.12A), as well as agenesis of a single testis in a bullfrog. There were a few eye abnormalities involving pupil size and formation (Figures 4.12B and 4.12C display a normal eye and an enlarged pupil, respectively). There were instances of missing limbs, including hemimely (Figure 4.12D), and it is not known if these are developmental or a result of trauma. Brachymely, or shorter limbs (Figure 4.12E), was the most common abnormality seen. In addition, skin lesions were observed that presented with white nodules. These white lesions were most likely opportunistic trematode skin infections (Figure 4.12F). Figure 4.13 demonstrates the incidence of gross abnormalities observed in captures by site level of contamination, which was comparable across sites.

In addition, dysgenesis of organs was also found histologically. This was especially true of the kidney. Figure 4.14 displays

some of the lesions observed in the kidney: tubular degeneration (Figure 4.14A), calcinosis (Figure 4.14B), encysted tubules (Figure 4.14C) and hyaline droplets of protein (Figure 4.14D). When compared across levels of contamination, renal dysgenesis was significantly more frequent at the medium contamination sites (Figure 4.15).

Inflammation. Focal areas of inflammation were common in the tissues of captures, particularly in the liver and kidney. As Figure 4.16 illustrates, hepatic inflammation was highest in sites classified as medium and was significantly different from the levels at low contamination sites. On the other hand, renal inflammation increased with increasing contamination and percent prevalence in the high contamination sites was significantly elevated (13.9%) compared to the low contamination sites (Figure 4.17). Incidence of inflammation across the years of collection decreased significantly (48.1-67.9%) in both tissues (see Figure 4.18 for hepatic inflammation and Figure 4.19 for renal inflammation) between years III (2005) and I (2003).

White Blood Cells. Because one of our contaminants, atrazine, has been identified as an immunosuppressant (Allran and Karasov 2001; Gendron et al. 2003), white blood cell content was calculated as a measure of immune responsitivity (Kiesecker 2002). White blood cell content in our captures was comparable (~5.2%) across site level of contamination with no significant differences apparent (Figure 1.20).

Discussion

Parasitism levels. Normally, parasites and their hosts exist in an equilibrium; however, anthropogenic influences like chemical contamination, as well as habitat alteration, introduced species and poor water quality due to acidification, influence parasite-host interactions (Sparling et al. 2000). Nematode, cestode and trematode infestations are often present, but their increase may be an additional burden that becomes significant under stressful conditions.

Nematodes are found in the lungs and gastrointestinal tracts of amphibians and can cause enteritis and anemia. Filariids can be found in blood vessels and lymph spaces with larval microfilaria in the circulation (Sparling et al. 2000). Signs of infection include lethargy and anorexia (Sparling et al. 2000). One of our captures, which presented with hemimelia, was extremely emaciated with slow movement.

Adult cestodes may cause gastrointestinal obstruction, while immature forms may occur as small aggregates beneath the skin, in muscle and in internal organs (Sparling et al. 2000), which were quite common in the kidneys of our captures. The observation that the prevalence of renal parasites increased with increasing contamination (Figure 4.6), suggests a role for

chemicals in infection rates and transfer, perhaps by increasing the susceptibility of the host animals.

Trematodes infect the intestines, bladder, lungs, kidneys and muscles (Johnson et al. 1999). Risk for trematode parasitism increases as pond systems are simplified and degraded because such conditions favor high numbers of aquatic snails infected with trematode miracidia (Sparling et al. 2000). Our observation that brain metacercariae infections decreased with increased contamination may reflect a direct toxic effect on the parasites or their first host, aquatic snails. Alternatively, insufficient host density would impede parasite transmission (Lafferty and Kuris 2005).

Trematodes rarely illicit an immune response; however, they may be responsible for limb deformities by mechanically disrupting limb formation and causing supernumerary of limbs (Johnson et al. 1999). Sessions and Ruth (1990) reported metacercarial cysts of trematodes in the cloacal and developing hindlimb regions of larvae. The authors speculate that the disruption was mechanical because they were able to reproduce the deformities with inert resin beads implanted in the developing hindlimb bud regions of the *Xenopus laevis*. Johnson et al. (1999) determined a correlation between exposure to cercaria and

numbers of deformities, in addition to an inverse relationship with survival.

Deformities. The broad spectrum of developmental abnormalities seen in amphibians likely results from multiple factors. Chemical contamination from agriculture, industry and urbanization may work synergistically with biotic and abiotic factors to give rise to the wide range of malformations (Ouellet et al. 1997). The common morphological abnormalities and injuries include ectromelia (limb agenesis), ectrodactyly (missing digits), polydactyly (multiple limbs), and abnormal webbing of the toes, which occur at low frequencies between 0 and 2% (Read and Tyler 1994). When it is higher, extrinsic factors like toxicants are suspected (Ouellet et al. 1997). The incidence of abnormalities at our sites was slightly higher than background, but was comparable across sites (Figure 4.13).

Damage to limb buds by predatory leeches can lead to ectromelia and ectrodactyly (Sparling et al. 2000). With the onset of metamorphosis, anurans lose the ability to regenerate (Sparling et al. 2000), but prior to that hyper-regeneration following injury may play a role in the observed polydactyly. Unilateral and asymmetrical lesions may be indicative of trauma, while bilateral and symmetrical lesions are more likely to be

developmental defects (Sparling et al. 2000). Agricultural pesticides, herbicides, insecticides, fungicides and fertilizers can cause deformities and subsequent mortality (Alvarez et al. 1995; Ouellet et al. 1997; Kiesecker 2002). In addition to the active ingredients, solvents as inert ingredients contribute to developmental toxicity resulting in axial skeleton and tail malformations such as hindlimb brachymely (shortened limbs), twisted epiphyses of the long bones, scoliosis, cutaneous fusions and joint dislocations of the hindlimbs (Cooke 1981; Alvarez et al. 1995). Futhermore, pesticides and parasites can interact to increase deformities (Relyea and Mills 2001). Hindlimb deformities are also more prevalent in amphibians exposed to agricultural runoff, which can potentially increase transmission rates of trematode infections (Lafferty and Kuris 2005). Developmental abnormalities can affect survivorship because they interfere with swimming, hopping, acquisition of prey items and avoidance of predators; and such abnormalities are likely to affect amphibians at the population level.

Agents involved in amphibian deformities may impact human health. Schwartz and LoGerfo (1988) found congenital limb reduction defects to be associated with parental agricultural work and residence in agricultural settings. Garry et al. (1996) reported an increased risk of birth anomalies in children

from parents who were private pesticide appliers or resided in agricultural regions. These anomalies included limb reduction defects, polydactyly, syndactyly (fusion of fingers) and adactyly (missing fingers). Nurminen (1995) indicated an increased risk of limb anomalies and orofacial clefts with environmental and occupational exposure to pesticides. Though pesticides are the most obvious agent, inorganic and organic fertilizers as well as specific pollens are also unique to the agricultural setting. Furthermore, estrogen plays an important role in normal development, and consequently, in abnormal development and resultant deformities (Timms et al. 2002). Since atrazine, one contaminant at our sites, acts through increased endogenous estrogen, it is conceivable that it can have a role in estrogen-mediated developmental effects.

It is important to note that limb abnormalities vary in frequency from year to year and site to site (Ouellet et al. 1997). Also, differences in mortality rates in deformed frogs due to predation may play a role, causing a bias toward a low number of observed deformities due to preferential predation.

Neoplasias of the skin, liver, kidney and other organs have been observed, and tumors are associated with contaminated habitats (Rose and Harshbarger 1977). In our captures, we saw

significant elevation of renal dysgenesis at sites characterized as medium contamination (Figure 4.15). Incidence of abnormal development may be reduced at high contamination sites due to decreased survivorship of affected larvae and metamorphs.

Though environmental pollutants and other anthropogenic stressors are suspected as important factors in morphological deformities, other important factors that influence survivorship and development, and may obscure interpretations in the field, are temperature, primary productivity and water chemistry (Harris et al. 1998). Freezing, as well as high temperatures enhance deformity rates (Cooke 1981). Consequently, causes of deformity rates are conceivably a complex interaction of factors. In addition, there may be a lag time from the presence of an agent to the later detection of deformities. The leading hypotheses in deformity formation include trauma, parasitic infection, and xenobiotics with interaction from UVB radiation and other as yet unidentified agents (Sparling et al. 2000).

Immunosupression. Amphibians maintain many of the same immune-related structures and functions as mammals. Bone marrow, spleen, thymus, lymphoid tissue and kidney produce cellular and humoral components (Carey and Bryant 1995). Like mammals, amphibians are able to mount antibody responses to initial and

subsequent antigen exposures (Carey and Bryant 1995). However, several factors influence the quality of the immune response: nutritional status, stress, age, season and especially temperature (Maniero and Carey 1997). When the animal's temperature drops below its preferred range, antibody production is reduced or even abolished. In addition, the immune system undergoes major changes during metamorphosis (Carey and Bryant 1995). This response varies with each species (Maniero and Carey 1997).

Contaminants can kill amphibians indirectly through altering immune function by suppression, rendering it unable to appropriately respond to disease (Carey and Bryant 1995; Carey et al. 1999; Pickford and Morris 1999; Taylor et al. 1999; Davidson et al. 2002; Kiesecker 2002; Blaustein and Johnson 2003). As a consequence, amphibians may experience lethal infections that are not normally fatal or may be killed by pathogens not historically lethal (Lips 1998). For example, overwintering is a stressful time as amphibians rely solely on cutaneous respiration, maintain slowed metabolisms, and utilize fat stores (Boutilier et al. 1997). Infections by *Aeromonas* sp. can be particularly detrimental to hibernating amphibians and contaminants in the environment that weaken, through reduced body condition and/or increased stress, an individual's immune

system can cause development of a lethal infection that would otherwise be controlled (Sparling et al. 2000).

Very few studies have looked at the interaction of contaminants, infectious agents and immune function. Tangredi and Evans (1997) studied Eastern box turtles (*Terrapene carolina*) infected with bacteria and fungi and found that the infections correlated with tissue concentrations of chlordane and its metabolites. It is possible that the toxicants suppressed the turtles' immune systems and left them vulnerable to opportunistic infections (Garner et al. 1997). Immunosuppression has been demonstrated in leopard frogs (*Rana pipiens*) exposed to an agricultural mixture that included atrazine (Gendron et al. 2003). Decreased immunocompetency against trematode infections was also shown in wood frogs (*Rana sylvatica*) with atrazine exposure (Kiesecker 2002). While our animals demonstrated increased inflammatory prevalence with chemical contamination (Figures 4.16 and 4.17), and no changes in white blood cell content (Figure 4.20), we did not study the effectiveness or responsiveness of the immune system in our captures.

Physiological links between neural pathways, the endocrine system and the immune system are being continually uncovered. For example, neuroendocrine peptide hormones modulate T and B

lymphocytes (Smith and Blalock 1988). Therefore, the interaction between toxicants and the immune system may involve the influence of other organ systems like the brain, autonomic nerve pathways and the endocrine system. Endocrine disruptors that act by increasing endogenous estradiol, like atrazine, are particularly effective as the sex organs and the brain are not the only entities responsive to sex hormones, the immune system is also (Bigsby et al. 1999). In addition to bone preservation, blood vessel integrity and brain function, estradiol also contributes to immune function (Akingbemi and Hardy 2001; Levin 2001). Therefore, changes in estradiol levels will affect the immune system and its ability to respond effectively. Likewise, many chemicals that contaminate our environment and act through endocrine disruption, can cause developmental abnormalities and alterations in immune function (Lips 1998).

Environmental changes may be sublethal, but acting singly or synergistically could stress amphibians enough to compromise their immune systems (Carey and Bryant 1995). As herbicides remove plant cover, amphibians receive increased exposure to UV-B and its damaging effects, which includes immunosuppression (Hurks et al. 1997). In addition, when stress rises due to overcrowding, increased competition, greater predation pressures and chemical contamination, release of immunosuppressive adrenal

cortical hormones increases; even short-term stress can lead to immune suppression lasting several days (Wilbur 1977). A depressed immune system favors transmission of helminths, viral, bacterial and fungal diseases and results in slowed growth, smaller size at metamorphosis, reduced reproduction, deformities and increased mortality (Carey and Bryant 1995).

Even if disease were the main cause of mortality, disease may not spring from novel pathogens, but from stress-induced changes in immune function brought about by changes in the environment, which are mostly anthropogenic (Carey 1993). If this is so, it is imperative for amphibian conservation, and possibly human health, that we identify and relieve the stressors involved. It is the combination of direct, indirect, lethal and sublethal effects of factors operating within a habitat that adversely affects populations and communities of amphibians and other organisms (Sparling et al. 2000). These interactions are complex, as Figure 4.21 demonstrates with the interplay of factors and their outcomes on immune function, parasitism and deformities. It is the study of these interactions that will prove beneficial in ameliorating effects on amphibians and the environment. Overall, chemical contamination has been shown to play a role in infection prevalence within a population. However, the extent of its influence will be determined in part

by other environmental factors present, many of which we do not know the effects.

Figure 4.1. Parasite histology
A, parasitic feet anchoring to mucosa of gut in a green frog male (50x). B, thorny hepatic parasite in a green frog male (200x). C, hepatic cell nests, possibly stemming from a tuberculosis infection, in a bullfrog male; note the futile cycle of inflammation with the presence of epithelial macrophages (50x). D, small dark foreign cells in hepatic blood vessel in a bullfrog female (200x). E, angiocentric parasite in the kidney of a green frog female (100x). F, rare testicular parasite in a green frog male (100x).

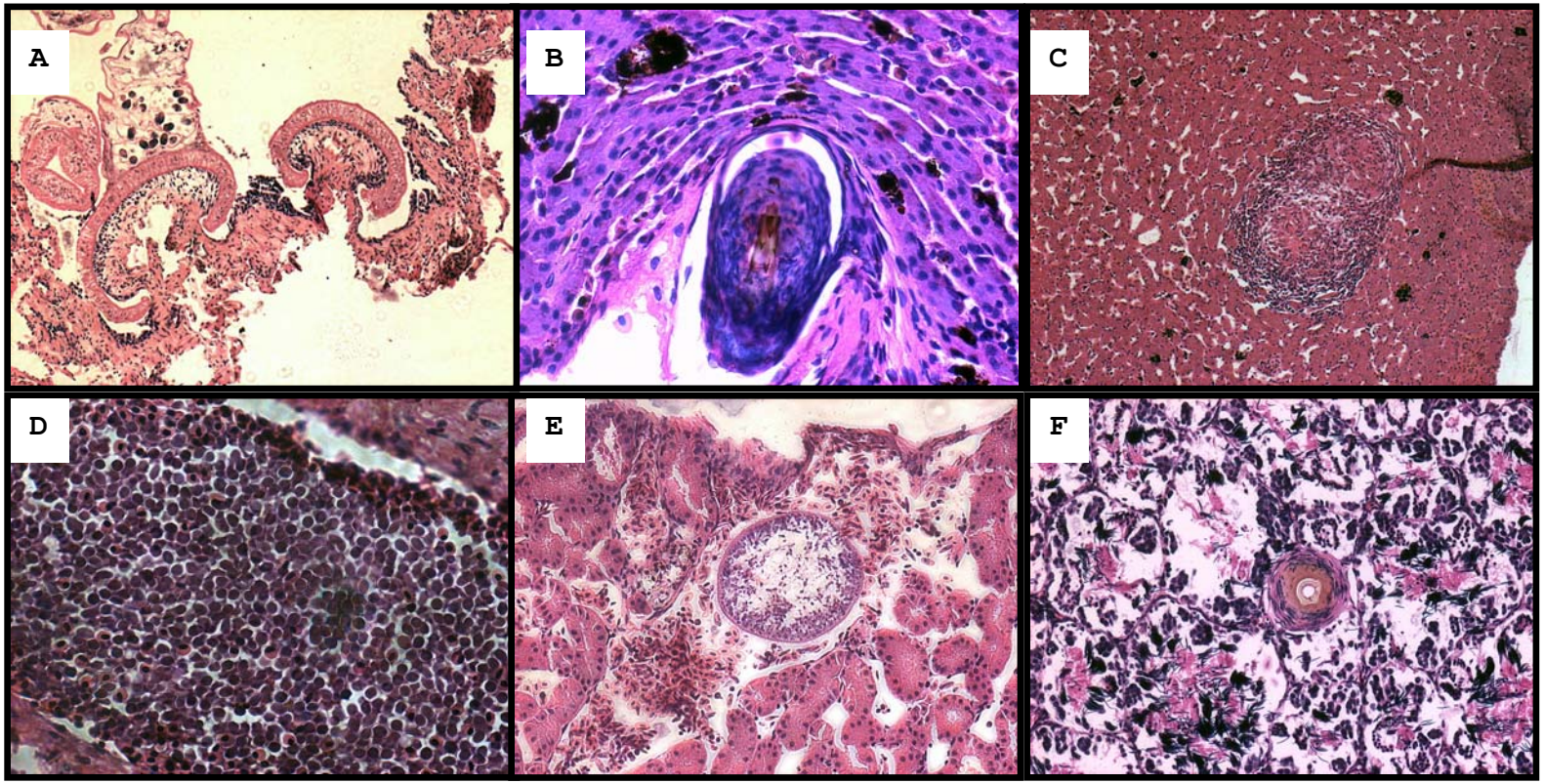
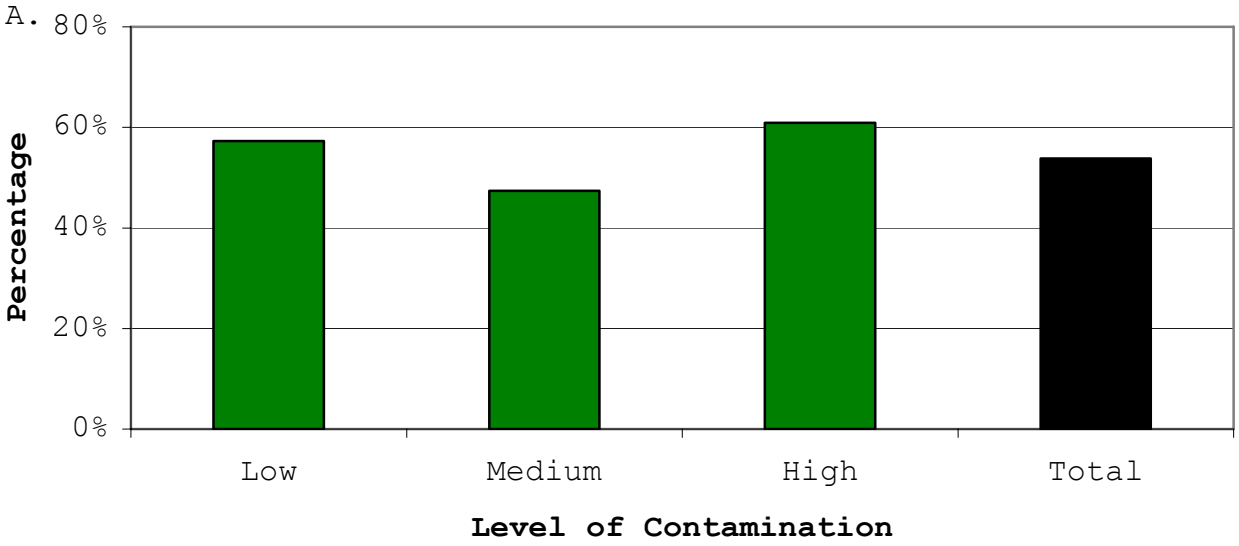


Figure 4.2. Parasitic prevalence
A, percent prevalence of parasites in captures by level of contamination. Pearson's chi-square analysis was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

A.

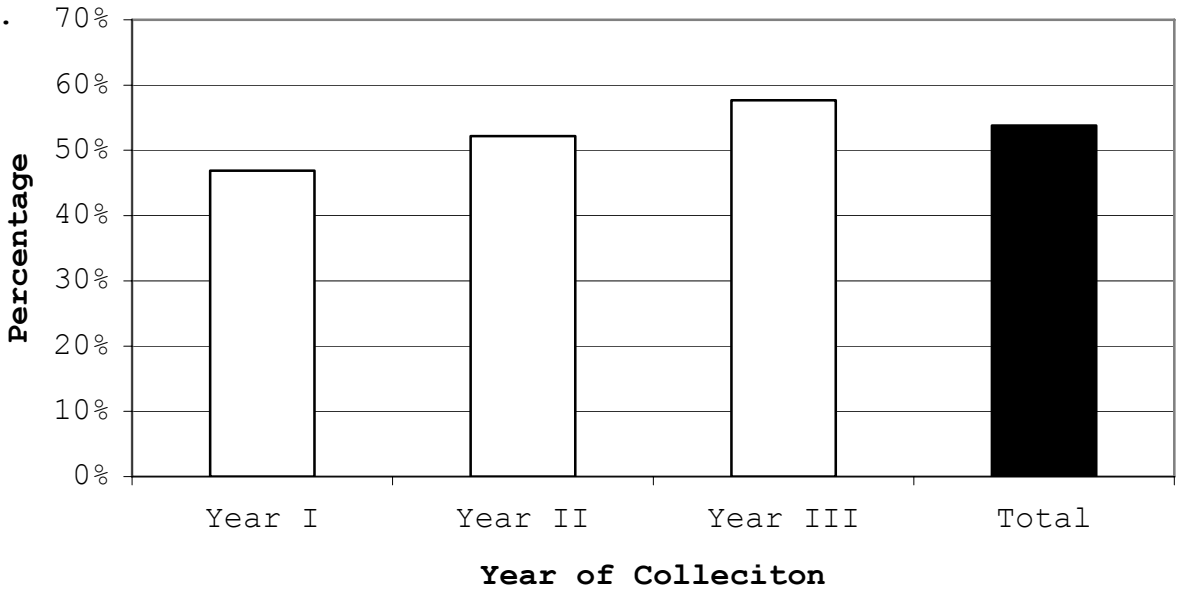


B.

Low Contamination	Medium Contamination	High Contamination	Total
56.25% (54/96)	47.40% (82/173)	60.90% (67/110)	53.56% (203/379)

Figure 4.3. Parasitic incidence
A, percent incidence of parasites in captures across collection years. Pearson's chi-square analysis was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

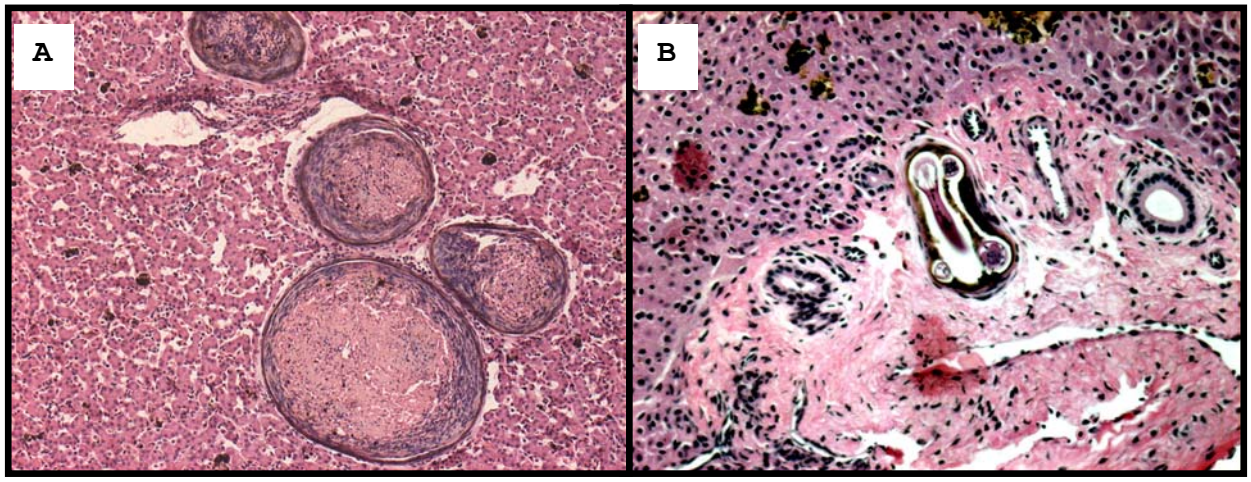
A.



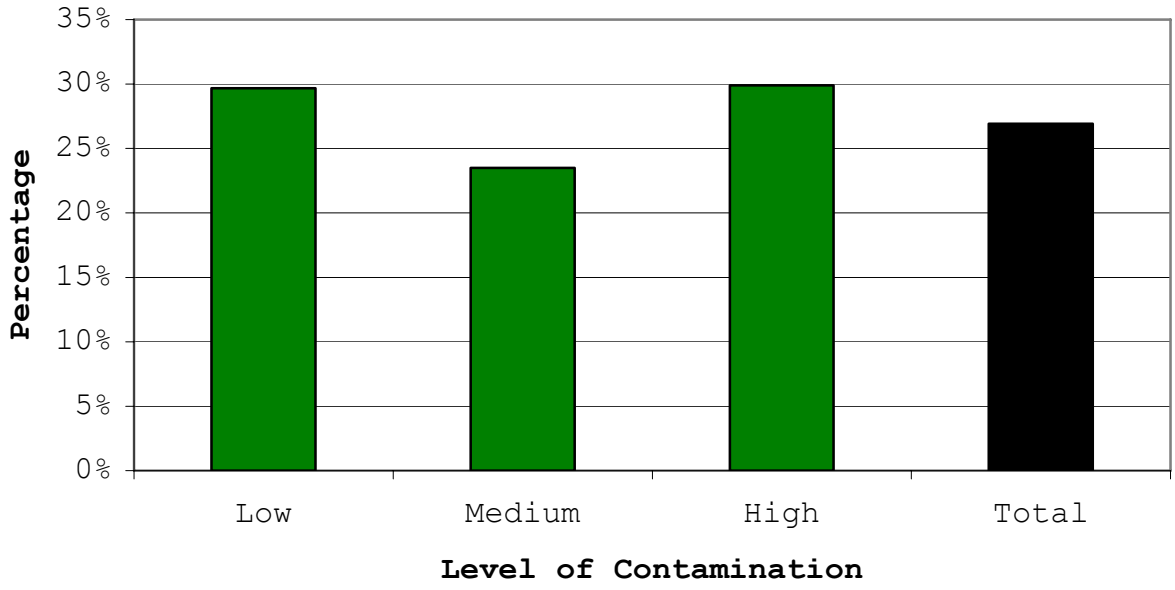
B.

Year I	Year II	Year III	Total
46.9% (15/32)	52.2% (107/205)	57.7% (82/142)	53.8% (204/379)

Figure 4.4. Hepatic parasite prevalence
A, larval trematodes in a bullfrog male (50x). B, fluke from a bullfrog female (100x). C, percent prevalence of hepatic parasites in captures by level of contamination. Pearson's chi-square analysis was used to test for significant differences between proportions. D, raw data with proportions in parentheses.



C.

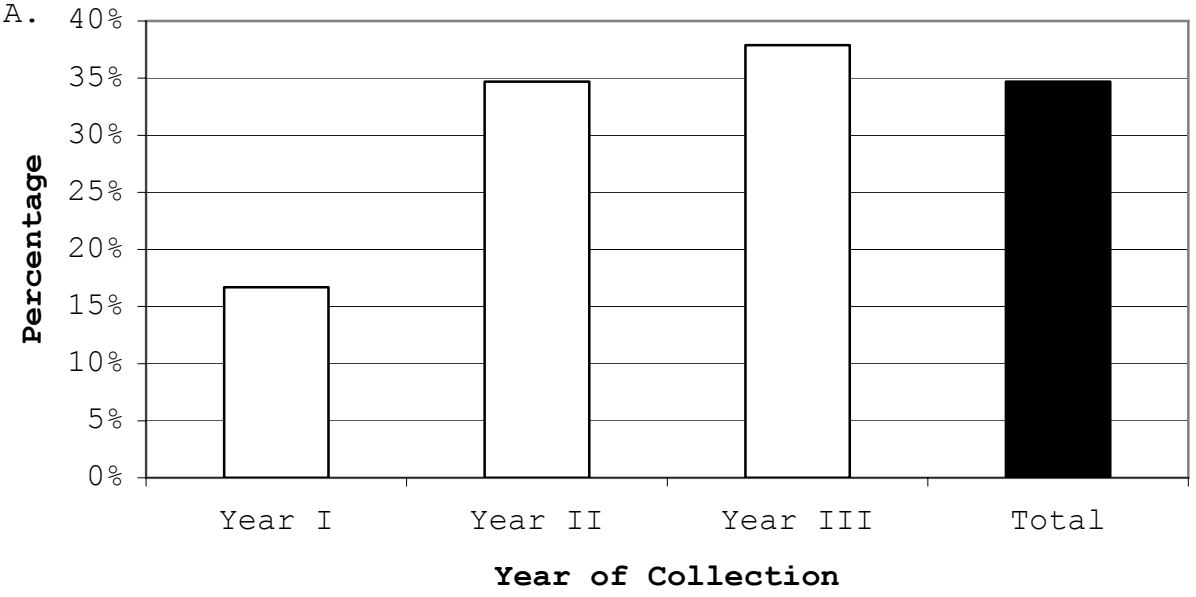


D.

Low Contamination	Medium Contamination	High Contamination	Total
29.67% (27/91)	23.49% (39/166)	29.90% (32/107)	26.92% (98/364)

Figure 4.5. Incidence of hepatic parasites
A, percent incidence of hepatic parasites across collection years. Pearson's chi-square analysis was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

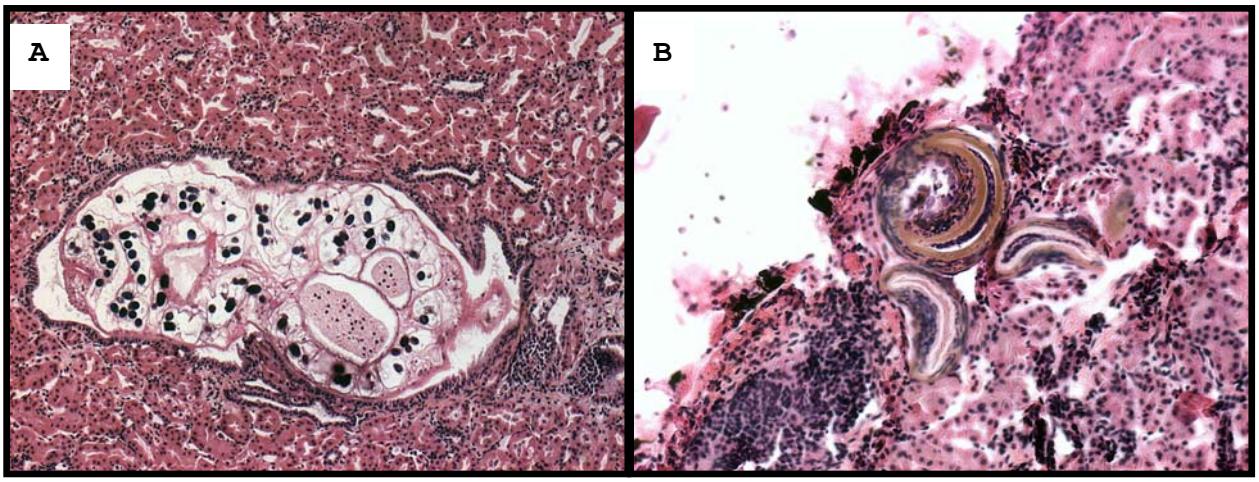
A.



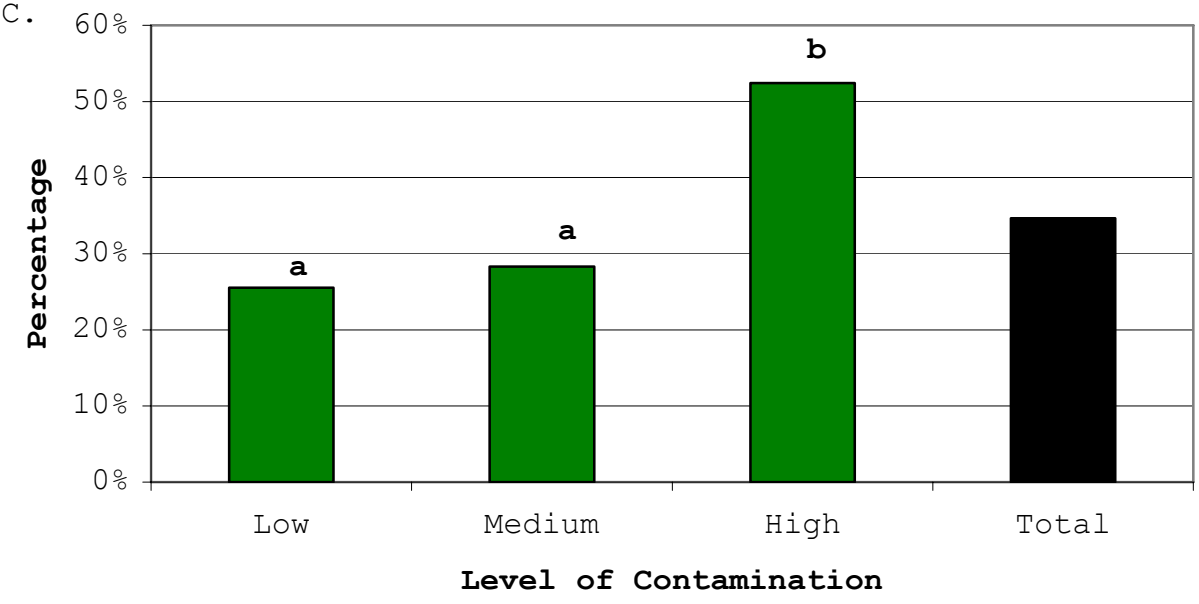
B.

Year I	Year II	Year III	Total
40.7% (11/27)	25.3% (50/198)	26.6% (37/139)	26.9% (98/364)

Figure 4.6 Renal parasite prevalence
A, round worm from a bullfrog female (50x). B, larval nematodes in a green frog male (100x). C, percent prevalence of renal parasites by level of contamination. Populations designated with different letters are significantly different ($p \leq 0.019$). Pearson's chi-square analysis was used to test for significant differences between proportions. D, raw data showing proportions in parentheses.



C.

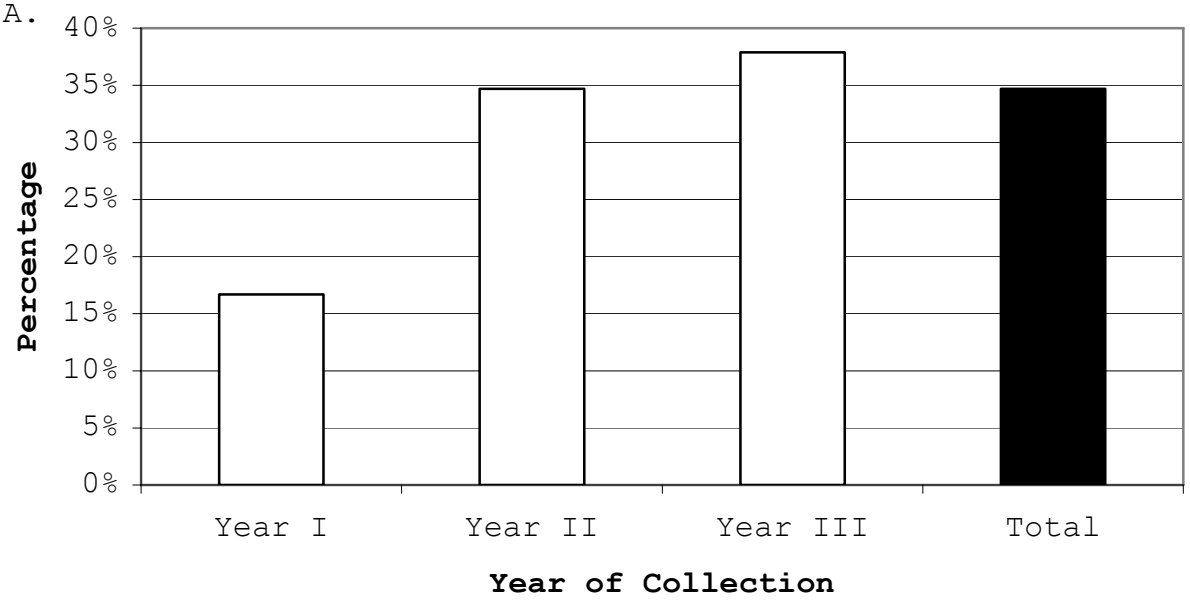


D.

Low Contamination	Medium Contamination	High Contamination	Total
25.56% (23/90)	28.30% (45/159)	52.43% (54/103)	34.66% (122/352)

Figure 4.7. Incidence of renal parasites
A, percent incidence of renal parasites across collection years. Pearson's chi-square analysis was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

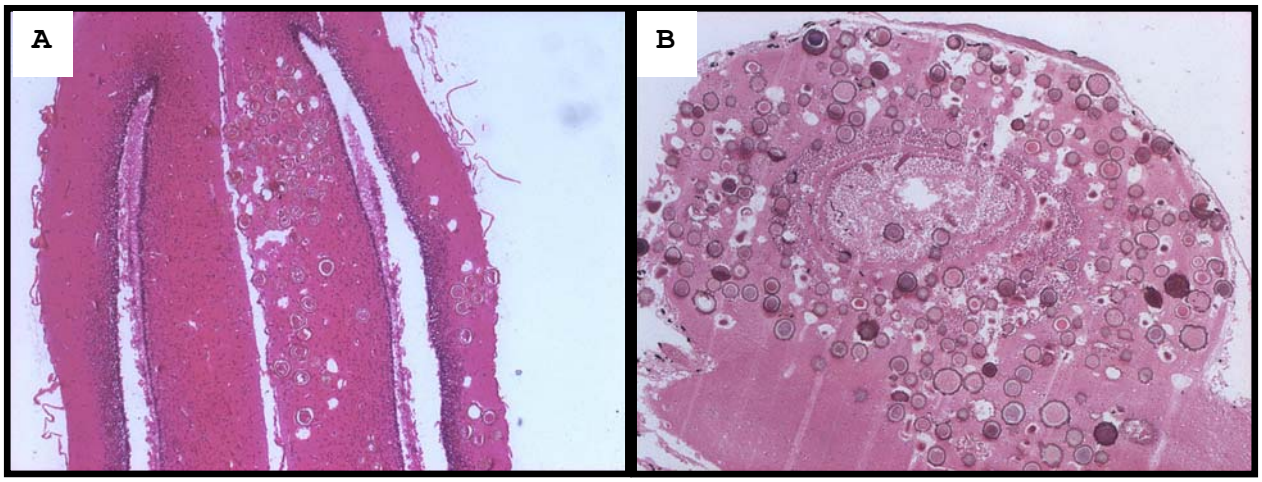
A.



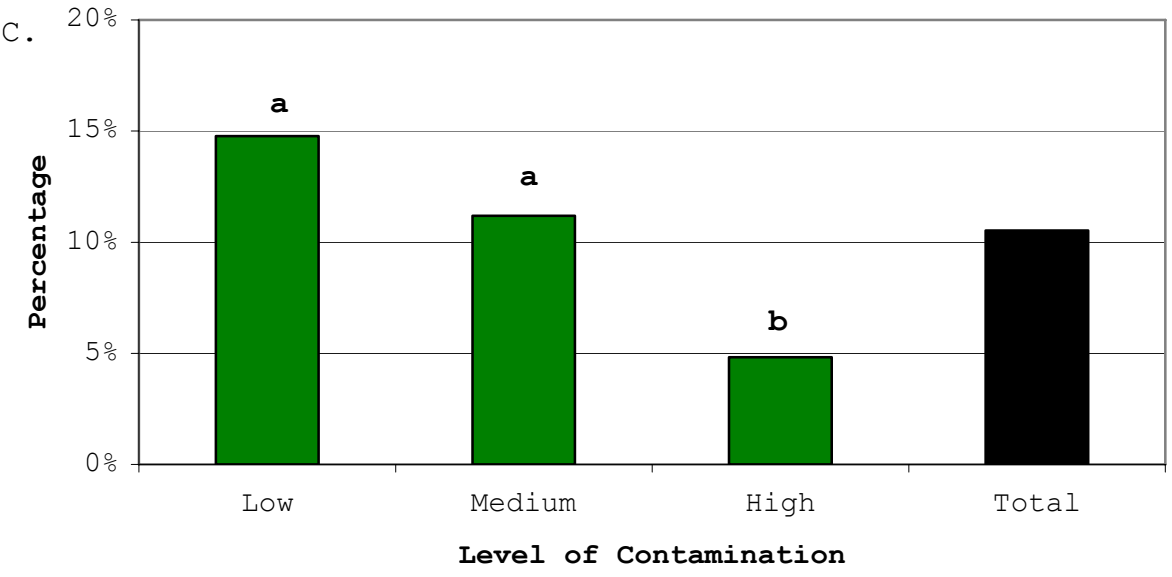
B.

Year I	Year II	Year III	Total
16.7% (4/24)	34.7% (68/196)	37.9% (50/132)	34.7% (122/352)

Figure 4.8. Metacercarial prevalence
A, metacercarial cyst infestation on one cerebral hemisphere of the brain in a bullfrog female (25x). B, metacercarial cysts in the optic lobe of a bullfrog female (25x). C, percent prevalence of metacercarial infection by level of contamination. Populations designated different letters are significantly different ($p \leq 0.046$). Pearson's chi-square analysis was used to test for significant differences between proportions. D, raw data showing proportions in parentheses.



C.

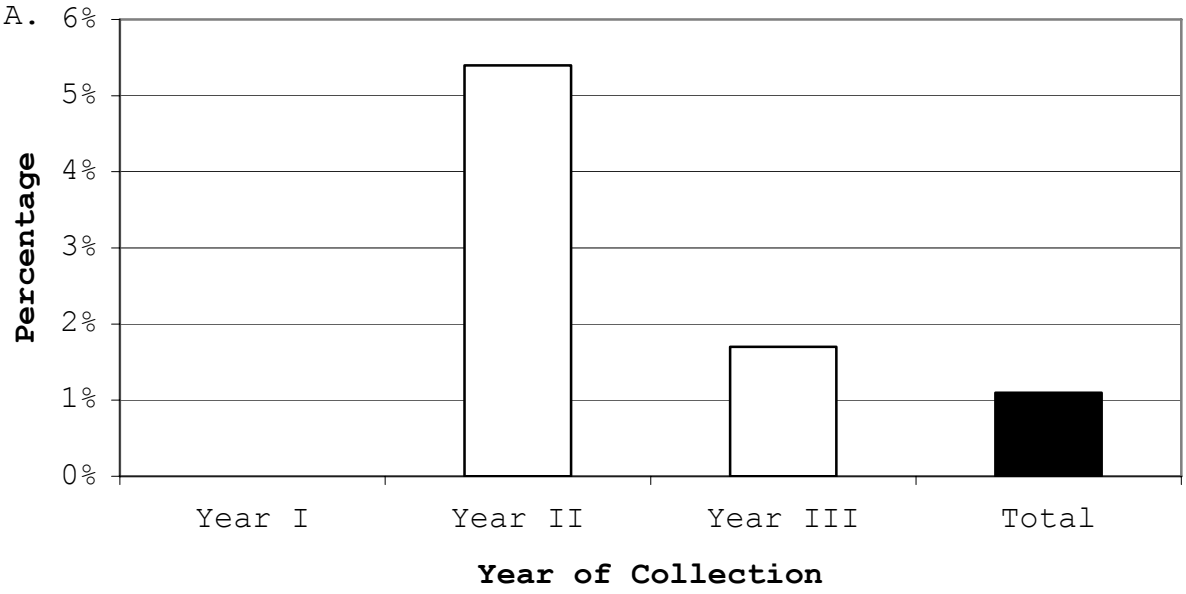


D.

Low Contamination	Medium Contamination	High Contamination	Total
14.77% (13/88)	11.18% (17/152)	4.82% (4/83)	10.53% (34/323)

Figure 4.9. Incidence of metacercarial cysts
A, percent incidence of metacercarial cysts across collection years. Pearson's chi-square analysis was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

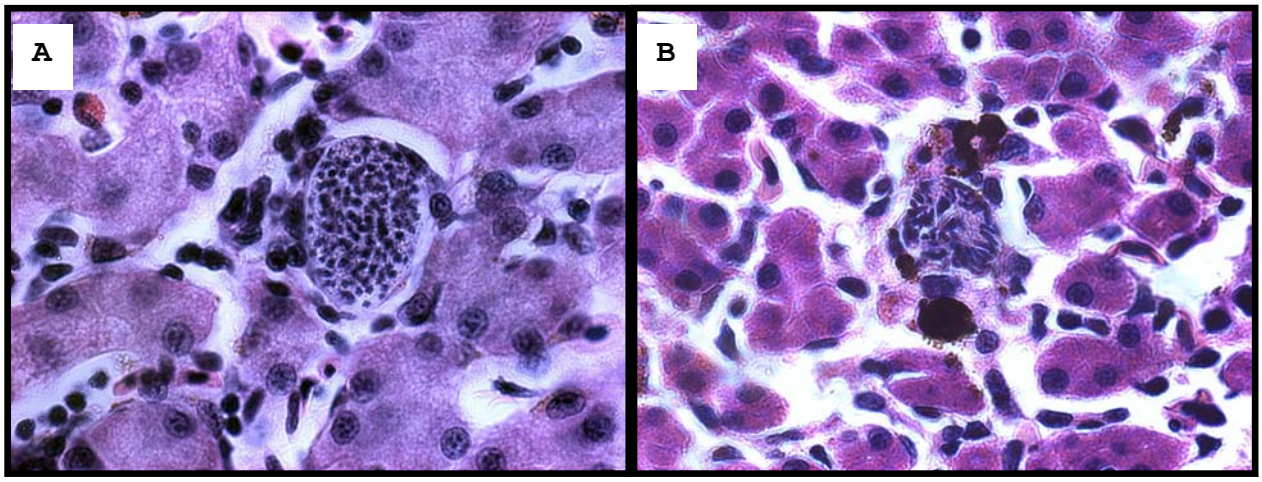
A.



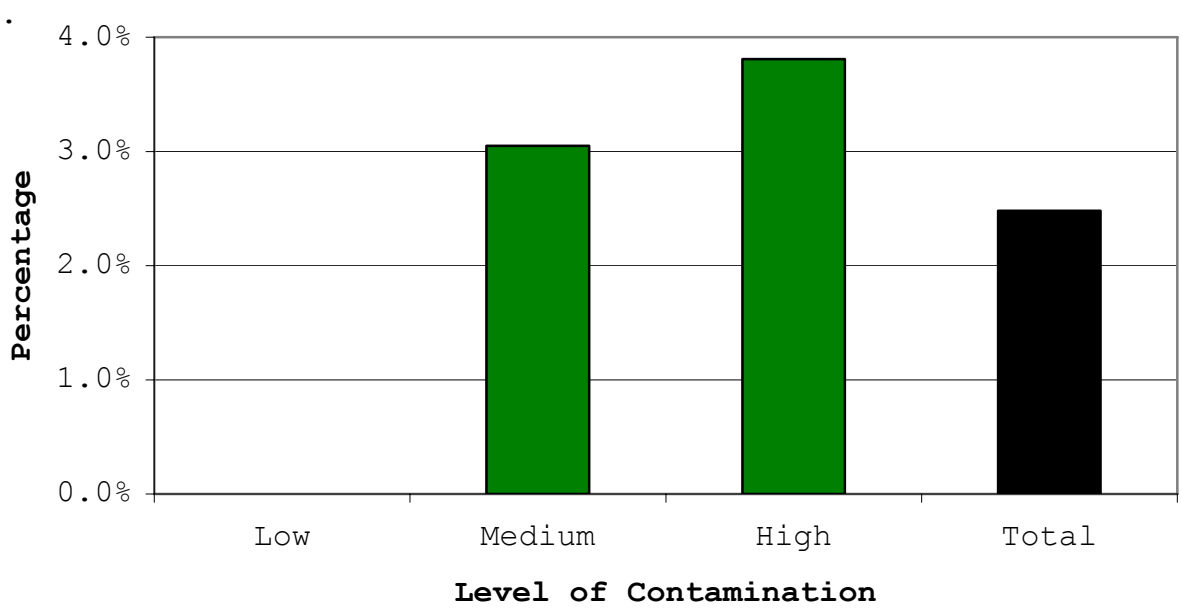
B.

Year I	Year II	Year III	Total
0.0% (0/0)	5.4% (10/186)	1.7% (23/136)	1.1% (34/323)

Figure 4.10. Protozoan prevalence
A, protozoan cyst in a bullfrog female (400x). B, protozoan cyst hatching in a bullfrog female (400x). C, percent prevalence of hepatic protozoan cysts by level of contamination. Pearson's chi-square analysis was used to test for significant differences between proportions. D, raw data with proportions in parentheses.



C.

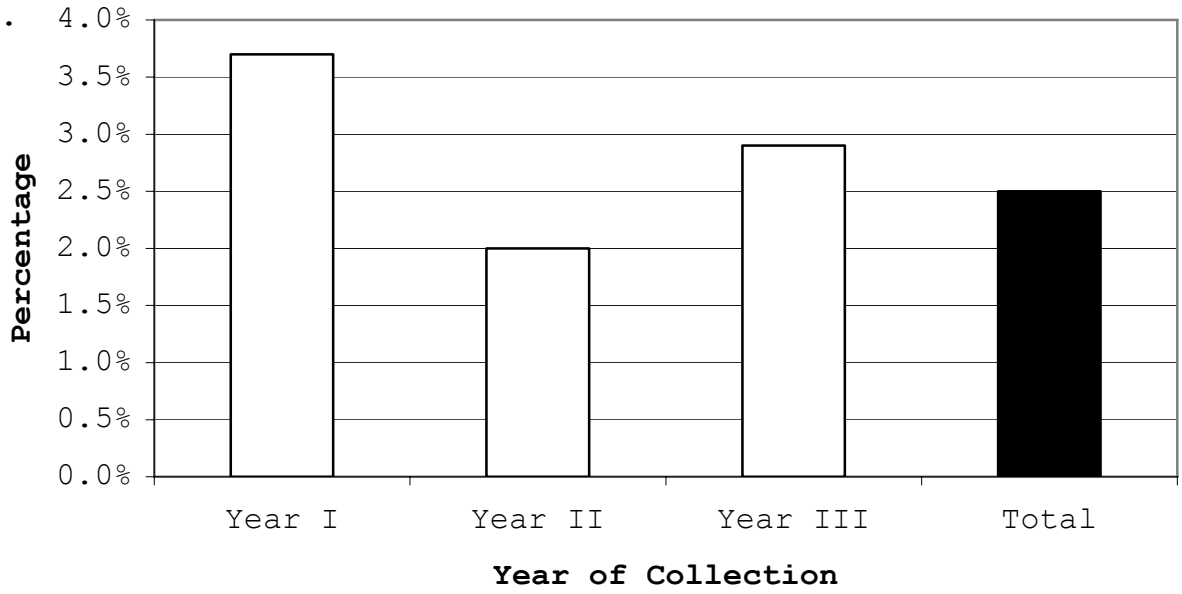


D.

Low Contamination	Medium Contamination	High Contamination	Total
0.00% (0/94)	3.05% (5/164)	3.81% (4/105)	2.48% (9/363)

Figure 4.11. Incidence of protozoon cysts
A, percent incidence of protozoon cysts across collection years. Pearson's chi-square analysis was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

A.



B.

Year I	Year II	Year III	Total
3.7% (1/27)	2.0% (4/198)	2.9% (4/138)	2.5% (9/363)

Figure 4.12. Deformities
A, displaysia in green frog testis (100x). B, normal pupil in left eye of male bullfrog. C, abnormally enlarged pupil in right eye of same bullfrog in (B). D, left leg hemimely in a bullfrog female. E, brachymely in a bullfrog female; note that the right leg is shorter than the left due to a shorter thigh. F, two skin lesions (arrows), possibly trematode cysts, on the dorsal surface of a bullfrog female.

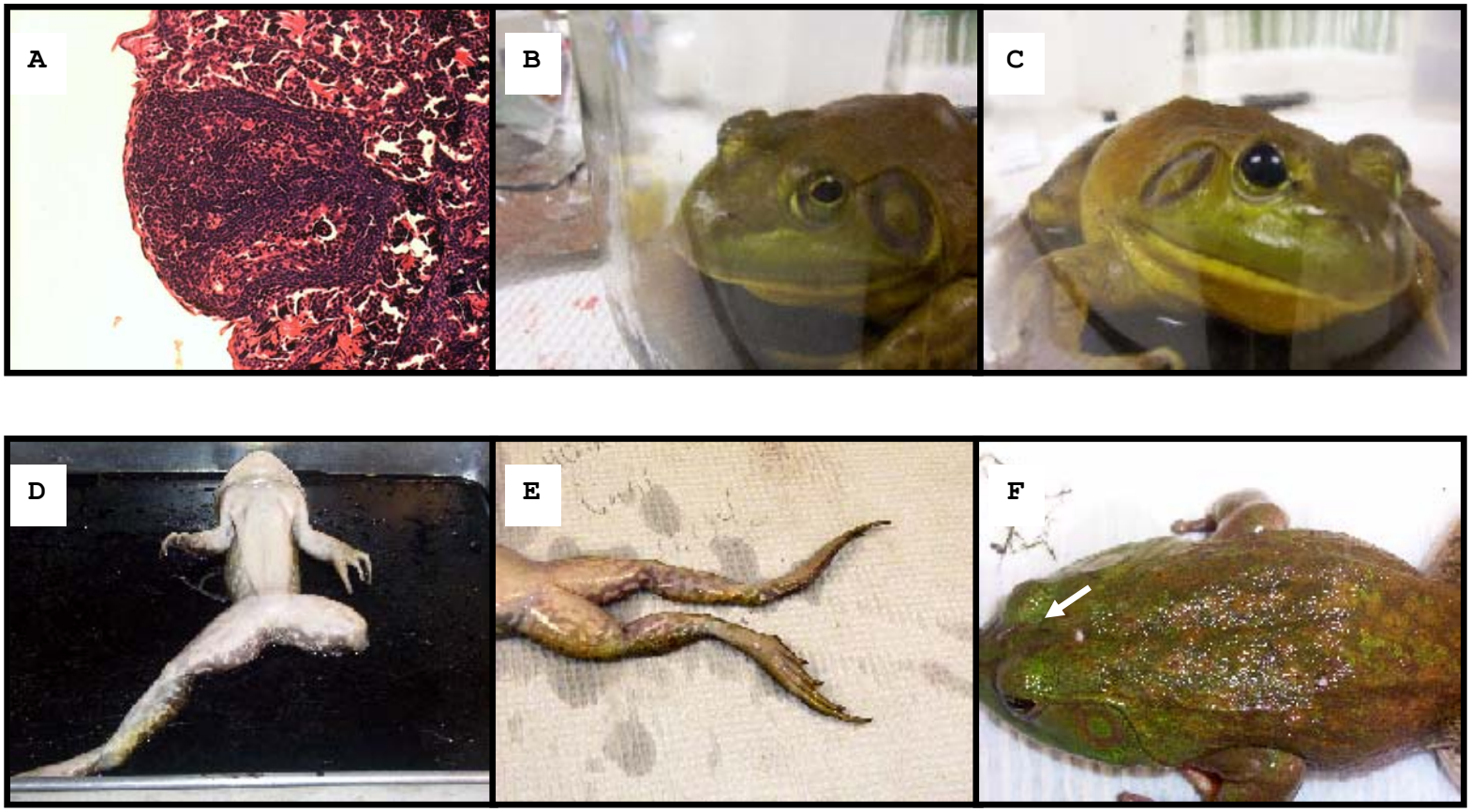
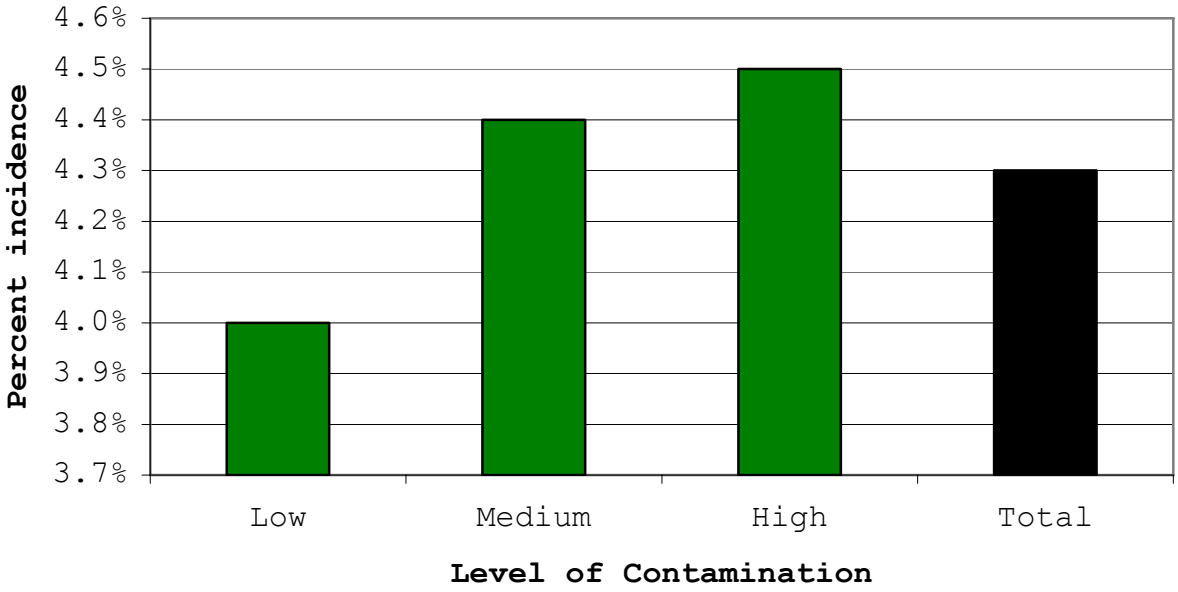


Figure 4.13. Frequency of deformities
A, percent prevalence of deformities by level of contamination. Pearson's chi-square analysis was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

A.



B.

Low Contamination	Medium Contamination	High Contamination	Total
4.0% (4/100)	4.4% (8/180)	4.5% (5/111)	4.3% (17/391)

Figure 4.14. Renal dysgenesis histology
A, tubular degeneration in a green frog male kidney (200x). B, nephrocalcinosis in a bullfrog male (100x). C, encysted tubules in a bullfrog female (200x). D, protein inclusion bodies in the kidney of an unsexed green frog juvenile (200x).

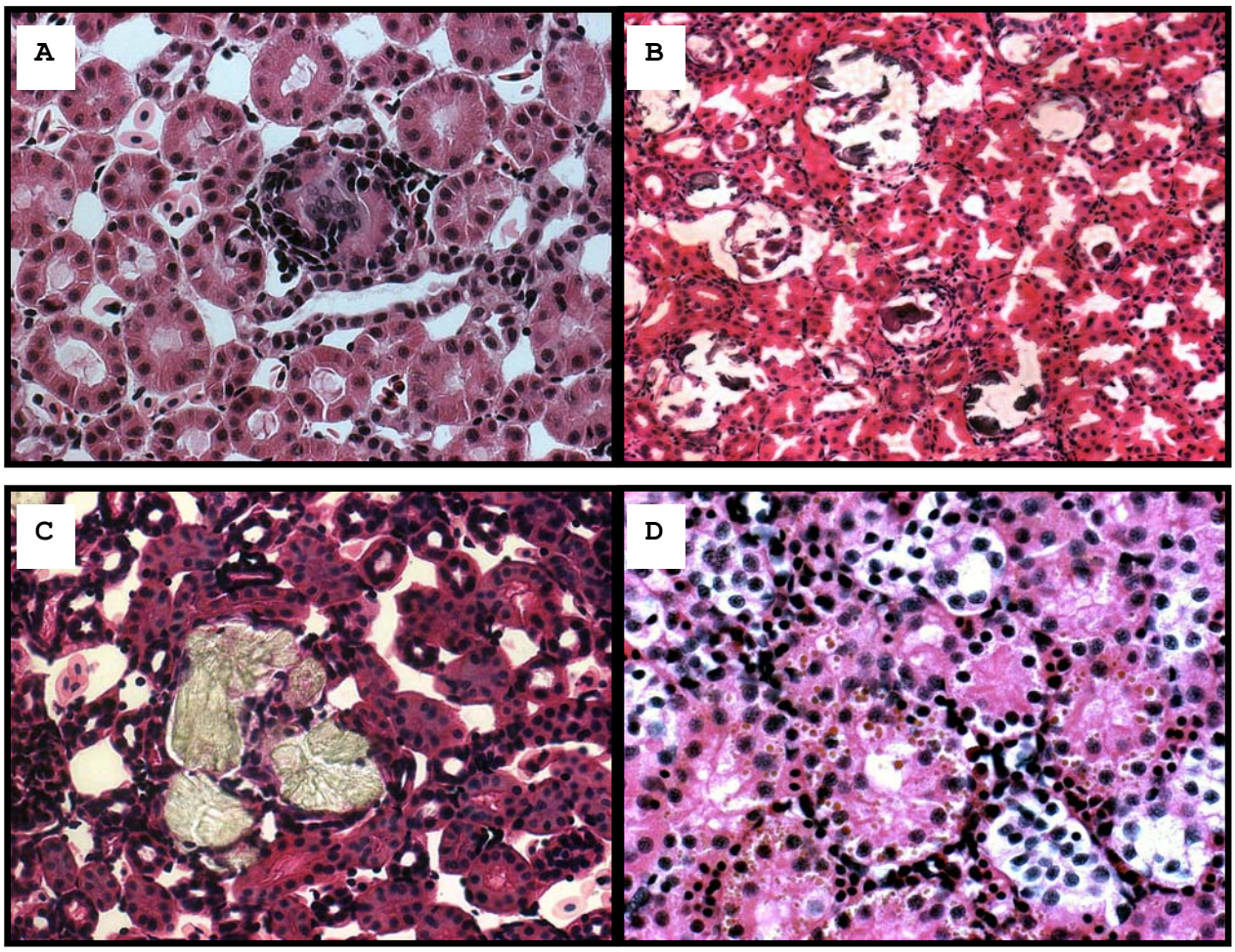
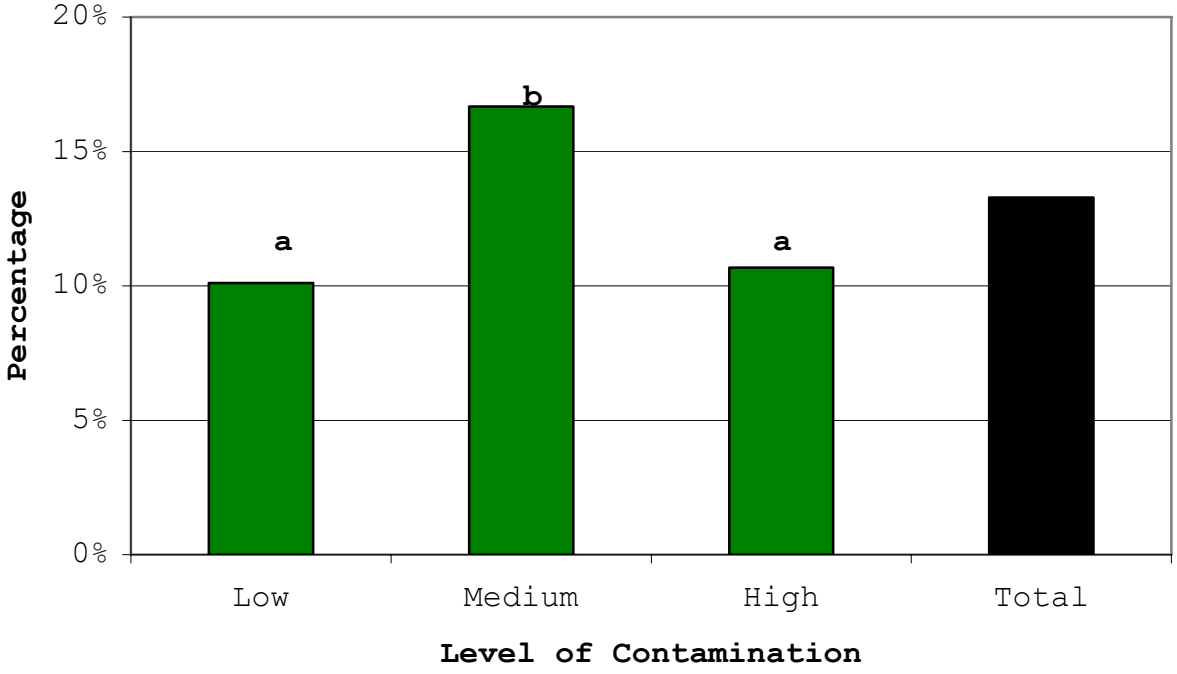


Figure 4.15. Renal dysgenesis frequency
A, percent prevalence of renal dysgenesis by level of contamination. Populations designated with different letters are significantly different ($p \leq 0.003$). Pearson's chi-square analysis was used to test for significant differences between proportions. B, raw data with proportions in parentheses.

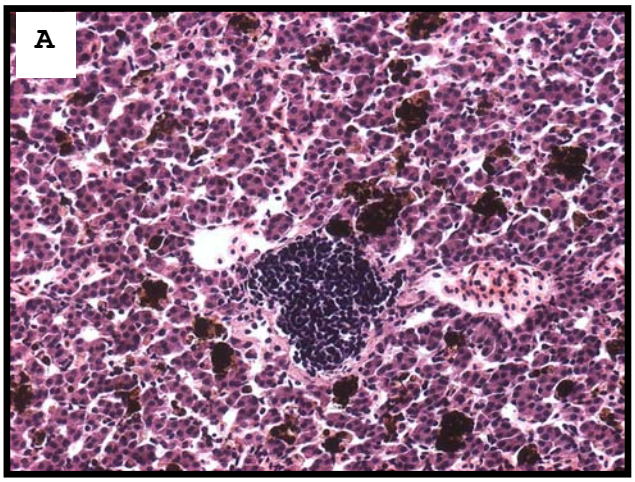
A.



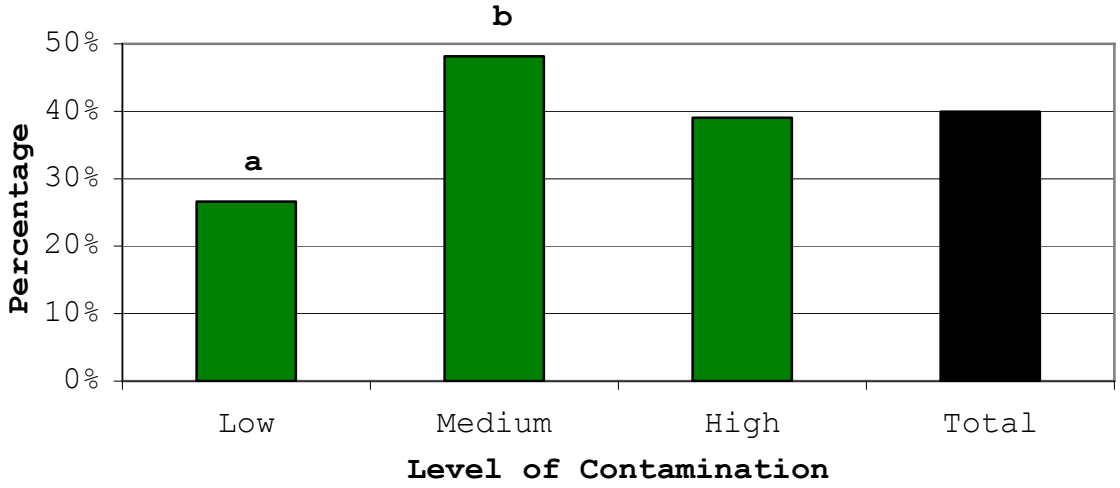
B.

Low Contamination	Medium Contamination	High Contamination	Total
10.11% (9/89)	16.67% (27/162)	10.68% (11/103)	13.28% (47/354)

Figure 4.16. Hepatic inflammatory prevalence
A, focal inflammation in a bullfrog male (100x). B, percent prevalence of hepatic inflammation by level of contamination. Populations designated with different letters are significantly different ($p = 0.036$). Pearson's chi-square analysis was used to test for significant differences between proportions. C, raw data where proportions are in parentheses.



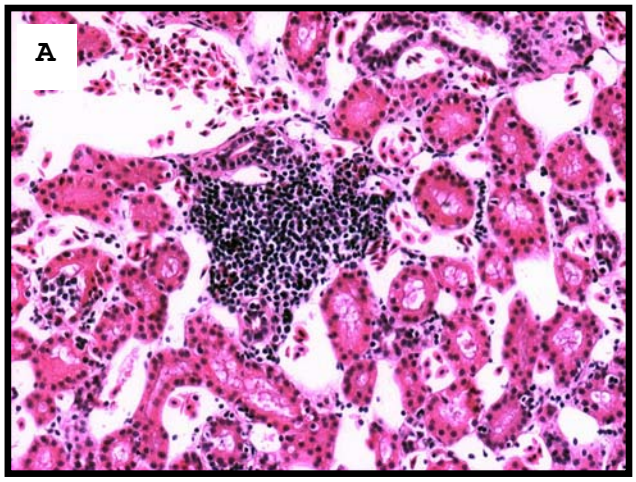
B.



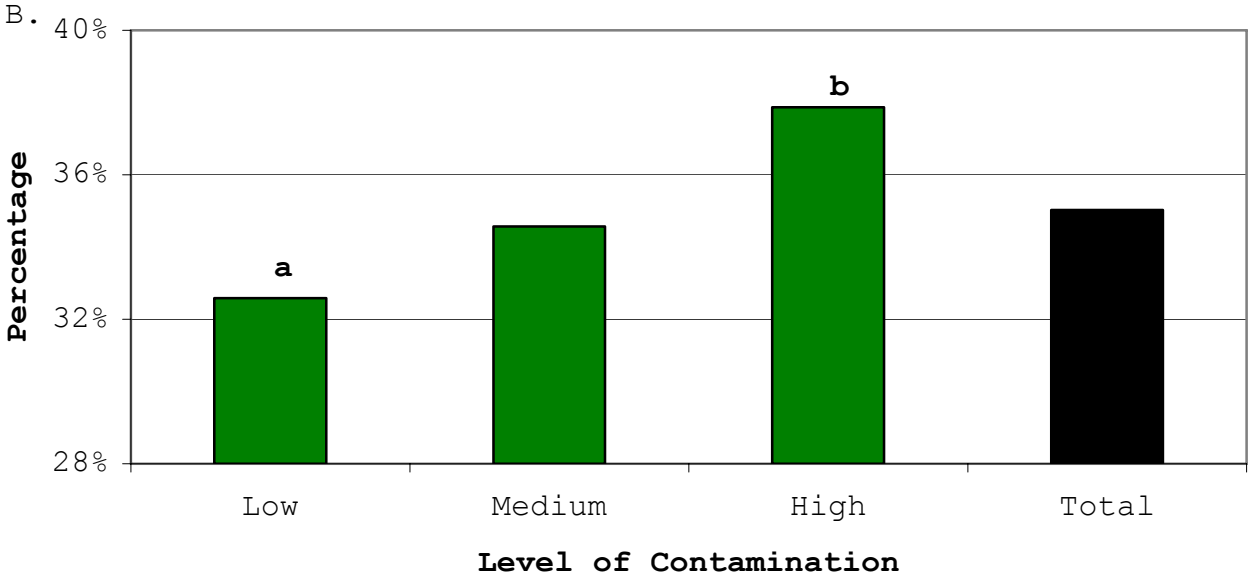
C.

Low Contamination	Medium Contamination	High Contamination	Total
26.60% (25/94)	48.17% (79/164)	39.05% (41/105)	39.94% (145/363)

Figure 4.17. Renal inflammatory prevalence
A, inflammation in a green frog male (100x). B, percent prevalence of inflammation by level of contamination. Populations designated with different letters are significantly different ($p = 0.048$). Pearson's chi-square analysis was used to test for significant differences between proportions. C, raw data where proportions are in parentheses.



B.

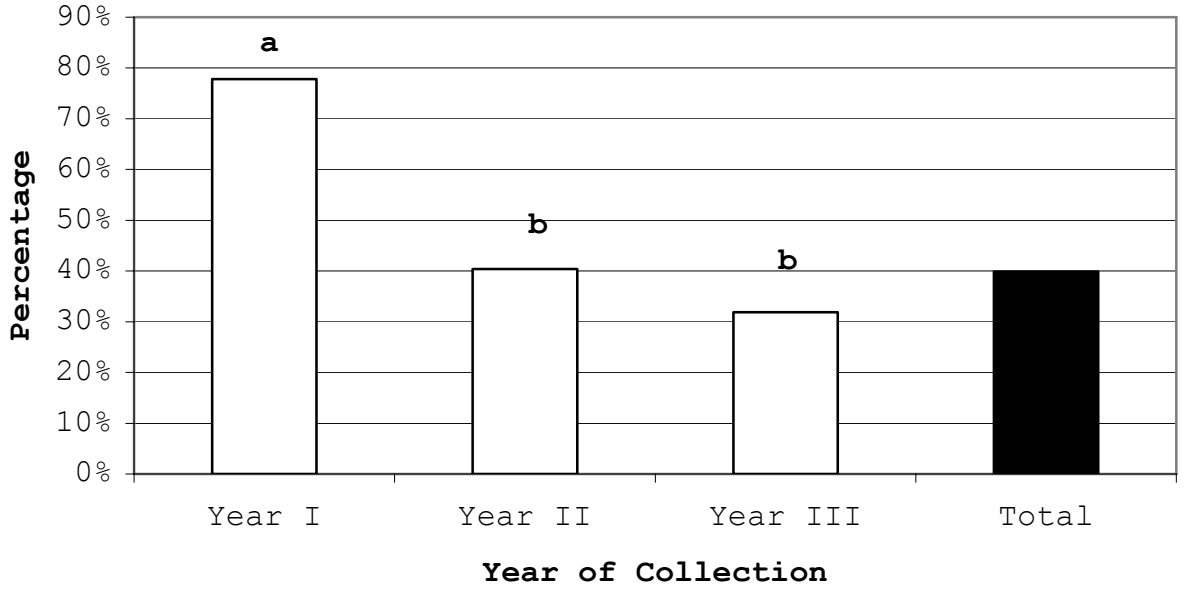


C.

Low Contamination	Medium Contamination	High Contamination	Total
32.58% (29/89)	34.57% (56/162)	37.86% (39/103)	35.03% (124/354)

Figure 4.18. Hepatic inflammation incidence
A, percent incidence of hepatic inflammation across collection years. Populations designated with different letters are significantly different ($p \leq 0.001$). Pearson's chi-square analysis was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

A.

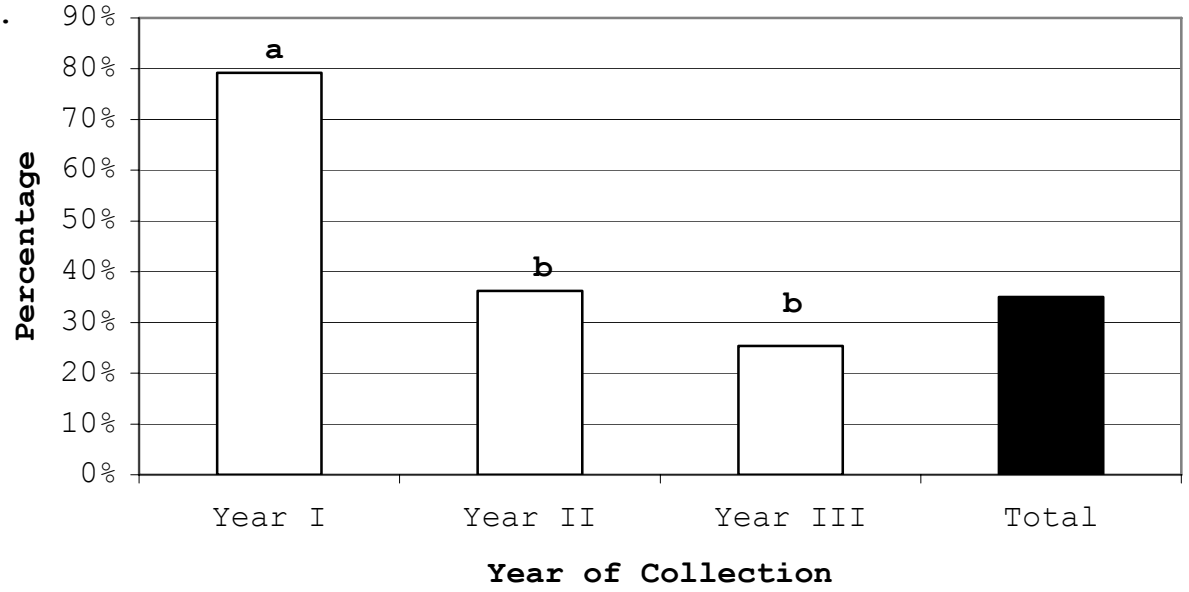


B.

Year I	Year II	Year III	Total
77.8% (21/27)	40.4% (80/198)	31.9% (44/138)	39.9% (145/363)

Figure 4.19. Incidence of renal inflammation
A, percent incidence of renal inflammation across collection years. Populations designated with different letters are significantly different ($p \leq 0.001$). Pearson's chi-square analysis was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

A.

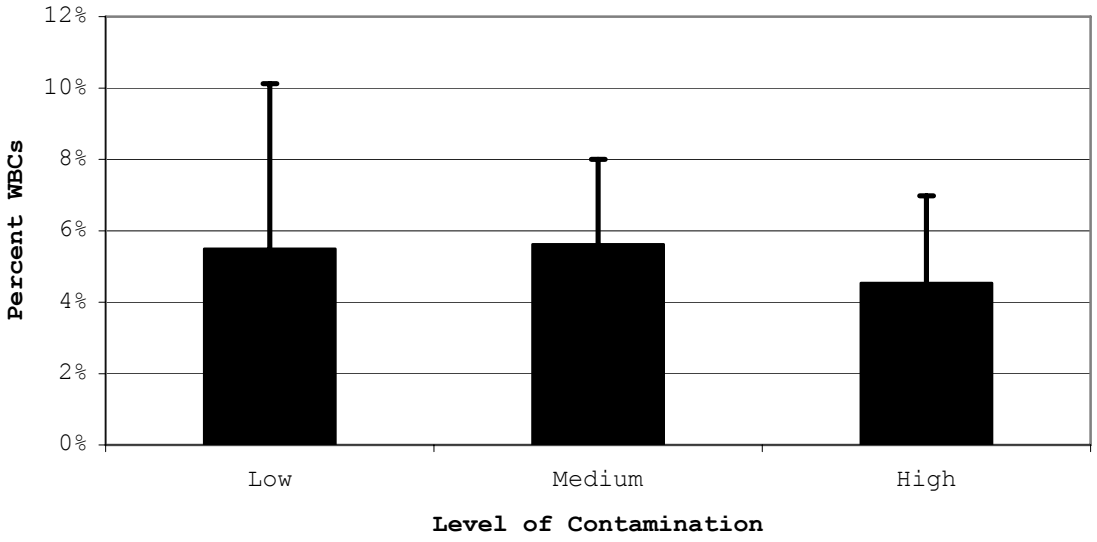


B.

Year I	Year II	Year III	Total
79.2% (19/24)	36.2% (71/196)	25.4% (34/134)	35.0% (124/134)

Figure 4.20. White blood cell percentages
A, average percent white blood cell content in blood smears by level of contamination, where error bars represent standard deviation. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw data where SD is standard deviation.

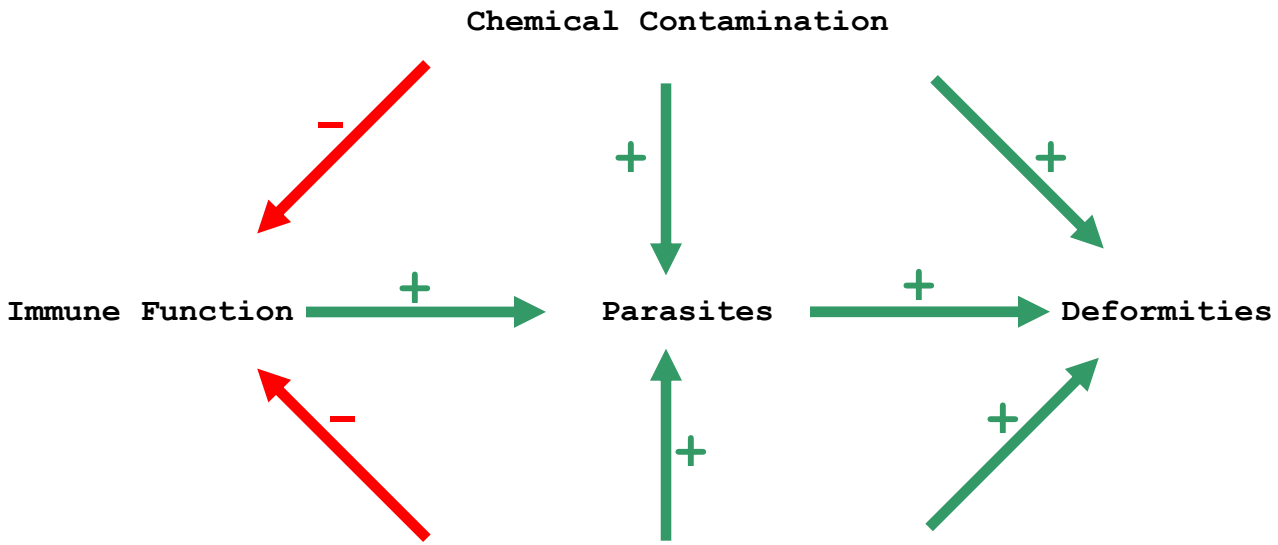
A.



B.

Level of Contamination	% WBC	SD
Low	5.49%	± 4.63%
Medium	5.62%	± 2.38%
High	4.54%	± 2.44%

Figure 4.21. Ecological interactions
Chemical contamination can directly increase parasitic prevalence and/or incidence, or do so through action on the immune system, making animals more susceptible to infection. Chemical contamination can also result in developmental defects either by direct action or by its role in increased parasitism (again, either directly or via immunosuppression). In addition to chemical contamination, other environmental factors can interact with one another and have their own influences on immune function, parasitism and deformities.



Effect of sublethal atrazine exposure on growth, metamorphosis and gonadal morphology in *Xenopus laevis* tadpoles

Chapter 5

Abstract

To determine whether observations in the field could be reproduced by atrazine in a controlled setting, larvae of the African clawed frog (*Xenopus laevis*) were exposed to environmentally relevant concentrations of atrazine (0, 1, 5, 20, 60, 120 µg/L) from first feeding stage to metamorphosis (~70 days). For many of the endpoints examined, the effects were non-monotonic. Tadpole survival to metamorphosis was significantly reduced only at 5 ppb atrazine. At 5 and 60 ppb atrazine, tadpoles that reached metamorphosis did so 6.7 and 12.3 days earlier than controls. The three middle atrazine concentrations (5, 20 and 60 ppb) yielded metamorphs of 31-39% lower weight than control. Metamorphs from 5 ppb atrazine also had 15% shorter body lengths, 18% shorter limbs and 22% less abdominal girth than controls. Abdominal girth was also reduced by 20% at the highest atrazine concentration tested (120 ppb). Malformations of the spine and limbs were 3.3- to 9.8-fold higher at 5 and 20 ppb than other treatment groups. Testicular

dysgensis (retarded testicular development), noted by abnormal morphology and histology, was 2-3 fold more frequent in select treatments than control. Though whole body testosterone and corticosterone levels were not significantly different following treatment, testosterone levels declined, while corticosterone levels increased with atrazine exposure. Our results indicate that low doses of atrazine affect developmental processes of *X. laevis* and support concerns regarding the herbicide's role in amphibian declines. The lowest observable adverse effect level (LOAEL) for testicular dysgenesis in this study was 1 ppb atrazine, within environmental concentrations.

Introduction

Chemical contamination is a global factor which may influence amphibian reproductive fitness and contribute to reported population declines (Howe et al. 1998; Allran and Karasov 2000; Carr et al. 2003). One chemical that has been studied as a potential cause in the loss of amphibian abundance is the herbicide atrazine. Atrazine's adverse endocrine effects on amphibians at low (3 to 200 ppb) doses have received little attention in the laboratory (Hayes et al. 2002b; Carr et al. 2003). The lower limit of this herbicide's toxicity needs to be further evaluated, as it has yet to be established.

Low doses of atrazine have been implicated in developmental effects in amphibians, particularly of the gonads (Hayes et al. 2002b; Tavera-Mendoza et al. 2002a, 2002b; Hayes et al. 2003). Tavera-Mendoza et al. (2002a) observed gonadal dysgenesis in African-clawed frogs (*Xenopus laevis*) exposed to 21 ppb atrazine over only 48 hours during sexual differentiation. In addition, Hayes et al. (2002b) observed hemaphroditism at concentrations as low as 0.1 ppb atrazine and reduced laryngeal size at 1 ppb atrazine in frogs exposed over their entire larval period. These studies indicate that atrazine's reproductive effects occur at concentrations far lower than those that yield acute toxicities, 0.41 to 127 ppm (Morgan et al. 1996; Sullivan and Spence 2003).

Our previous work utilizing field studies at agricultural sites containing agrochemical contamination included atrazine, but also included many other variables including other sources of chemical contamination. Controlled laboratory studies were carried out to confirm the effects of atrazine and to determine if observations in the field could be reproduced. In the current study we looked at the effects of low dose atrazine exposure (0, 1, 5, 20, 60, 120 ppb) on the *Xenopus laevis*. Our objective was to determine the effects of atrazine at environmentally relevant concentrations on growth and

developmental parameters during the larval stage of *X. laevis*. Survival, growth, and proportion of larvae with malformations and testicular abnormalities were determined. In addition, whole body testosterone and corticosterone levels were calculated. This experiment and resulting analyses were designed to evaluate atrazine's ability to act as an endocrine disruptor in amphibians and to cause retardation of testicular tissue at doses seen in our environmental surveys.

Materials and Methods

Test animals and animal care. *Xenopus laevis*, the African-clawed frog, was chosen as the laboratory animal on the basis of its fast growth, with complete metamorphosis in nine weeks (Beck 1994; Sullivan and Spence 2003), its large size, and because it is an established, commercially available laboratory animal that survives well in captivity (Palmer et al. 1998; Pickford and Morris 1999). In addition, there exists an extensive body of literature on this species, which has been used extensively in hormonal experiments of this type (Hayes and Licht 1993), and its response to sex steroids are well documented (Hayes et al. 2002b). For example, the introduction of estrogen causes tadpoles to turn into females and the presence of androgens leads to larger larynges, a secondary male trait (Hayes et al. 2002b). *Xenopus laevis* are also completely aquatic, making them

particularly suitable for studies with waterborne chemicals (Palmer et al. 1998).

The proper permit (SH27017) for the scientific holding of the exotic species *Xenopus laevis* in New Jersey was obtained from the Wildlife Permits Unit of the New Jersey Department of Environmental Protection's Division of Fish and Wildlife.

Xenopus laevis embryos were purchased from Nasco (Fort Atkinson, WI). There was a two-day acclimation period prior to treatment to prevent undue stress (Howe et al. 1998; Veldhoen and Helbing 2001; Rohr et al. 2003). After acclimation, tadpoles were transferred to testing aquaria using wide-mouth disposable plastic pipettes. Tadpole tank assignments were determined by random number generation (Reeder et al. 2005, see Table 5.1 for assignments). Tadpoles were held in 10 gallon glass tanks, initially containing six liters of system water. System water was municipal water filtered and dechlorinated by an automatic sand filter, two 25 mm particle filters, and an activated carbon filter. Water was allowed to aerate for at least 24 hours prior to use in order to degas and allow it to come to test temperature {Help, 2000 #1049}. Following 24 hours in six liters of system water, tadpoles received an additional six liters of system water and treatment commenced. The final

density was three tadpoles per gallon in order to allow uninhibited growth (Skelly and Kiesecker 2001). While denser populations tend to yield smaller juveniles, this experimental density avoids outright mortality and prevents delays in metamorphosis (Belden et al.; Hayes 1997; Britson and Threlkeld 1998). In addition, healthy immune systems (Carey et al. 1999) and intended exposures (concentrations become diluted with tadpole numbers, Hayes 1998) are maintained at this lower density, and aggression is ameliorated (some species are territorial and will not tolerate the presence of other members, Beck 1994). In this manner, a stressor variable is removed.

A 12:12 hour light:dark photoperiod (eNasco; Gray and Janssens 1990; Noriega and Hayes 2000; Rohr et al. 2006), with lights on from 7am to 7pm and no external lighting, was maintained throughout the experiment. Amphibians were kept under aeration, with an air stone at either end of each tank, to keep organic content low (Taylor and Pedretti 1992; Rohr et al. 2006). All tanks had secure screen covers to prevent escape yet allow air circulation (Verhoeff-de Fremery and Griffin 1987). The bottom and a single side of the tanks were painted black to reduce stress and escape behavior (Taylor and Pedretti 1992; Rohr et al. 2003). Gloves were worn when handling animals and during husbandry operations, as oils in human skin can be harmful to

frogs (Taylor and Pedretti 1992). Nets were used gently when necessary to avoid injury to animals (Beck 1994). All equipment was sanitized with a dilute bleach solution followed by several rinses with hot water prior to housing tadpoles (Taylor and Pedretti 1992).

Animals were fed a commercial diet provided by Nasco, manufactured by Purina specifically for *Xenopus*. Feed was 1-1.5g of tadpole food per tank per feeding (Fort et al. 1999; Fort et al. 2000) beginning at start of experiment (three to four days post fertilization), when animals had reached the free swimming and eating stage (stage 25, Gosner 1960). Previously, the animals fed on their yolk while independent feeding structures form (Gosner 1960; Hatch and Burton 1998). At this stage in development, the operculum (flap of skin that covers the internal gills) is developing and the external gills are degenerating (Gosner 1960; Feder and Burggren 1992; Hatch and Blaustein 2000). Feed contents were fish meal, meat meal, soybean meal, corn meal, wheat flour, dried yeast, distillers solubles, whey, wheat germ meal, salt and vitamin supplements-diacalcium phosphate (~44% protein, 11% ash, 6% fat and 2% fiber) with a caloric content of 2.8 Kcal/g (eNasco). Food was prepared by taking 1-1.5 g of the powder and making a paste using system water. The paste was formed into a ball and

dropped into the tank for access by the tadpoles. The animals were fed this protein-based mix three times a week (e.i., Sundays, Tuesdays and Thursdays). Feedings occurred the evening prior to water changes when uneaten food was removed. Animals were fed sufficient amounts of food to prevent delays in metamorphosis and to ensure proper growth (Larson et al. 1998) and immune function (Rollins-Smith 1998; Carey et al. 1999).

To reduce accumulation of fouled food and feces, tanks were maintained regularly. Fifty percent static water changes (Metcalfe et al. 2000; Fort et al. 2001; Mackenzie et al. 2003; Coady et al. 2004) were performed on Mondays, Wednesdays and Fridays (Kloas et al. 1997; Carr et al. 2003; Mackenzie et al. 2003; Rohr et al. 2003). The water changes were done with an aquarium pump using a dry application with inlet and outlet tubing to remove 50% of aquarium contents. Inlet tubes were fitted with mesh to prevent tadpoles from being sucked into the pump or otherwise harmed. The outlet tube drained into a waste container. Renewal treatments were administered utilizing a separate aquarium pump using a wet application with only an outlet tube. During water removal, bottoms of the tanks were cleared of uneaten food and other debris. The interiors of the tanks were wiped down with soap-free, glass algae scrapers as

needed to remove algae and any dry material, typically once a week, just prior to water changes. Each tank had dedicated and color-coded pumps, tubing and equipment to prevent cross contamination (Carr et al. 2003). Tanks were rotated once a week to prevent positional effects (Hayes et al. 2002b; Rohr et al. 2003). For a summary of test conditions, see Table 5.2. All animal husbandry and procedures were approved by Rutgers University's Care and Use of Laboratory Animals Committee in protocol #04-040.

Test chemical. Atrazine (CAS# 1912-24-9) of 98% purity was purchased from ChemService (Chester, PA). Formulation is an important factor in these experiments and by using 98% pure atrazine, we were able to evaluate atrazine's effects in the absence of inert ingredients, like surfactants, that are present in commercial formulations of the pesticide. Treatment solutions were made in acetone so that the final concentration of acetone in each tank was 0.004% (v/v, Mackenzie et al. 2003; Sullivan and Spence 2003; Rohr et al. 2006). Table 5.3 displays calculations for atrazine treatments. Stock solutions were kept cold in a 4°C refrigerator in amber glass bottles (Thornton 2000). All treatment solutions were allowed to come to room temperature before being added to the rearing medium to prevent any temperature fluctuations and undue stress on the animals

(Storrs and Kiesecker 2004). All stock solutions (made just before water changes) and mixing containers were color-coded, as with water change equipment, to indicate treatment and prevent cross contamination (Carr et al. 2003). Disposal of used atrazine occurred via oxidation using sufficient sodium hydroxide to raise the pH to >14. Atrazine was allowed to hydrolyze for at least 24 hours. The solution was then brought to neutral pH with hydrochloric acid, diluted with excess water and washed down the drain (ATSDR 2003). Atrazine disposal followed the hazards protocol approved by Rutgers Environmental Health and Safety for animal protocol #04-040.

Water chemistry. Atrazine concentrations were confirmed by gas chromatography with a mass spectrometry detector following solid-phase extraction (see Chapter 3 for details on water analysis). Samples were collected immediately following the mixing of treatment solutions.

The water quality measurements listed below were taken to ensure proper environmental and organism health in the laboratory. Temperature was monitored daily with thermometers submerged in the rearing media. Two thermometers recorded current, high and low temperatures. One was maintained in the system water tank, while the other was maintained in Tank #1 (red) containing 20

ppb atrazine. Temperature was maintained at 20°C {Help, 2000 #1049;Chang, 1956 #98;Beck, 1994 #1043;eNasco, #1045;Express, #1046}. According to the animal suppliers, this temperature is within the animals' optimal range for bodily functions like movement, digestion and feeding (eNasco). Temperature is a major factor influencing larval growth, development and immune function (Boone and Bridges 1999; Kiesecker et al. 1999) and was therefore kept constant with little fluctuation to prevent adverse effects. The following parameters were monitored three times a week with instrumentation used in parentheses: dissolved O_2 (Oakton® Dissolved Oxygen Meter), pH (Oakton® Acorn™ Meter Kit), ammonia, nitrate (LaMotte Nitrate Test Kit Model NCL), nitrite (Orion AQUAfast II AQ2046) and chlorine (Hanna Instruments Free Chlorine Test Kit). Also, hardness, alkalinity (LaMotte Alkalinity Test Kit Model WAT-DR) and conductance (Oakton® Acorn™ CON 5 Conductivity Meter) were measured weekly.

Exposures. To determine amphibian response to atrazine, *Xenopus laevis* tadpoles were exposed to the following nominal concentrations of atrazine: 0, 1, 5, 20, 60 and 120 ppb. Treatments occurred over the entire larval period, and tadpoles were exposed to atrazine via immersion in the rearing medium containing the herbicide (Clark et al. 1998). Exposure via immersion was employed to mimic exposure routes in the aquatic

environment (Clark et al. 1998; Palmer et al. 1998). In this manner, atrazine was absorbed and metabolized as it would be in the environment (Palmer et al. 1998) with uptake across the skin (Sohoni et al. 2001).

Twenty animals were used per treatment group (120 animals total; see Table 5.3 for treatment assignments for each tank). This range of atrazine concentrations includes environmentally relevant values from surface waters across the country (e.g., 224 ppb in Midwestern streams, Hayes et al. 2002b), as well as in New Jersey (25 ppb from field water samples, see Chapter 3). The lower concentrations of this range represent typical exposure from contaminated waters, while the higher concentrations represent runoff events near application sites (Rohr et al. 2003). Furthermore, at these concentrations an effect should be seen (Tavera-Mendoza et al. 2002a, 2002b; Carr et al. 2003; Sullivan and Spence 2003). For example, Hayes et al. (2002b) reported effects (including smaller vocal organs and lower testosterone levels) at 0.1 ppb, thirty times lower than the EPA drinking water standard of 3 ppb (Sanders 2002) and ten times below our lowest concentration. These concentrations were nonlethal to the amphibians (Allran and Karasov 2001) because the concentrations for this experiment were well below atrazine's LC_{50} range of 0.41 to 126 mg/L listed for several frog

species, including *X. laevis* at the high end of this range (Birge et al. 1983; Morgan et al. 1996; Howe et al. 1998; Carr et al. 2003).

General measurements. During the experiment, the following data were recorded to evaluate the developmental progress of the organisms: survival (with dead or moribund animals removed and stored in formalin), growth and general health, activity level (observed during feedings and water changes), and observations of any developmental abnormalities (Cooke 1981; Britson and Threlkeld 1998; Fort et al. 2001).

Following metamorphosis (marked by complete resorption of the tail, Gosner 1960), animals were anesthetized in a 1% (1 g/L) solution of MS-222 (Metcalfe et al. 2000). The date of complete metamorphosis was recorded and the following measurements taken: weight, snout-vent length, hind limb length (average of both right and left limbs), and abdomen girth. Additionally, any observed developmental malformations were recorded. All animals were assigned a unique accession number, which was determined as follows: TankTreatmentCodeMetamorph#; e.g., R06 was from the red treatment group (20 ppb atrazine) and was the sixth metamorph.

Animals were sacrificed and tissue samples taken as described for collected wild frogs (see Chapter 3 for details) with the following modifications listed below. The alimentary canal (from behind the stomach to the end of the rectum) was dissected out and measured as "gut length" (Gray and Janssens 1990). Tissues (gonads, liver, kidney and brain) were fixed in Bouin's (Herman and Kincaid 1988; Coady et al. 2004; Anway et al. 2005) for six hours. Animals for radioimmunoassay analysis were flash frozen whole in liquid nitrogen. Individuals were assigned to be analyzed by histology or radioimmunoassay based on random number generation (see Table 5.4 for analysis assignments based on order of metamorphosis). Based on gonadal morphology, individuals were sexed as male, female or intersex. Gonadal structure was then evaluated histologically for evidence of endocrine disruption. Tissues for histological examination were processed, embedded, sliced and stained as detailed in Chapter 3 with a single modification: the kidney and attached gonads were transversely cut in order to look at renal and gonadal tissues concurrently.

Whole body radioimmunoassay. Whole body radioimmunoassay (RIA) with extraction and column chromatography for sample purification (Belden et al.) was performed. Tadpoles were homogenized in wet ice with a glass pestle and mortar (size 21,

Kimble Kontes, Vineland, NJ) with 3-4 volumes of methanol. Decanted homogenates were kept in wet ice, spiked with ^{3}H-testosterone and –corticosterone, and ethyl acetate added. Samples were vortexed and left at 4°C overnight to allow sample and tritiated hormones to reach equilibrium. Samples were centrifuged (12 minutes, 2500 rpm at room temperature), supernatant transferred and dried in a 37°C water bath under an ultra-purified air pump. Dried samples were redissolved in 10% ethyl acetate-isooctane and then loaded onto prepared celite (diatomaceous earth, Sigma-Aldrich, St. Louis, MO) columns. Hormones were separated and eluted under nitrogen gas using isooctane with increasing levels of ethyl acetate. Testosterone was eluted with 20% ethyl acetate-isooctane and corticosterone was eluted with 50% ethyl acetate-isooctane. Eluted samples were dried (as before) and reconstituted in phospate-buffered saline with gelatin (PBSG). Approximately 36% and 40% of reconstituted testosterone and corticosterone, respectively, were assayed and whole body levels (ng/g) obtained as described in Chapter 3. Detection limit: 6.6 pg (~1 ng/g).

Statistical analyses. Logistic regression analysis and Fisher's exact test were performed for survival, and prevalence of malformations and histological abnormalities. Comparisons of means were performed by one-way analysis of variance (ANOVA) for

days to metamorphosis, body weight, SVL, HLL, abdomen girth, gut length, and whole body testosterone and corticosterone. Significance for each test was set at $p < 0.05$. For each analysis, p values are provided in the results section.

Results

Water chemistry. Actual atrazine concentrations (with target concentrations in parentheses) were as follows: 1.32 ± 0.56 µg/L (1.0), 4.96 ± 0.25 µg/L (5.0), 21.07 ± 0.88 µg/L (20.0), 55.72 ± 1.89 µg/L (60.0) and 119.24 ± 0.94 µg/L (120.0), with no detection of atrazine in control treatments. Representative chromatograms for each treatment are displayed in Figure 5.1, showing relative atrazine peaks at ~26.8 minutes. Water temperatures averaged 19.78 ± 1.70°C. Mean values for water quality measurements taken three times a week were 8.2 ±0.6 dissolved O_2, 7.54 ± 0.13 pH, <0.1 ppm ammonia, 0.76 ± 0.12 ppm nitrate, <0.01 nitrite and <0.01 ppm chlorine (with an n = 39 for each analysis). Values for water quality parameters measured weekly were 72.8 ± 19.6 ppm hardness as $CaCO_3$ (calcium carbonate), 116.5 ± 17.3 ppm alkalinity and 514.42 ± 22.2 µmhos/cm conductance (with an n = 13 for each analysis. (See Table 5.5 for summary of water quality measurements.)

General Measurements. Percent of tadpoles surviving to metamorphosis ranged from 93.75% to 100% and all treatment groups were comparable to control except 5 ppb atrazine where survival was significantly reduced at 57.89% (Figure 5.2). Of the tadpoles that reached metamorphosis, those treated with 5 and 60 ppb atrazine reached the end of their larval period the fastest, averaging 64.0 and 58.4 days respectively (Figure 5.3). Significant differences were also seen in weight at metamorphosis following atrazine treatment, though not in a dose-dependent manner. The body weights of animals treated with 5, 20 and 60 ppb atrazine were 38.8%, 30.8% and 32.6% reduced compared to controls (Figure 5.4). In addition to yielding metamorphs of reduced weight, 5 ppb atrazine also resulted in significantly reduced length measurements. Metamorphic snout-vent length (Figure 5.5), hind-limb length (Figure 5.6) and abdominal girth (Figure 5.7) were reduced 14.8%, 17.9% and 21.7% compared to control. Abdominal girth was also significantly reduced (20.4%) at 120 ppb atrazine compared to control (Figure 5.7). Alimentary canal, or gut length, was measured for each frog undergoing histological analysis. Though no significant differences were observed, gut length became progressively shorter with increasing atrazine treatment from 1 to 20 ppb (Figure 5.8). However, the parameter began to increase at the

two highest atrazine concentrations, displaying a non-monotonic response known as a U-shaped response.

Abnormalities. Several malformations were observed among treated animals (Figure 5.9). These deformities were mostly forward curvature of the spine (lordosis) and several abnormalities of the limbs: ectromelia (absence of a limb), synmely (limb fusion) and subluxations (partial joint dislocations). These spinal and limb malformations were often concurrent within single individuals. There was also a single case of cloacal prolapse, where the intestines passed through the cloacal opening and the tadpole subsequently died. Tadpoles displaying the severest forms of these deformities succumbed to death before metamorphosis was complete. The prevalence of both spinal and limb malformations were highest in the 5 and 20 ppb atrazine treatment groups (Figure 5.10). Malformations of the spine were 8.1- and 7.7-fold higher than control for 5 and 20 ppb atrazine respectively. For limb deformities, 9.8- and 8.5-fold increases over control were observed for 5 and 20 ppb. No significant differences were detected for deformity prevalence between other treatment groups and control.

Early amphibian tadpoles have undifferentiated gonads with an inner medulla and outer cortex. The medulla differentiates into

testicular tissue, and the cortex differentiates into ovarian tissue with regression of the opposite tissue (Reeder et al. 1998). Figure 5.11 illustrates normal morphology and histology of gonadal tissues. Testes lie on the anterior portion of the kidney's ventral surface, are non-pigmented, smooth and short. Figure 5.11A illustrates normal testicular morphology with arrows indicating the rostral (the right side of the photomicrograph) and caudal (left side) extent of the gonads (Carr et al. 2003; Hayes et al. 2003). Ovaries are pigmented (by melanophores), are much longer than testes (extending posteriorly down the kidneys), and have a lobed structure (Figure 5.11B, Carr et al. 2003; Hayes et al. 2003). Figures 5.11C and 5.11D illustrate the normal histology of the testes and ovaries, respectively. Note the presence of an ovarian cavity where medullary tissues regressed.

Abnormalities were observed in the testes of treated animals both morphologically and histologically. Morphological changes in the testes included elongation of the caudal end of the tissue, as illustrated in Figure 5.12A. This elongated morphology resembles ovarian tissue but lacks the lobed structure. Figures 5.12B and 5.12C display photomicrographs of an underdeveloped testis from 60 ppb atrazine and a bi-lobed testis from our lowest concentration tested, respectively.

Figure 5.13 shows the morphology and histology of the single gonad that could not be differentiated as either testes or ovaries. The kidneys of this individual from 5 ppb atrazine were underdeveloped, as were the gonads. Because the gonads appear smooth (Figure 5.13A), they are likely to be elongated testes. However, the histology of the gonads (Figure 5.13B) reveals a cavity in one gonad typical of ovarian tissue along with the presence of small oogonia. Figure 5.14 displays the prevalence of both morphological and histological abnormalities of the testes by atrazine treatment. Abnormal morphology was significantly elevated in all treatments by 2- to 3-fold compared to control, except 20 ppb atrazine. Histological evidence of testicular anomalies was only elevated at 60 ppb atrazine, which was 3-fold higher than control.

Liver, kidney and brain tissues were also histologically evaluated. Very little pathology (the following two out of 41 histologically-evaluated animals) was observed in these organs. However, from the 20 ppb atrazine treatment, a necrotic liver was observed (Figure 5.15A), and from 60 ppb atrazine, a single case of renal calcinosis was detected (Figure 5.15B).

Whole body radioimmunoassay. New metamorphs were too small from which to obtain blood; therefore, whole body hormone analyses

were performed for testosterone and corticosterone. Figure 5.16 displays whole body testosterone levels above detection (~1 ng/g) for new metamorphs. Though levels resulting from atrazine treatment were not significantly different from control, levels showed a nearly dose-dependent reduction. Whole body corticosterone levels similarly did not elicit any statistically significant differences due to atrazine exposure (Figure 5.17); however, levels increased with increasing atrazine concentration.

Discussion

At 5 ppb atrazine, tadpoles had reduced survival and decreased time to metamorphosis. The shorter larval period, possibly selected for by high mortality, likely resulted in decreases in weight at metamorphosis, snout-vent length, hind limb length, and abdominal girth. All of these parameters are related as growth indices, indicating an overall inhibition of growth. At 60 ppb atrazine, larval period and metamorphic weight were significantly reduced which may reflect the strategy of utilizing energy preferentially toward the process of metamorphosis rather than weight gain. This treatment group was also hyperactive and exhibited hiding behavior. Tadpoles treated with 120 ppb atrazine experienced a longer larval period, yet weighed less and were thinner (reduced abdominal

girth) than their control counterparts. Tadpoles in this treatment displayed loss of appetite and sluggish movement; this loss of energy assimilation may reflect direct atrazine toxicity. Howe et al. (1998) also saw sluggishness and reduced swimming speed in leopard frogs (*Rana pipiens*)and American toads (*Bufo americanus*) exposed to relatively high concentrations of atrazine (2.8 - 23 ppm). In this situation, the larval period may have been extended in an effort to achieve a critical weight to undergo metamorphosis. Relyea (2001a) also found decreased body width associated with longer larval periods as a response to predator cues. Consequently, smaller metamorph size and weight may be a general stress response.

The high proportions of malformations in the 5 and 20 ppb atrazine treatments were unexpected, as no reports of skeletal anomalies due to atrazine exposure were found in the literature. However, a study by Iguchi et al. (2001) found skeletal malformations and suppressed organogenesis in *Xenopus laevis* tadpoles treated with 10 µM estradiol. These abnormalities were only induced if treatment started prior to onset of metamorphosis (stage 39, Gosner 1960). Similarly, the malformations observed in this experiment were not apparent until the onset of metamorphosis. Based on the timing of skeletal anomalies, it is possible that increased estradiol

levels result from atrazine exposure and interferes with cell-to-cell adhesion and cell migration during metamorphosis as hormonal cues become too skewed to effectively direct development (Birge 1959; Howe et al. 1998). This may be why skeletal elements that differentiate earlier are more resistant to defects than those that differentiate later (Sparling et al. 2000).

Abnormalities seen in testicular tissues of treated animals could reflect the loss of testosterone observed. Plasma testosterone levels in exposed male frogs were expected to be depressed compared to controls as a ten-fold decrease was observed in adult male *X. laevis* exposed to 25 ppb atrazine was reported by Hayes et al. (2002b). While levels did decrease in our study following atrazine treatment, differences were not significant. This lack of statistical significance does not necessarily indicate a lack of biological significance, for only small changes are needed to elicit a biological response.

We did observe disruption in testicular development at both the gross and cellular levels. Prevalence of morphological changes was higher than histological changes, which may reflect alterations in spatial development. The testes develop anterior to posterior, and prior to complete development the anterior

portion would be more developed than the posterior (Hayes et al. 2003). Consequently, delays in development due to depressed hormonal cues can result in a posteriorally underdeveloped gonad at the cellular level.

Pathologies in non-gonadal tissues were rare. The single necrotic liver observed at 20 ppb atrazine occurred in a metamorph displaying both spinal and limb deformities. The dysgenesis in the liver may reflect global developmental defects due to abnormal hormonal milieu. The renal calcinosis from an animal treated with 60 ppb may be a consequence of the rapid growth experienced by this treatment group, which left tissues underdeveloped.

Dose pattern. In this study, the low doses of atrazine, especially 5 ppb, had the most dramatic effects on *X. laevis* larvae. A non-monotonic dose response curve (NMDRC) is one where the response reverses in a U or inverted-U shape with increasing chemical concentration (Davis and Svendsgaard 1990; Storrs and Kiesecker 2004). NMDRCs are typical for endpoints of growth, longevity, and reproductive parameters and are typically displayed by endocrine disruptors (Calabrese and Baldwin 1999; Akingbemi and Hardy 2001; Rohr et al. 2006). We saw a U

response for survivorship and growth parameters. We also observed an inverted-U response for malformations.

With chemicals that display an NMDRC, it is possible for very low concentrations to be without measurable effect, while intermediate doses are the most effective at eliciting a response, and higher concentrations have a decreasing effect (Chen 2001). Such responses have been previously reported with atrazine exposure. Storrs and Kiesecker (2004) observed mortality in several species of tadpoles to be highest at 3 ppb atrazine, compared to both control and higher doses of atrazine. Rohr et al. (2006) found the greatest decrease in post-exposure survival rates of streamside salamander (*Ambystoma barbouri*) larvae at 4 ppb atrazine. These reports were confirmed by our studies, where we found higher mortality at 5 ppb atrazine. Furthermore, Coady et al. (2004) found that green frogs (*Rana clamitans*) exposed to 10 ppb atrazine (lowest dose tested) were smaller in length and weight at metamorphosis as well as older compared to controls. This data coincides well with our growth profiles following atrazine treatment.

Many endocrine disruptors do not have a definable threshold level, or a level below which effects are negligible (Welshons et al. 2003). Therefore, very small amounts of the agent can

alter cellular responses, especially in reproductive organs (Schönfelder et al. 2002a). This phenomenon is the consequence of a biologically active system that is already above threshold; therefore, any input to the system elicits a response (Bigsby et al. 1999; Sheehan et al. 1999; Welshons et al. 2003). Endocrine disruptors can have effects below analytical detection limits as a result (Russell et al. 1997). Hayes et al. (2003) reported more pronounced effects of gonadal dysgenesis and sex reversal in leopard frogs (*Rana pipiens*) due to atrazine exposure at the lower dose studied (36% and 29% at 0.1 ppb versus 12% and 8% at 25 ppb, respectively). In addition, diethylstilbestrol, a synthetic nonsteroidal estrogen, enhanced prostate growth and branching and androgen binding activity at concentrations as small as 0.1 and 0.5 pg/ml in organ culture (Gupta 2000b). Consequently, low environmentally relevant concentrations of chemicals with estrogenic activity, like atrazine, may cause perturbations in reproductive parameters (Sheehan et al. 1999). In the current study, changes in testicular morphology and histology were detected at the lowest concentration of atrazine tested, 1 ppb. Overall, the doses at which we observed the greatest effects were consistent with those previously reported and our observations support the hypothesis that atrazine's effects are non-monotonic and are evidence of endocrine disruption.

Influences on metamorphosis. The process of metamorphosis is regulated by the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4). However, other hormones modulate thyroid hormone expression and/or activity and therefore play a role in determining the timing of metamorphosis (Hayes 1997). Both steroid and adrenal hormones affect T_3 and T_4 and these influence the process of metamorphosis.

Both testoserone and estradiol inhibit metamorphosis by inhibiting the conversion of T_4 to T_3, the more potent of the two hormones (Richards and Nace 1978; Hayes 1997; Nishimura et al. 1997). The proposed mechanism of atrazine's endocrine effects is through the induction of aromatase, which converts testosterone to estradiol. At 120 ppb atrazine, three tadpoles failed to complete metamorphosis within the treatment period. Levels of testosterone were reduced the greatest at this concentration (64.2% compared to control), though not statistically, and could indicate a concomitant rise in estradiol. This rise in endogenous estradiol parallels studies done with exogenous estradiol yielding large non-metamorphosing tadpoles (Nishimura et al. 1997). Under our conditions, the level of increased estradiol would not be expected to prevent metamorphosis, but to delay it as observed at 120 ppb atrazine.

A general response to stress in vertebrates is increases in the adrenal glucocorticosteroid, corticosterone (Belden et al.). Like testosterone, corticosterone levels in this study did not change significantly in treatment regimes from control. However, the elevation seen in corticosterone levels (26.3% at the highest atrazine concentration compared to control) may have biological significance. Rise in this stress hormone inhibits both growth and development during early larval stages (Hayes et al. 1993; Hayes 1995; Hayes and Wu 1995; Hayes 1997). In later larval stages, corticosterone accelerates conversion of T_4 to T_3 and promotes metamorphosis (Hayes 1997). Since growth and development are tightly coupled, larvae will not progress in development until critical size at each stage is reached. For example, larval tiger salamanders (*Ambystoma tigrinum*) exposed to 75 ppb atrazine took a longer time to reach stage 4 (middle) of metamorphosis, but did so at the same size and weight. However, exposure to 250 ppb atrazine resulted in stage 4 achievement at the same time as controls, but at a smaller weight and size (Larson et al. 1998). A chemical that can activate an animal's stress response, through corticosterone release, can compromise the animal's ability to respond to stress (Gendron et al. 1997; Hayes et al. 1997). Corticosterone plays a role in inhibiting non-essential functions in order to

allow the animal to survive in the face of changes in its environment (Belden et al.). Inhibition could be of growth and/or reproduction and would account for some of the effects seen on growth and development in this study.

Effect on energy budget. Growth and reproductive potential are defined in terms of energy budget processes (Rowe et al. 1998). Resource energy taken in is allocated to maintenance costs associated with basic physiological processes. Any surplus is then applied to growth, reproduction and/or storage. Chemical exposures can increase energy used, by increased metabolic rate and/or protein synthesis, or decrease energy assimilation, by decreased foraging efficiency and create a deficit in energy balance (Rowe et al. 1998; Casper and Hendricks 2006). Corticosterone and thyroxine interact to regulate metamorphosis based on energy assimilation and fitness: if energy assimilation is limited, differentiation is slowed in favor of growth; if assimilation falls below a critical level, differentiation is maximized and growth is compromised (Larson et al. 1998; Diana et al. 2000). As atrazine toxicity increases, the metabolic costs to counteract its effects overwhelm homeostatic measures (Calabrese and Baldwin 1999, 2001). In such a state, the organism is in allostatic overload; that is, the cost to maintain system stability renders the

animal unable to maintain positive growth; therefore, corticosterone increases and metamorphosis occurs (Belden et al.). As the higher concentrations of a chemical like atrazine saturate normal detoxifying pathways, toxic effects result (Calabrese and Baldwin 1999) and the allostatic load threshold is reached during the larval stage and corticosterone rises in response (Belden et al.), so the animal can escape the adverse conditions. In our study, atrazine concentrations may have been below the threshold for allostatic overload. However, the processes of energy utilization may have been influenced by atrazine (either directly or indirectly) and contributed to changes in larval period length and eating behaviors.

In support of this energy budget model, larger tadpoles tend to have lower corticosterone levels than their smaller counterparts (Belden et al.). Larger tadpoles, therefore, can undertake larger allostatic loads as their body condition (larger fat reserves) allows them access to energy stores other tadpoles may not have (Belden et al.). This difference in body condition may explain the variation we observed in our corticosterone levels. Figure 5.18 illustrates body weight distribution as compared to corticosterone levels for all treatments in our study. Although the r-square value is low, there is a slight trend showing that corticosterone decreases with increasing body weight. Figure

5.19 indicates treatment for each individual. Within certain treatment groups (1, 5 and 60 ppb atrazine), a clearer trend toward lower corticosterone with increased body weight is seen. Among control metamorphs however, the opposite trend is observed, corticosterone levels are higher for animals with greater body weight.

Consequences of altered development. Prolonged larval period and reduced size and weight at metamorphosis are both cause for concern. A longer time to metamorphosis will lead to animals emerging from their breeding waters late in the season, possibly resulting in mortality from the colder temperatures (Larson et al. 1998). Being smaller at metamorphosis is associated with reduced survival, greater predation by gape-limited predators, inability to utilize larger food items, and lower fecundity {e.g., greater predation by gape-limited predators; inability to utilize larger food items; \Hayes, 1993 #298;Werner, 1986 #1195;Werner, 1994 #1194}. Both an extended larval period and reduced metamorph size lead to overall reduced adult fitness. This complex hormonal system results in varied responses to chemical stressors, which depend on the balance of conditions.

Both corticoids and sex steroids affect the length of the larval period by interacting with the thyroid axis. In addition,

developmental rates and timing of metamorphosis are influenced by environmental factors like temperature, competition and contamination (Kiesecker and Blaustein 1998). The dose-response curve observed in this study could be explained by changes in hormone ratios; that is, relative levels of thyroidal, gonadal and adrenal hormones. Indeed, endocrine disruptors can alter hormone function through several mechanisms (e.g., steroid feedback, metabolic activity) and this phenomenon explains why they may be associated with multiple and seemingly contradictory effects, even within the same system or organ (Akingbemi and Hardy 2001). There appears to be multiple thresholds or windows of responsiveness for the different effects of atrazine.

Conclusions and considerations. Larvae that transform at a smaller size do not survive as well and are less fit than their larger counterparts (John-Alder and Morin 1990). Parameters such as time to first reproduction, fecundity, dispersal, and adult survivability are dependent on growth and size at metamorphosis (Smith 1987; Semlitsch and Gibbons 1988). Accordingly, as metamorphs become smaller, these parameters decrease. Consequently, low doses of a chemical that result in decreased metamorphic size can leave populations vulnerable to decline.

In addition to reductions in survival and growth profiles, low doses of atrazine also resulted in greater gross malformations. Deformities greatly reduce an animal's ability to escape predation, are associated with increased disease susceptibility and can be lethal, as evidenced by our observation that tadpoles displaying the most severe malformations subsequently died.

Our data on gonadal anomalies in *X. laevis* most correlate with our field observations of retarded testicular development in bullfrog (*Rana catesbeiana*) males. Both animals display a demasculinization effect resulting from a loss of testosterone influence. In addition, these data indicate that the no observable adverse effect level (NOAEL) for atrazine action on testicular development is at or below 1 ppb, our lowest concentration tested, which yielded testicular effects at both the morphological and cellular levels. This observation is important in determining endocrine disruption risk for amphibians in relatively pristine areas exposed to very low maintained levels of atrazine due to atmospheric deposition (1 to 20 ppb, Drost and Fellers 1996; Roberts 2006).

It should be noted that the *Xenopus* species tends to be more tolerant of environmental contaminants than other species. This is especially true in regards to atrazine, for which *Xenopus*

laevis has an LC_{50} of 126 ppm (Morgan et al. 1996), while bullfrogs display an LC_{50} of 410 ppb (Birge et al. 1983). It can be expected that if bullfrogs are more sensitive than *Xenopus* to its lethal effects, they will also be more susceptible to atrazine's sublethal effects. Though a large genetic distance separates laboratory *Xenopus laevis* from wild species, developmental pathways are highly conserved (Sparling et al. 2000). However, conditions used in this experiment do not mirror those in the field. *Xenopus* were exposed to a single static contaminant, whereas in the field, animals experience a complex mixture of agents, that are constantly changing, all of which may exert individual effects on fitness and health. Effects in the field from atrazine exposures will be heavily dependent on timing, as the critical window of sensitivity may be sexual differentiation. This window of susceptibility could be studied using the present effective concentrations at different time periods and life stages of the animal.

The non-monotonic response patterns observed in our study supports previous research showing that atrazine's greatest impacts on survival occur between 3-4 ppb, the lowest doses tested (Storrs and Kiesecker 2004; Rohr et al. 2006). These responses are supportive of hormonal involvement. Skeletal malformations that presented at onset of metamorphosis in 5 and

20 ppb atrazine may reflect increases in estradiol (Iguchi et al. 2001). These abnormalities are a major source of reduced survivorship, even for larvae that complete metamorphosis. In addition, our analysis of testicular tissues indicates that atrazine's LOAEL is at or below 1 ppb (lowest dose tested) and supports theories of atrazine's ability to affect amphibian reproduction even at very low doses. Furthermore, the changes in testosterone and corticosterone hormones indicate effects, albeit subtle, on hormone axes. Overall, atrazine exhibits endocrine disruptor properties at very low doses easily attainable in the field. This herbicide's action may be time and tissue specific and has the ability to transform a population to one of reduced reproductive fitness. Such a state will leave populations susceptible to declines as other stressors impinge upon them.

Table 5.1. Tadpole tank assignments
Random number generation table for tadpole assignments to atrazine treatment (e.g., the 31st tadpole collected was transferred to tank #5, blue). Random number generator provided by (Urbaniak and Plous 1997-2007).

Tadpole Assignments					
Red Tank 1	Green Tank 2	Orange Tank 3	Pink Tank 4	Blue Tank 5	Teal Tank 6
66	7	94	74	88	10
81	9	87	56	97	36
113	67	54	80	23	104
30	102	95	117	111	44
8	13	100	76	15	53
50	33	49	51	92	22
85	41	112	6	101	108
107	16	12	17	61	90
26	118	1	14	21	4
84	46	89	19	43	91
32	24	62	120	2	93
47	70	103	3	119	73
25	34	75	27	99	109
58	116	78	71	37	57
18	72	68	45	98	79
39	69	60	38	86	64
48	28	55	42	29	105
106	65	115	110	20	83
35	5	59	52	114	11
63	96	82	40	31	77

Table 5.2. Exposure conditions for *Xenopus laevis* larvae

Physical Parameters	
Exposure paradigm	six atrazine concentrations (0, 1, 5, 20, 60, 120 ppb) 0.004% acetone (v/v)
Controls	one solvent control
Replicates	one per treatment
Test Chamber Size	10 gallon glass aquarium
Test water	24 liters system water*
Number per treatment	20 tadpoles per aquarium
Feed	Nasco Frog Brittle Tadpole Powder (3x/week)*
Water Changes	50% static renewal 3x/week*
Aeration	two air stones per aquarium
Test Duration	entire larval period (not to exceed 75 days)
Environmental Parameters	
Test organism	*Xenopus laevis* (African clawed frog)
Age of Organisms	tadpoles → metamorphs
Density	~3 tadpoles per gallon
Temperature	20 ± 1.5 °C (monitored daily)
Photoperiod	12:12 (lights on at 7am)
Light Quality	ambient laboratory illumination

*see text for descriptions

Table 5.3. Atrazine concentration calculations
Atrazine treatment stock solutions and concentrations. Calculations were performed as follows. In order to have a final concentration of 0.004% acetone, 480 µL of acetone needed to be added per 12 liters of system water (volume in every 50% water change). As an example, for a final treatment concentration of 1 µg/L, a concentration of 25 mg/L atrazine is needed in the stock solution (12L * 1 µg/L / 480 µL = 25 mg/L). Also, since approximately 33 water changes were performed (11 weeks at 3 changes a week), ~16 mL of acetone (33 * 480 µL) was needed for the entire experiment. To account for any errors, stock concentrations were made to 20 mL. To attain 25 mg/L atrazine in 20 mL, 0.5 mg atrazine was needed (25 mg/L * 20 mL). When weighed out, 0.49 mg of atrazine was obtained, therefore the volume of acetone added was adjusted to 19.6 mL (0.49 mg / 25 mg/L) in order to obtain a stock solution of 25 mg/L.

Tank	Tank #	Tank Atrazine Concentration (µg/L)	Volume to Tank (µL)	Stock Atrazine Concentration (mg/L)	Desired Stock Atrazine Weight (mg)	Actual Stock Atrazine Weight (mg)	Stock Volume (mL)
Pink	4	0	480	0	0	0	20
Green	2	1	480	25	0.5	0.49	19.6
Teal	6	5	480	125	2.5	2.55	20.4
Red	1	20	480	500	10	10.02	20.04
Blue	5	60	480	1500	30	29.96	19.97
Orange	3	120	480	3000	60	59.99	20

Table 5.4. Metamorph analysis assignments
Random number table used to assign metamorphs to an analysis, either histology (Histo) or radioimmunoassay (RIA). Highlighted cells represent metamorphs used in either histological or RIA analysis. Random number generator provided by (Urbaniak and Plous 1997-2007).

0 ppb		1 ppb		5 ppb		20 ppb		60 ppb		120 ppb	
Histo	**RIA**	**Histo**	**RIA**	**Histo**	**RIA**	**Histo**	**RIA**	**Histo**	**RIA**	**Histo**	**RIA**
6	1	16	3	11	17	10	18	11	13	7	15
15	10	12	11	19	3	9	11	8	3	11	5
17	19	8	15	8	1	17	13	4	10	2	4
2	12	4	7	14	6	20	5	18	2	8	10
8	4	19	20	4	12	16	6	1	17	3	12
7	3	9	2	16	5	3	1	12	19	1	20
13	14	1	5	13	9	12	7	15	6	9	19
5	20	14	17	20	18	14	15	7	9	17	18
9	11	6	13	10	15	19	8	20	14	13	6
20	16	18	10	7	2	2	4	5	16	14	16

Figure 5.1. Atrazine chromatograms
Comparative gas chromatography chromatograms for each atrazine treatment. The atrazine peak (at ~26.8 minutes) is highlighted.

0 µg/L

1 µg/L

5 µg/L

20 µg/L

60 µg/L

120 µg/L

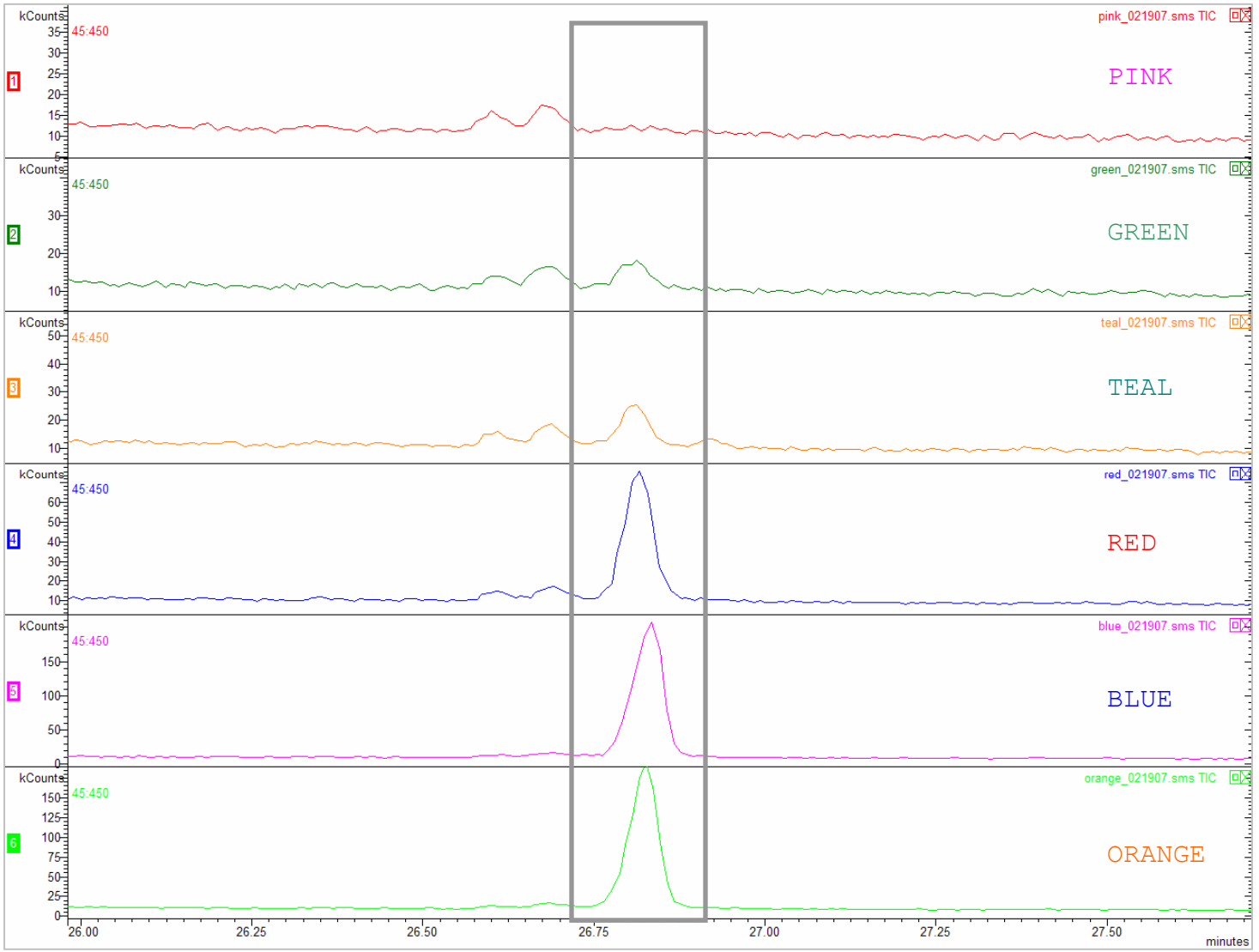
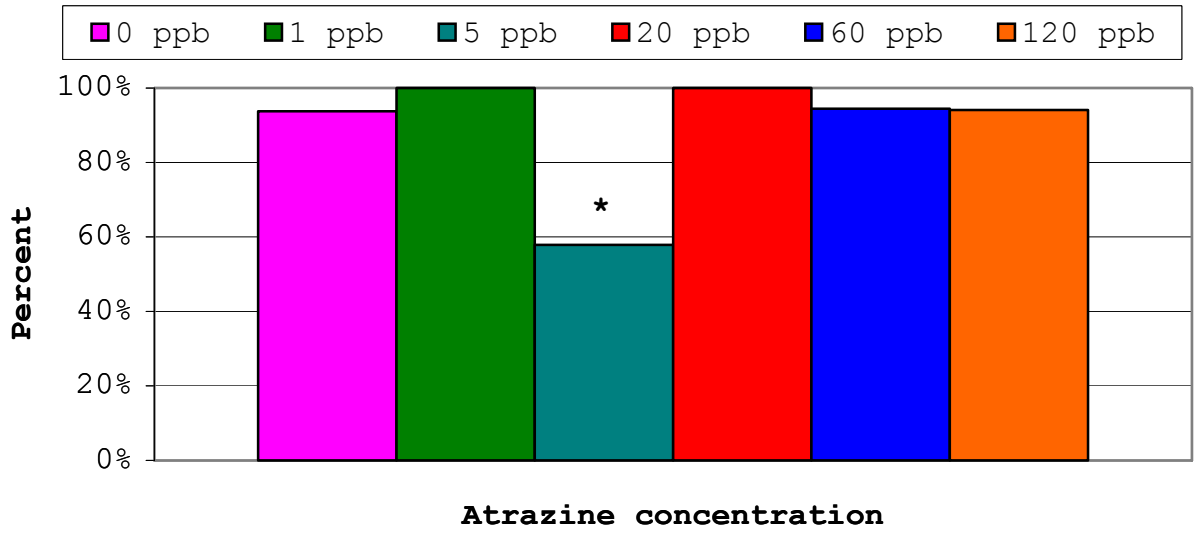


Table 5.5. Water quality measurements
Summary of water quality measurements taken throughout experiment.

Water Quality Measurements		
Daily	temperature	19.78 ± 1.70°C
3x/week	dissolved O_2	8.2 ± 0.6 ppm
	pH	7.45 ± 0.14
	ammonia	<0.1 ppm
	nitrate	0.76 ± 0.12 ppm
	nitrite	<0.01 ppm
	chlorine	<0.01 ppm
Weekly	hardness ($CaCO_3$)	72.8 ± 19.6 ppm
	alkalinity	116.5 ± 17.3 ppm
	conductance	514.42 ± 22.21 µmhos/cm

Figure 5.2. Percent survival
A, percent survival among larvae by increasing atrazine concentration. Treatment designated with a symbol is significantly different from control ("*" p = 0.018). Fisher's exact test was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

A.

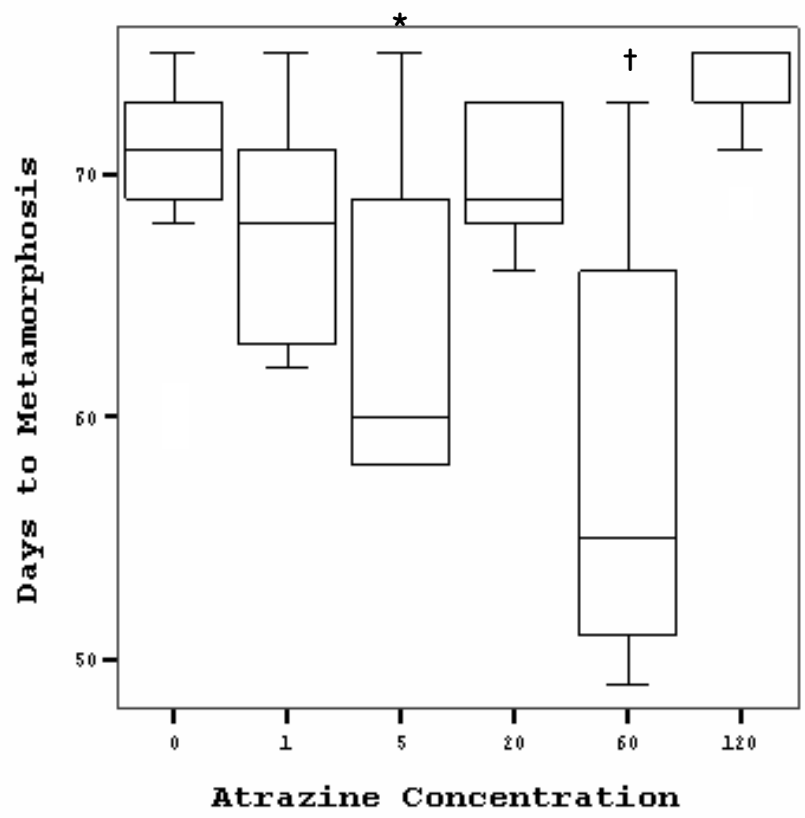


B.

Pink (0 ppb)	**Green** (1 ppb)	**Teal** (5 ppb)	**Red** (20 ppb)	**Blue** (60 ppb)	**Orange** (120 ppb)
93.75% (15/16)	100.00% (16/16)	57.89% (11/19)	100.00% (17/17)	94.44% (17/18)	94.12% (16/17)

Figure 5.3. Days to metamorphosis
A, days to metamorphosis among larvae by increasing atrazine concentration. Lines in boxplots represent the 50th percentile, or median, of the data. Treatments designated with a symbol are significantly different from control ("*****" p = 0.014; "**†**" p < 0.001). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

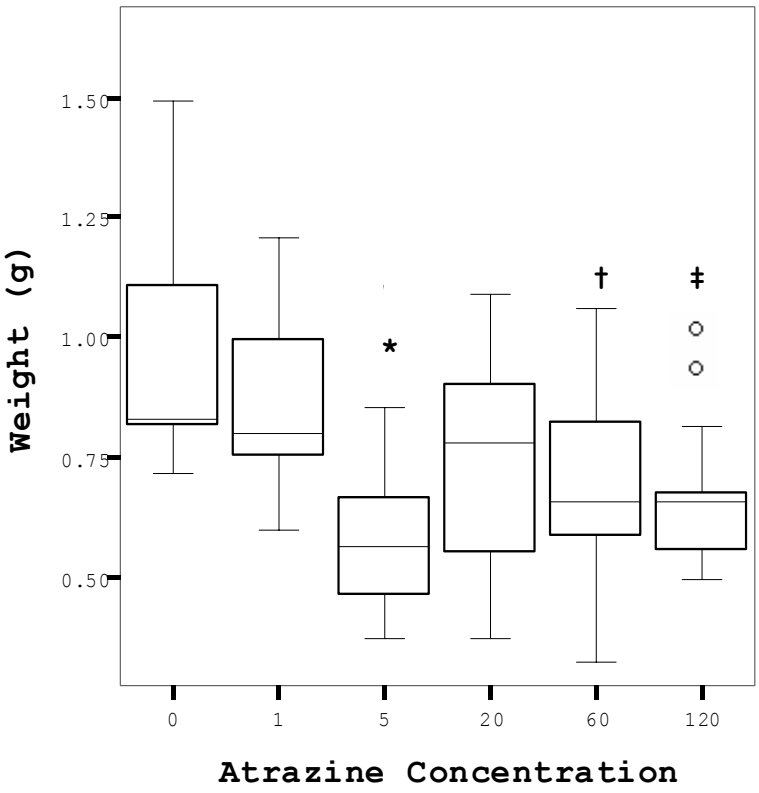


B.

Atrazine (ppb)	Days to metamorphosis	SD
0 (13)	70.7	± 4.0
1 (18)	68.2	± 4.2
5 (11)	64.0	± 6.6
20 (17)	70.3	± 2.8
60 (17)	58.4	± 7.8
120 (16)	73.1	± 1.7

Figure 5.4. Weight at metamorphosis
A, weight at metamorphosis among larvae by increasing atrazine concentration. Lines in boxplots represent the 50th percentile, or median, of the data. Treatments designated with a symbol are significantly different from control ("*****" p = 0.001; "**†**" p = 0.007; "**‡**" p = 0.006). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

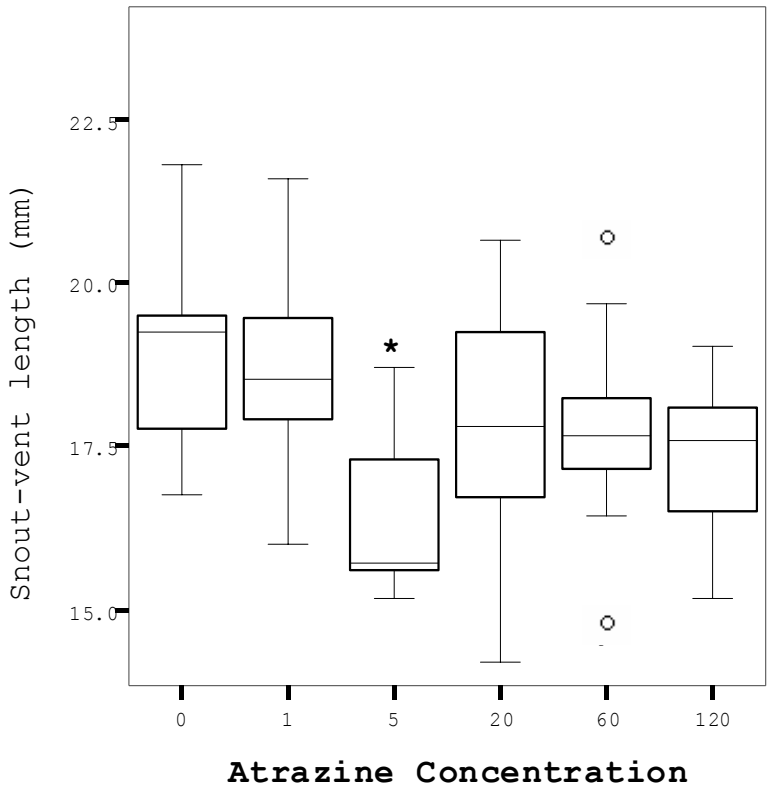


B.

Atrazine (ppb)	Weight (g)	SD
0 (10)	0.9966	± 0.3182
1 (14)	0.8721	± 0.2157
5 (10)	0.6103	± 0.2071
20 (17)	0.7418	± 0.2147
60 (17)	0.6897	± 0.2002
120 (14)	0.6720	± 0.1459

Figure 5.5. Snout-vent length
A, SVL at metamorphosis among larvae by increasing atrazine concentration. Lines in boxplots represent the 50th percentile, or median, of the data. Treatment designated with a symbol is significantly different from control ("*****" p = 0.003). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

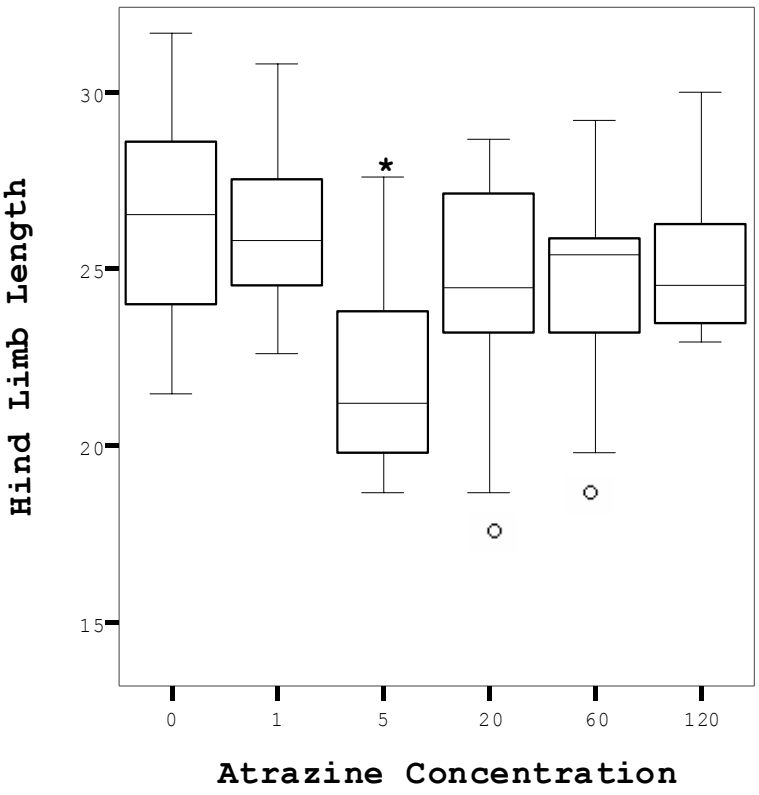


B.

Atrazine (ppb)	SVL (mm)	SD
0 (9)	19.35	± 2.1013
1 (15)	18.78	± 1.4080
5 (10)	16.49	± 1.3433
20 (17)	17.84	± 1.7867
60 (17)	17.74	± 1.6208
120 (13)	17.51	± 1.4840

Figure 5.6. Hind limb length
A, HLL at metamorphosis among larvae by increasing atrazine concentration. Lines in boxplots represent the 50th percentile, or median, of the data. Treatment designated with a symbol is significantly different from control ("*****" p = 0.010). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

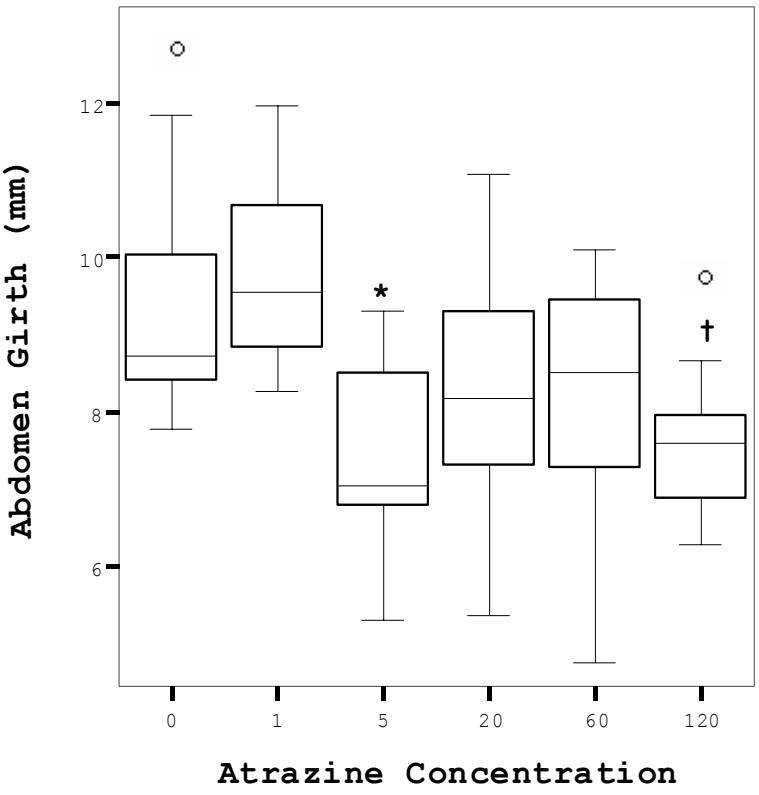


B.

Atrazine (ppb)	HLL (mm)	SD
0 (10)	26.60	± 3.3468
1 (15)	26.05	± 2.4258
5 (10)	21.84	± 2.7792
20 (16)	23.99	± 4.2042
60 (17)	24.50	± 2.6225
120 (14)	25.04	± 1.9557

Figure 5.7. Abdomen girth
A, abdomen girth at metamorphosis among larvae by increasing atrazine concentration. Lines in boxplots represent the 50th percentile, or median, of the data. Treatments designated with a symbol are significantly different from control ("*****" p = 0.024; "**†**" p = 0.021). One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.

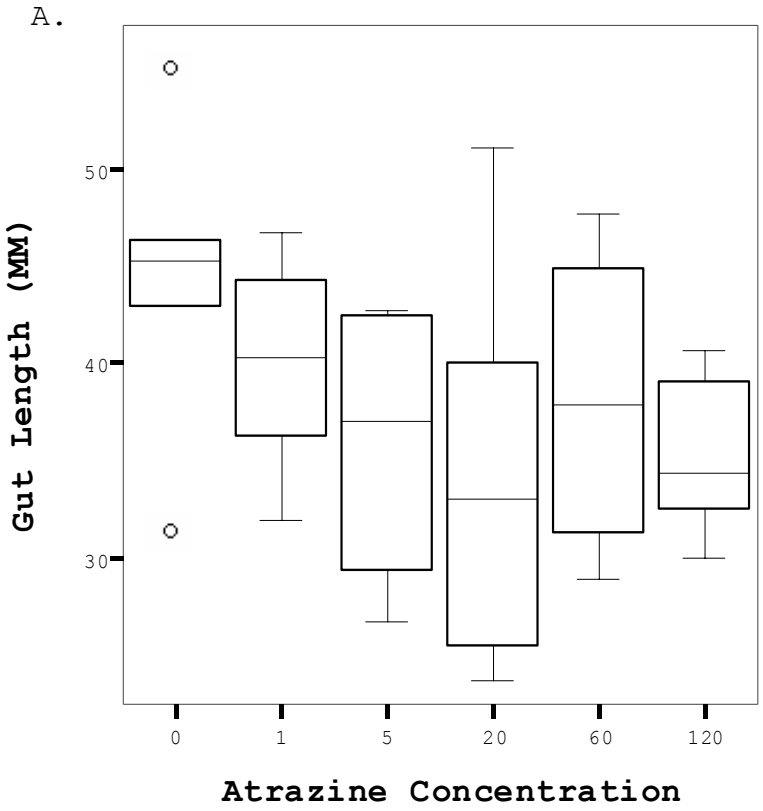


B.

Atrazine (ppb)	Abdomen girth (mm)	SD
0 (9)	9.46	± 1.7828
1 (15)	9.81	± 1.1484
5 (10)	7.41	± 1.2221
20 (17)	8.14	± 1.4738
60 (16)	8.25	± 1.5497
120 (14)	7.53	± 0.9264

Figure 5.8. Gut length
A, gut length at metamorphosis among larvae by increasing atrazine concentration. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.



B.

Atrazine (ppb)	Gut length (mm)	SD
0 (5)	44.40	± 8.8168
1 (8)	40.08	± 5.2638
5 (4)	35.91	± 7.8364
20 (8)	33.98	± 9.4426
60 (4)	38.07	± 8.4216
120 (7)	35.48	± 4.2006

Figure 5.9. Malformations
A, spinal deformity in a premetamorph treated with 5 ppb atrazine. The sternum protrudes (arrow) outward. B, limb deformities in a metamorph treated with 20 ppb atrazine. Both legs exhibit several joint malformations. C, malformations in a metamorph treated with 5 ppb atrazine. The upper portions (arrow) of the limbs are fused together (synmely). Note the abdominal edema (arrow head).

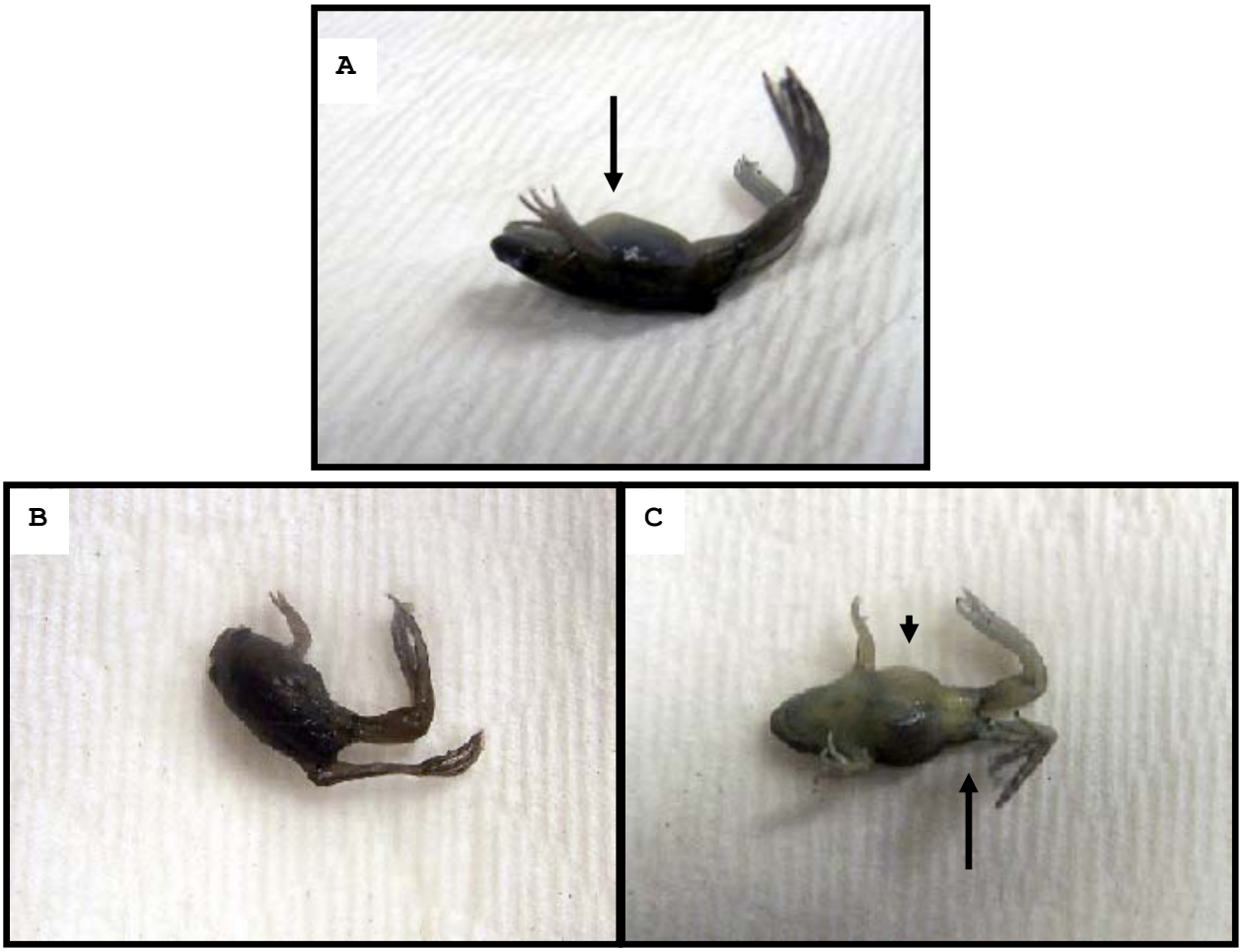
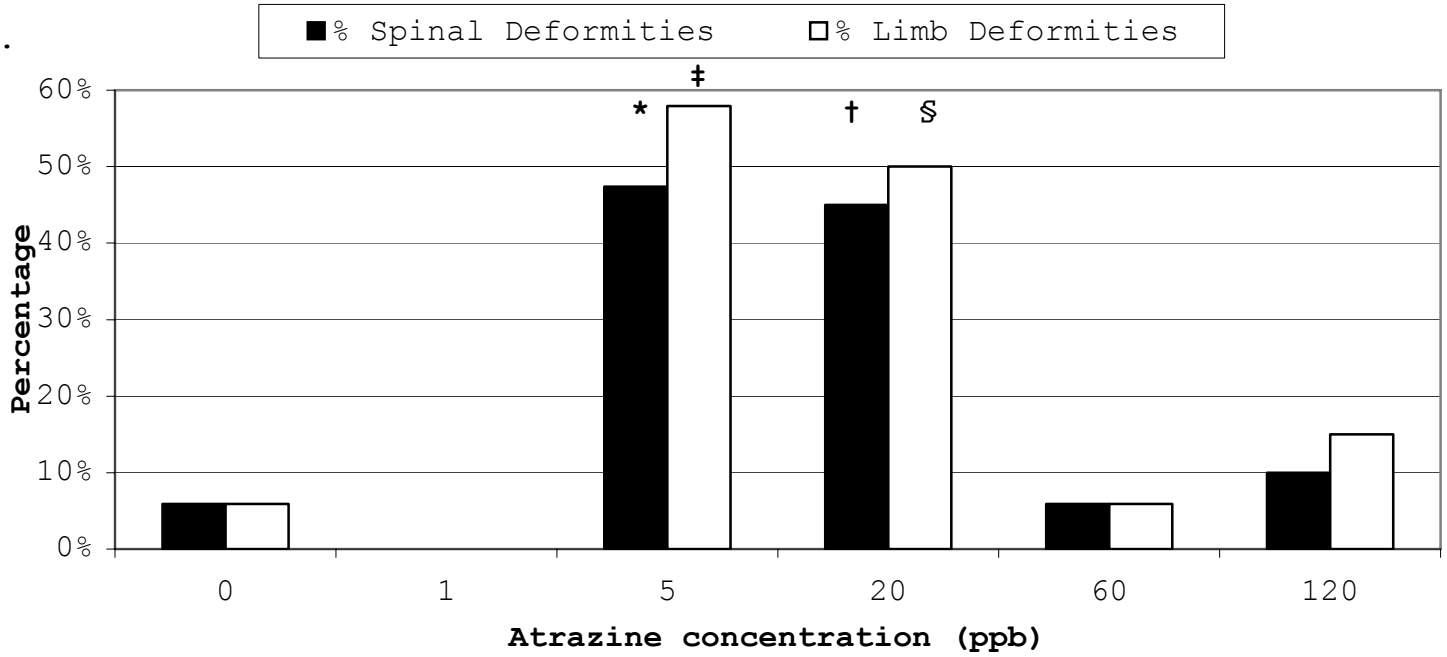


Figure 5.10. Prevalence of malformations
A, prevalence of spinal and limb deformities among larvae by increasing atrazine concentration. Treatments designated with a symbol are significantly different from control ("*" p = 0.007; "†" p = 0.010; "‡" p = 0.001; "§" p = 0.004). Fisher's exact test was used to test for significant differences between proportions. B, raw data showing proportions of spinal and limb deformities for each treatment in parentheses.

A.



B.

Atrazine Concentration	0 ppb	1 ppb	5 ppb	20 ppb	60 ppb	120 ppb
Spinal Deformities	5.88% (1/17)	0.00% (0/19)	47.37% (9/19)	45.00% (9/20)	5.88% (1/17)	10.00% (2/20)
Limb Deformities	5.88% (1/17)	0.00% (0/19)	57.89% (11/19)	50.00% (10/20)	5.88% (1/17)	15.00% (3/20)

Figure 5.11. Normal gonadal structure
A, normal testicular morphology; note that the testes are unpigmented and short. B, normal ovarian morphology; note that the ovaries are pigmented (melanophores) and much longer than testes. C, normal testicular histology (100x) with no obvious cortical tissue. D, normal ovarian histology (100x) with ovarian cavity (OC). Arrows demarcate the rostral (right of photomicrograph) and caudal (left of photomicrograph) ends of the gonads.

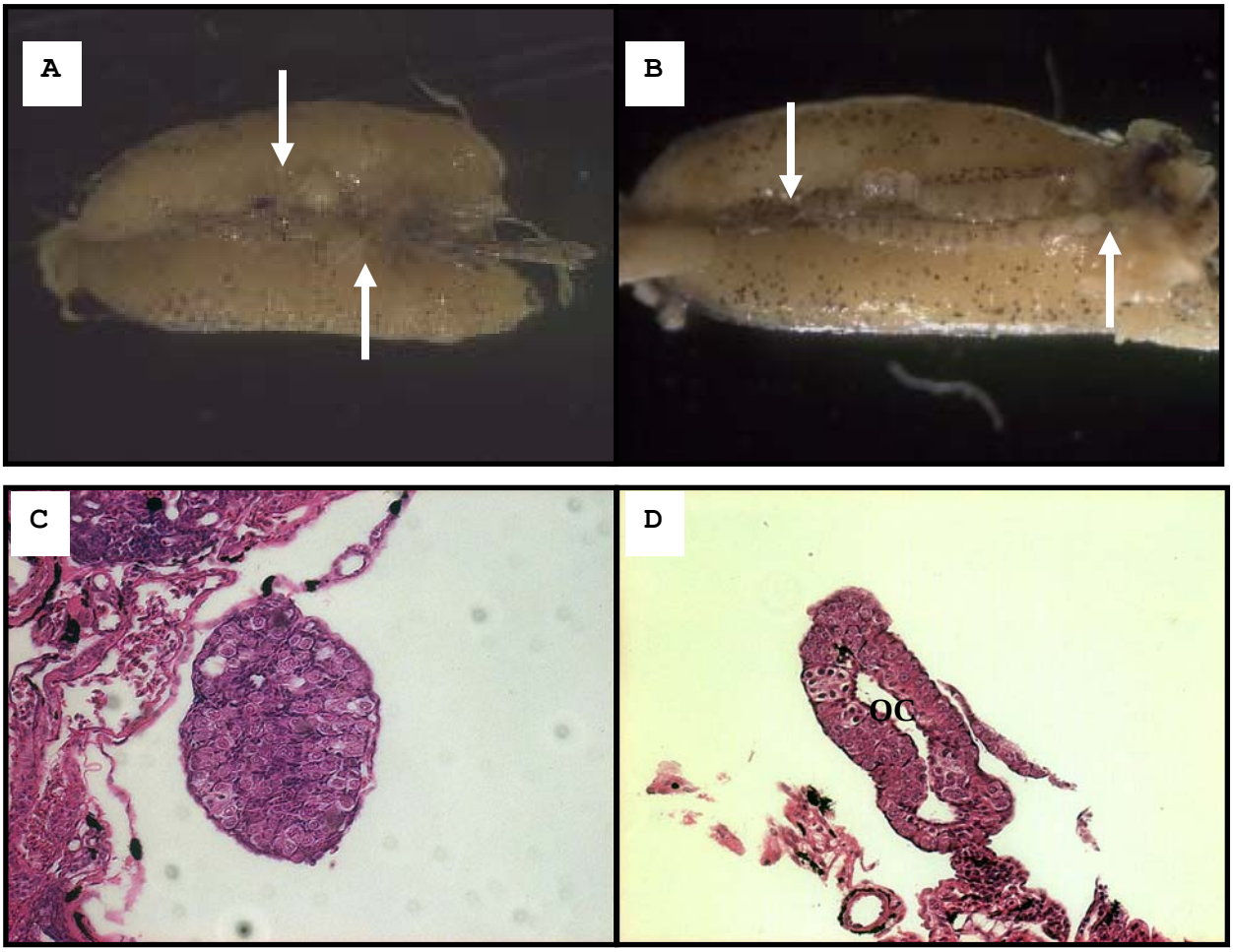


Figure 5.12. Abnormal testes
A, elongated testes from 120 ppb atrazine treatment. B, abnormal testis from 60 ppb atrazine treatment (200x). C, bi-lobed testes from 1 ppb atrazine treatment (25x). Arrows demarcate the rostral (right of photomicrograph) and caudal (left of photomicrograph) ends of the gonads.

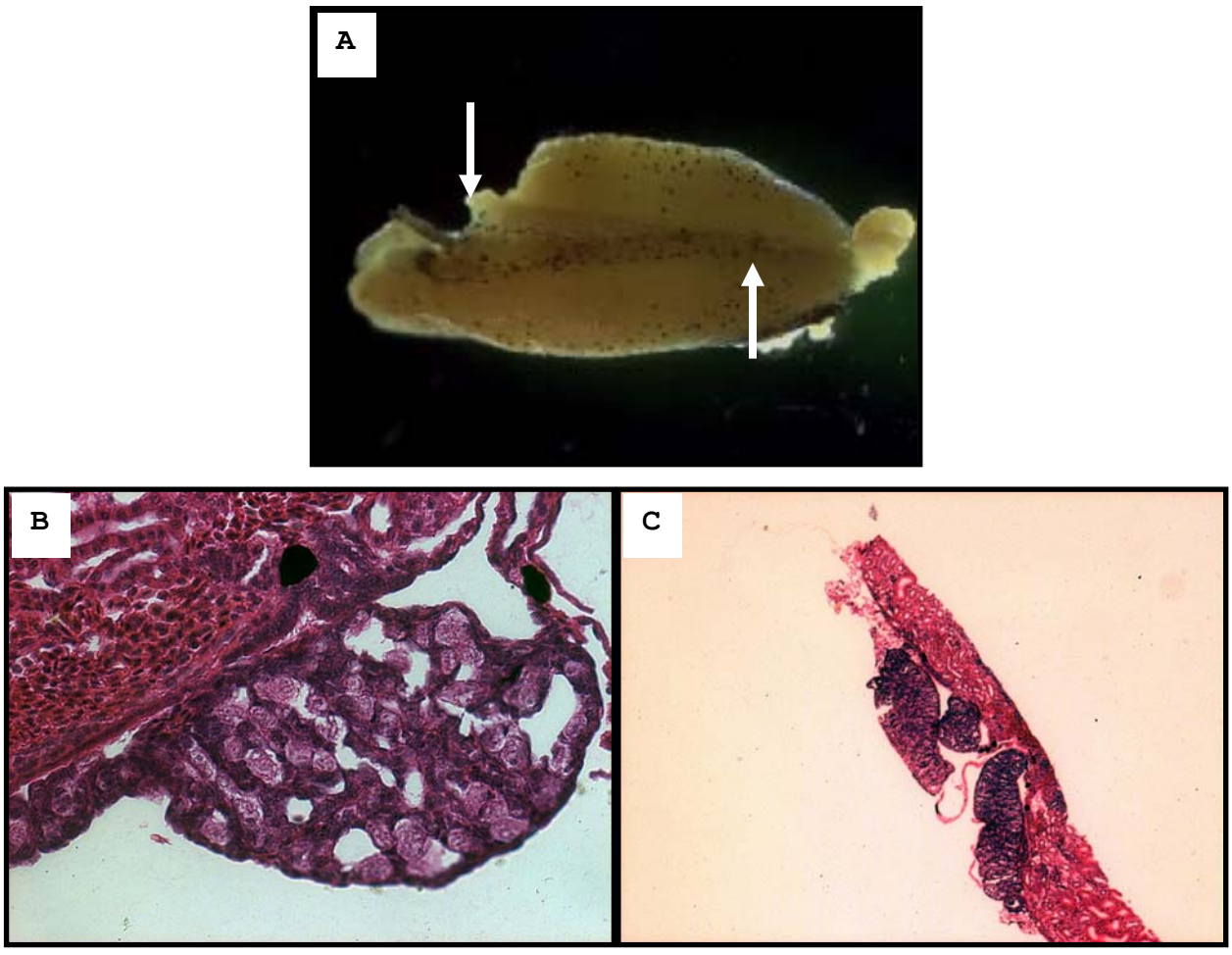


Figure 5.13. Ambiguous gonads
A, underdeveloped kidney and gonads from 5 ppb atrazine treatment. B, histology of "A" (100x); note the cavity (C) in the left gonad. Arrows demarcate the rostral (right of photomicrograph) and caudal (left of photomicrograph) ends of the gonads.

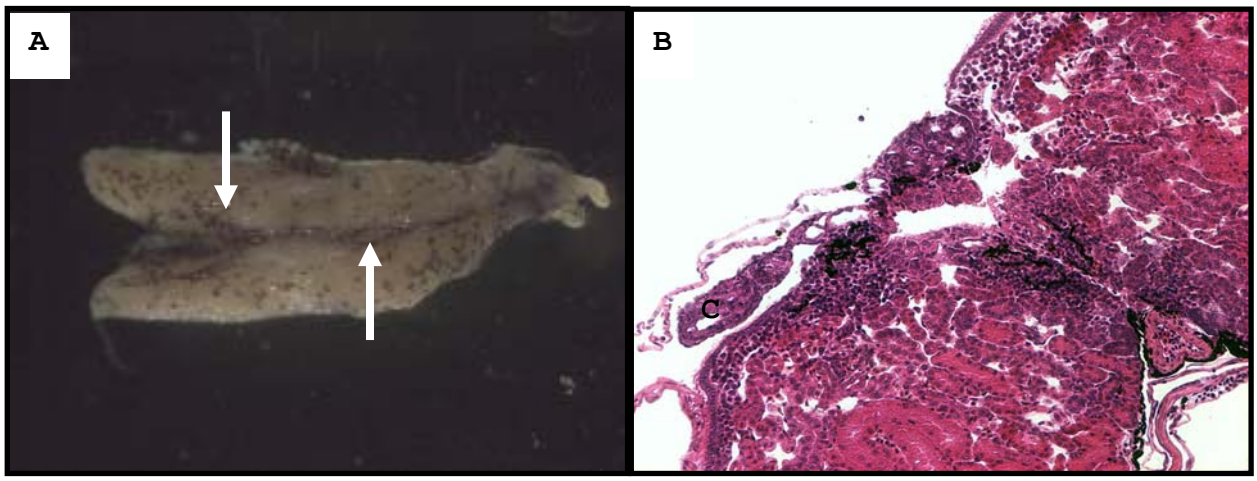
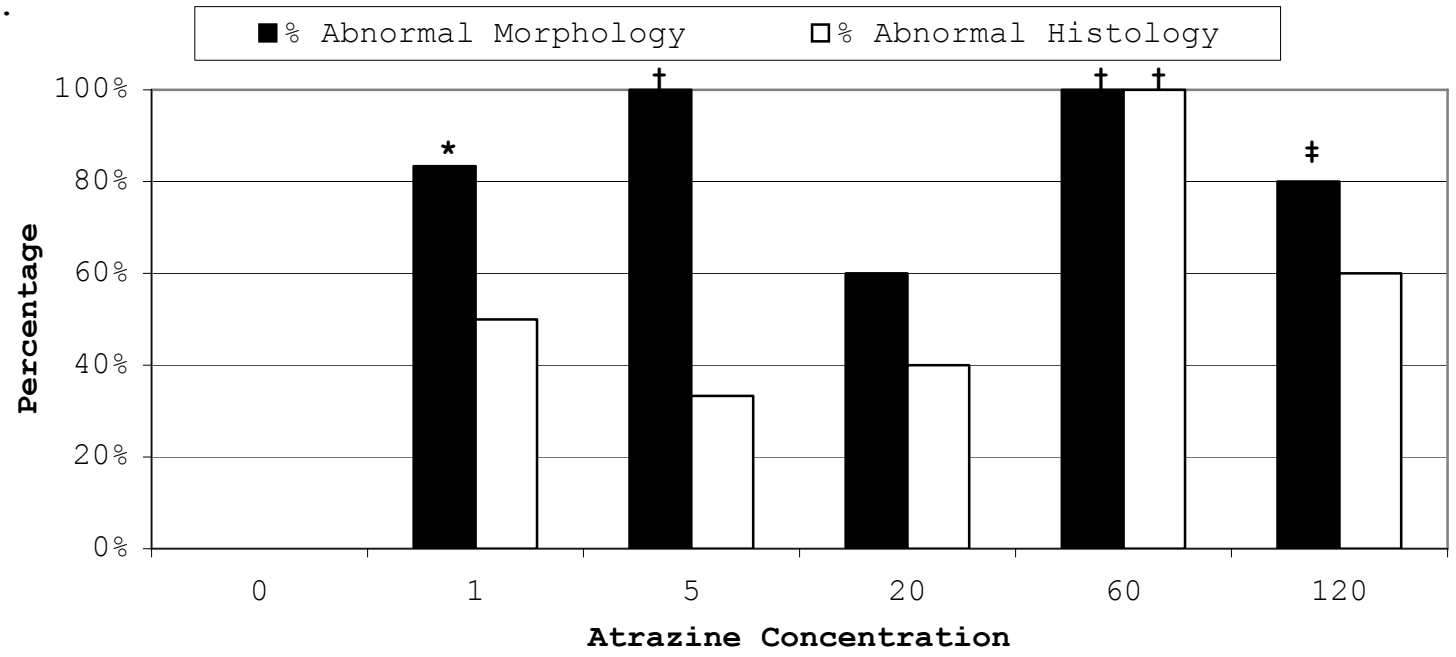


Figure 5.14. Prevalence of testicular dysgenesis
A, prevalence of abnormal morphology and histology of the testes among larvae by increasing atrazine concentration. Treatments designated with a symbol are significantly different from control ("*" p = 0.018; "†" p = 0.014; "‡" p = 0.028). Logistic regression analysis with coding was used to test for significant differences between proportions. B, raw data showing proportions in parentheses.

A.



B.

Atrazine Concentration	0 ppb	1 ppb	5 ppb	20 ppb	60 ppb	120 ppb
Abnormal Morphology	0.00% (0/3)	83.33% (5/6)	100.00% (3/3)	60.00% (3/5)	100.00% (3/3)	80.00% (4/5)
Abnormal Histology	0.00% (0/3)	50.00% (3/6)	33.33% (1/3)	40.00% (2/5)	100.00% (3/3)	60.00% (3/5)

Figure 5.15. Tissue pathologies
A, necrotic liver from 20 ppb atrazine treatment group (50x).
B, renal calcinosis from 60 ppb atrazine treatment group (200x).

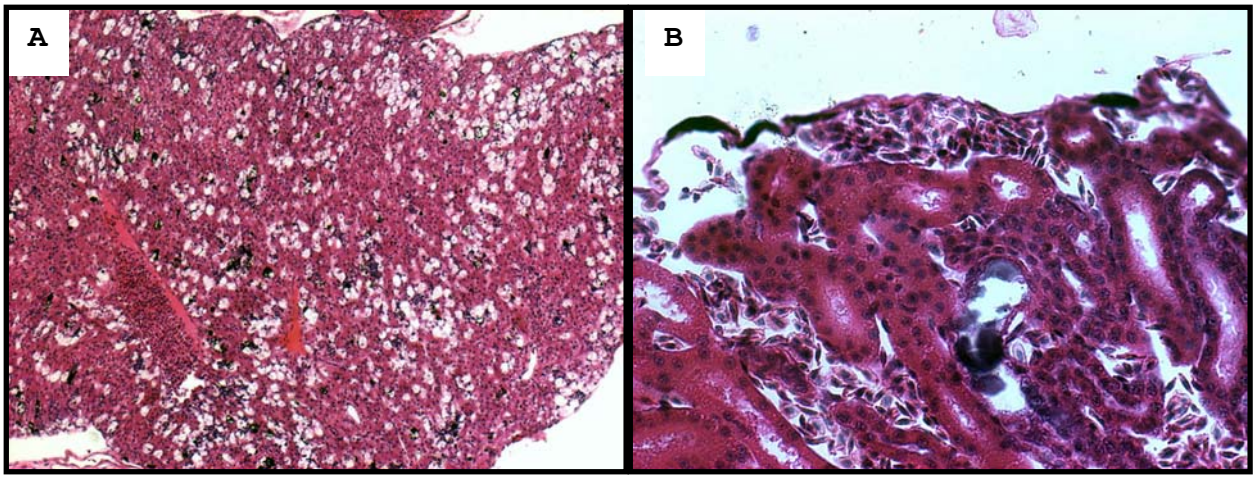
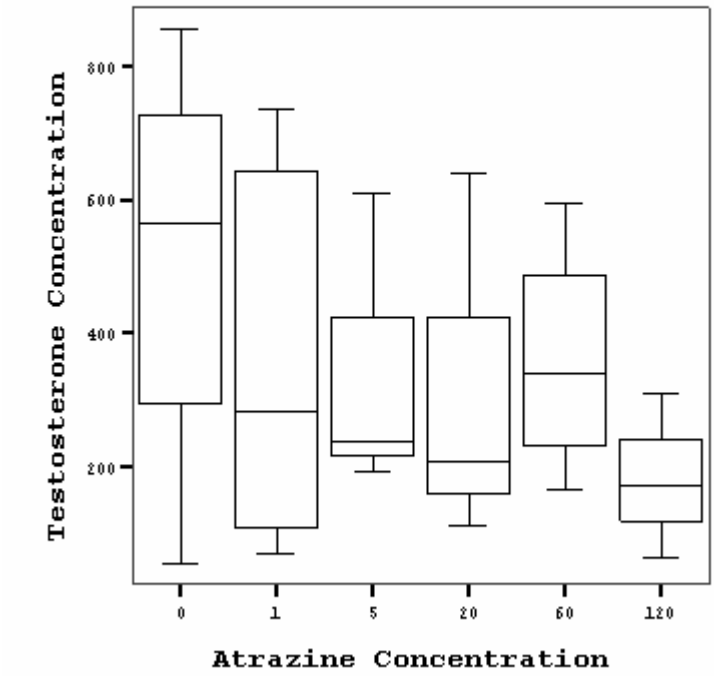


Figure 5.16. Male whole body testosterone
A, testosterone detected at metamorphosis among larvae by increasing atrazine concentration. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses as a fraction of total frogs assayed for hormone levels.

A.

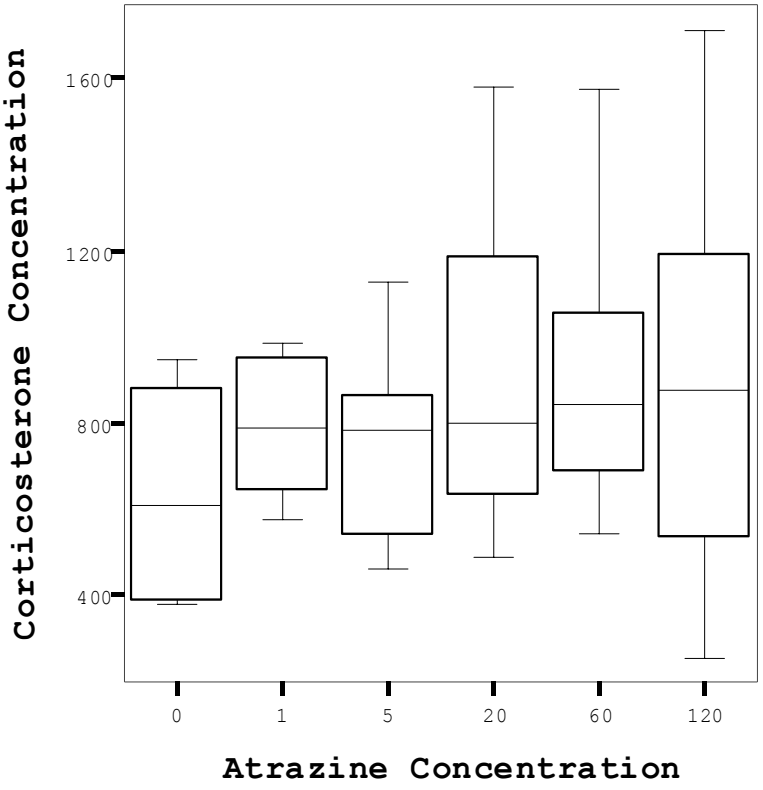


B.

Atrazine (ppb)	Testosterone (ng/g)	SD
0 (4/6)	509.89	± 333.85
1 (5/8)	367.56	± 305.81
5 (3/5)	347.57	± 228.41
20 (3/9)	319.99	± 281.11
60 (4/9)	360.19	± 179.91
120 (3/7)	182.42	± 122.59

Figure 5.17. Whole body corticosterone levels
A, corticosterone detected at metamorphosis among larvae by increasing atrazine concentration. Lines in boxplots represent the 50th percentile, or median, of the data. One-way analysis of variance with Bonferroni correction was used to test for significant differences between means. B, raw numerical data of means where SD is standard deviation and number of frogs included in each analysis is in parentheses.

A.



B.

Atrazine (ppb)	Corticosterone (ng/g)	SD
0 (6)	635.60	± 247.81
1 (7)	882.43	± 363.25
5 (5)	754.70	± 265.57
20 (9)	960.52	± 423.54
60 (9)	900.06	± 351.43
120 (7)	862.48	± 545.71

Figure 5.18. Corticosterone levels by body weight
Corticosterone levels and body weight plotted for each individual frog.

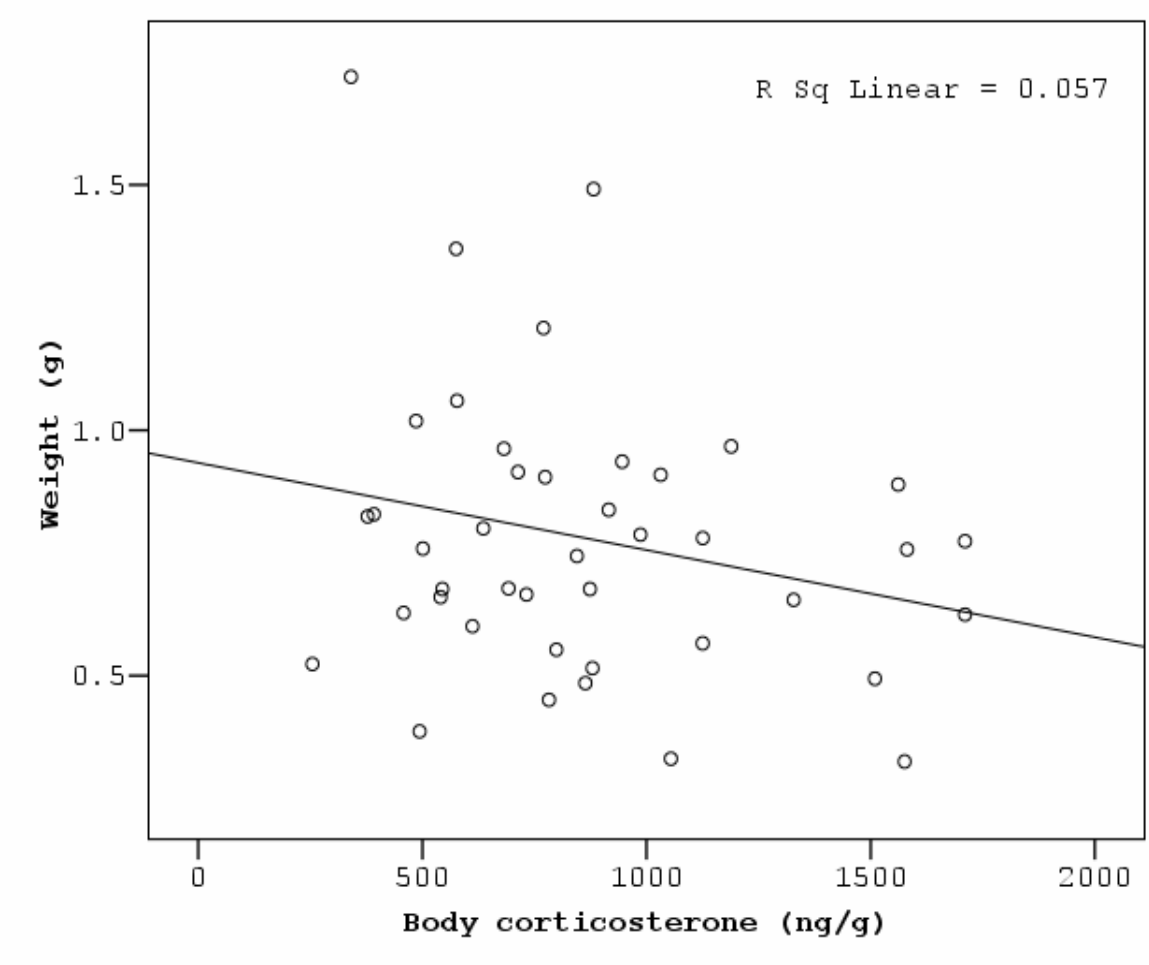
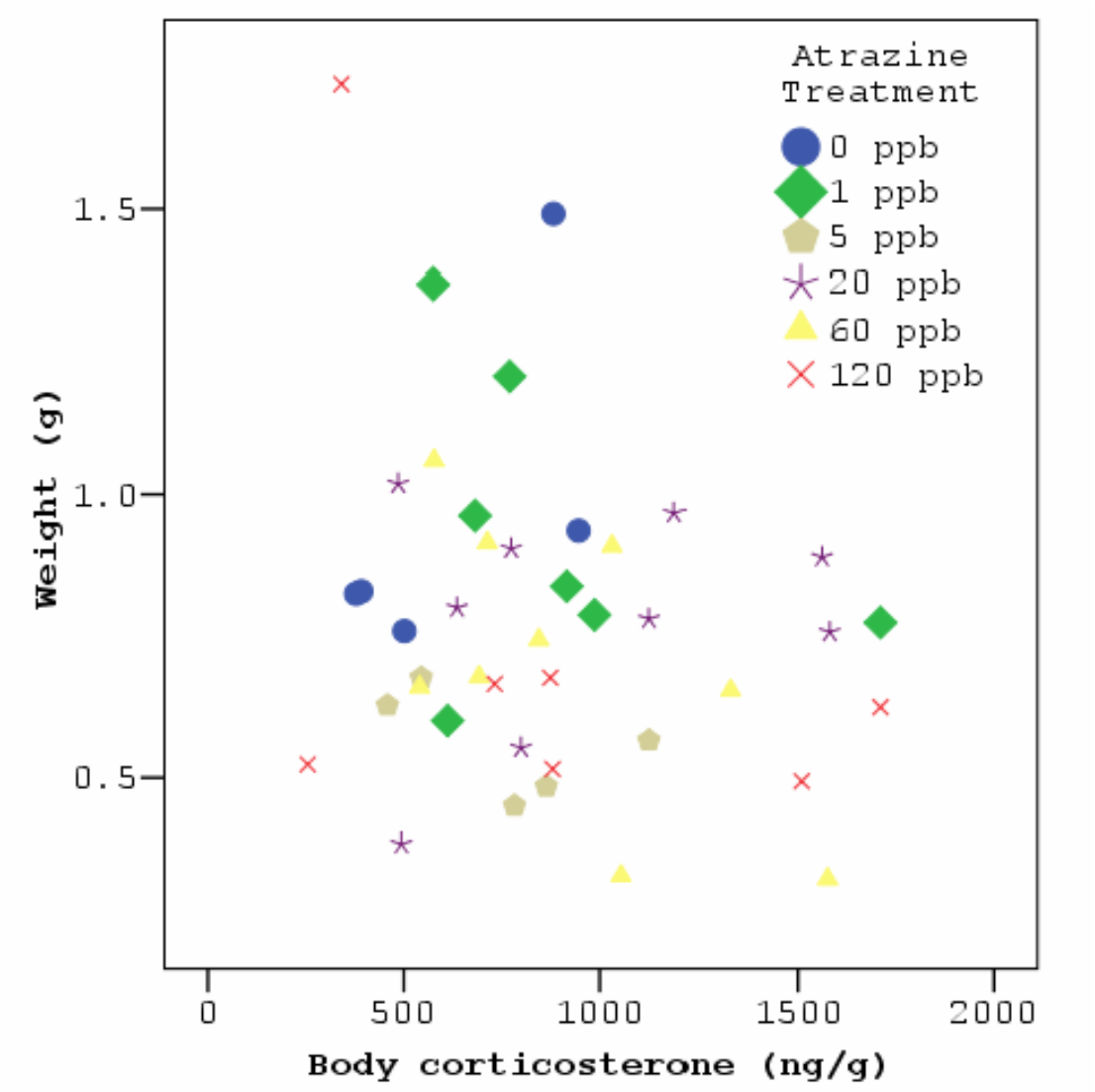


Figure 5.19. Corticosterone levels by weight and treatment Corticosterone levels and body weight plotted for each individual frog for each treatment group.



Conclusions
Chapter 6

We undertook a comprehensive study utilizing both field and laboratory experimentation to determine the impact of environmental atrazine contamination on amphibians. Our overall hypothesis was that atrazine exposure in anuran amphibians alters reproductive and survival capabilities. In the field component of the study, we evaluated variations in secondary sexual characteristics, disruptions of the gonads and other tissue structures, alterations in plasma testosterone levels, changes in parasitism, malformations and inflammation, and shifts in blood components. These endpoints were assessed in the native New Jersey species bullfrog (*Rana catesbeiana*) and green frog (*Rana clamitans melanota*) captured from agricultural sites with various levels of pesticides as determined by pesticide use histories and water analyses. In addition, to more clearly identify the role of atrazine in the pathogenesis of field-observed developmental effects, we exposed *Xenopus laevis* (African clawed frog) tadpoles to a wide range of environmental atrazine concentrations (0, 1, 5, 20, 60, 120 ppb) during their entire larval period. During this time, we

recorded survival and growth patterns, whole body testosterone and corticosterone levels, and gonadal morphology and histology.

Study Findings

Display of secondary sex traits (Chapter 2)

Quantification of the sexing technique utilized in the field revealed a convergence of eye-to-ear ratios between bullfrog males and females as site chemical contamination increased. This trend could result from at least two causes: (1) a higher percentage of immature, or sub-adult, animals were captured from sites with greater chemical contamination compared to sites with less contamination, and/or (2) chemical contamination present at collection sites delays, or perhaps disrupts, presentation of male secondary sexual characteristics in exposed animals. Because bullfrog body weight and snout-vent length were comparable across levels of contamination, the latter cause seems more probable. Secondary sex traits are used to display male sexual maturity and reproductive fitness to reproductively mature females for breeding. The absence of these traits may reflect poor reproductive ability, due to either a delay in sexual maturity or failure to achieve sexual maturity. Consequently, populations containing mostly males deficient in these characteristics will experience reduced breeding success and may undergo decline.

Disruption of reproductive development (Chapter 3)

Our field analyses indicate an endocrine disruptive effect on populations of bullfrogs (*Rana catesbeiana*) and green frogs (*Rana clamitans melanota*). Both species presented with reduced testicular weights (as normalized to kidney weight), and increased prevalence of dysgenesis of the kidney and testes. However, responses were site- and species-specific. Reduced testicular weight among bullfrogs occurred at high contamination sites and mostly correlated with atrazine exposure. In green frogs, decreased testicular weights were at medium contamination sites impacted with the herbicide metolachlor.

At the histological level, several abnormalities were observed in the kidney and testes. The renal dysgenesis and pathologies found (sperm aggregation, tubular degeneration, nephrocalcinosis, encysted tubules and protein inclusion bodies) was mainly observed in green frogs at sites characterized as medium contamination. This increased prevalence of renal dysgenesis correlates with the depressed testicular weights among green frog males also at medium sites.

Similarly, the frequency of testicular injury (retarded development, hyperplasia, dysplasia, edema and calcification)

was also highest at sites of medium contamination levels. However, the majority of testicular injury cases occurred in bullfrogs, rather than in green frogs. Since development of the testes occurs along with the kidneys and the two organs share the same mesodermal tissue, there may be some level of interdependence or similar sensitivity to hormonal influence.

In addition, since this pattern of pathology is at medium contaminated sites, the prominent pesticide detected, metolachlor, may be playing a role. This hypothesis is supported by reproductive effects observed in rats, which when exposed to metolachlor underwent testicular atrophy (EXTOXNET 1996). There also may be species-specific organ sensitivity, since renal dysgenesis was prominent in green frogs, while testicular dysgenesis was observed in bullfrogs. Because effects were not detected in the liver between sites, there is no evidence of general organodysgenesis with respect to this pattern of environmental contamination.

The testicular dysgenesis revealed differential sensitivity to estrogenic versus antiandrogenic effects between the two species. Green frogs presented with testicular oogenesis in three of our sites, two of which were characterized as high contamination (containing methoxychlor, metolachlor and

atrazine). The formation of ovotestes indicates feminization due to an estrogenic influence. Feminization also supports the dysgenesis of the kidney, which may result from estrogen-mediated pathology. On the other hand, bullfrog testicular analysis yielded retarded development as evidenced by the absence of active spermatogenesis. This pathology is a result of demasculinization, or the loss of androgenic influence required for proper testicular function. These different responses from the two species indicate that both estrogenic and antiandrogenic agents may exist in their environment. However, each species appears to be preferentially sensitive to one, and not the other.

Our field data support the hypothesis that endocrine disruption is experienced by frog populations resident in habitats with agricultural contamination. However, our observations could not substantiate atrazine as the source of the disruption. Instead, evidence of endocrine disruption best correlated with metolachlor use and exposure.

Parasites, malformations, and blood components (Chapter 4)

Changes in parasitic frequencies were dependent on the specific tissue. Renal parasites increased, while brain metacercarial cysts declined with level of contamination. Parasite ecology is

complex and many factors influence increases and/or decreases in parasitic prevalence. These factors include impacts on the health of the hosts and the parasites themselves. The percentage of malformed frogs was slightly above background (4.3% versus 0-2%, Read and Tyler 1994) and may be associated with the low pH (4.0-5.0) of our ponds. Prevalence of inflammation was also tissue-specific, which was highest in hepatic tissues at medium-contaminated sites and in renal tissues at high-contaminated sites. Again, a myriad of environmental factors influence immune function and have varying effects on this system. Hematocrit was reduced among female bullfrogs in high contamination in contrast to both low and medium contamination. Literature exist describing atrazine's effects on red blood cell count (Prasad et al. 1991; Hussein et al. 1996) and this property may be shared by other agro-chemicals present in pond waters.

Low-dose response to atrazine (Chapter 5)

In our laboratory-controlled experiments with *X. laevis*, many of our measured endpoints (survival and growth parameters) presented with non-monotonic responses. Though this type of response pattern is unusual, it supports previous studies that also found the greatest impact on survival at 3 - 4 ppb (lowest doses tested) of atrazine (Storrs and Kiesecker 2004; Rohr et

al. 2006). Spinal and limb malformations were unexpected and deformities presented at onset of metamorphosis. At this time hormonal levels, especially thyroidal and adrenal hormones, reach critical levels and the animal begins to undergo major structural and biochemical changes. This transformation is a stressful time for the animal with already elevated hormone levels, and additional overlapping burdens can be especially detrimental. The malformations observed at 5 and 20 ppb atrazine that were severe resulted in the death of the affected tadpoles and appear to have been the major source of reduced survivorship in the 5 ppb treatment. Mild malformations allowed tadpoles to complete metamorphosis; however, animals displaying limb and spinal deformities would most likely not survive long in natural settings. Such animals would be unable to escape predation or effectively compete for resources.

The effect of atrazine exposure on testicular tissues was dramatic. Even at our lowest dose tested, 1 ppb atrazine, abnormal gross structure (elongated testes) and histology (small, underdeveloped testes) were observed in metamorphs. This low effective concentration is well within environmentally relevant doses of atrazine and supports hypotheses regarding the herbicide's ability to negatively impact amphibian reproduction. In addition, our hormone analyses showed trends whereby

testosterone was reduced with atrazine exposure and corticosterone was elevated with treatment. These changes in hormone may provide clues to the mechanisms of atrazine action. Overall, our studies suggest that current environmental levels of atrazine are capable of adversely affecting wild amphibian populations by altering endocrine processes.

Male sensitivity to endocrine disruptors

Reports from the literature, as well as the data presented in this dissertation, agree that endocrine disruptive effects are more pronounced in males than females. One possible explanation is sex-specific energy allocation. Up to 70% of a female frog's body weight is dedicated to primary reproductive function (Casper and Hendricks 2006). A decrease in energy availability (due to either decreased energy assimilation or shifts in energy utilization; e.g., due to increased metabolic demand for protein production related to increased stress) would decrease energy for reproductive function (what is remaining after homeostatic and other necessary functions). However, this decrease in available energy would need to be considerable before an effect on reproductive function would be seen in the female, since energy requirements are few. In the male, however, energy demands for reproductive-related activities are far more numerous. In addition to the primary testicular action of

spermatogenesis and maintenance of related structures and functions, the male frog must also maintain secondary sex traits. These characteristics include coloration of the throat, an enlarged tympanic membrane, a large larynx able to produce advertisement calls, as well as the ability to maintain and defend breeding territory from intruder males. For males, a reduction in energy availability limits energy to be used for all the aforementioned functions, not just primary sexual function. Consequently, decreased energy input has a larger impact in males due to the reproductive strategies of these two studied species.

Another possible explanation for enhanced male sensitivity is the male's dependence on androgens for its male-specific traits. These traits are sensitive to the ratio of androgen to estrogens. Changes in relative levels of sex hormones can have drastic consequences in males, as their tissues are extraordinarily sensitive. In females, large changes in estradiol need to occur before significant alterations result in relative ratios since estradiol content is already much higher than testosterone.

The cause of these sex-specific effects may also be due to sexual dimorphism in water absorption. Males of the Japanese

tree frog (*Hyla arborea japonica*) were shown to absorb more water than female frogs (Iguchi et al. 2001). By absorbing more water, males receive greater exposure to water-borne chemicals than females and may therefore be more susceptible to effects.

Implications of multiple effects

Atrazine has been shown in the literature and in this study to have multiple effects: demasculinization and feminization of male gonads, lowered hematocrit, hypophagia, reduced survival, reductions in growth parameters, increased prevalence of malformations, reduced laryngeal size and depressed testosterone levels. These multiple effects may come from multiple modes of action. It has been shown that atrazine interacts with the androgen binding protein (ABP, Danzo 1997), competitively inhibits phosphodiesterases (Roberge et al. 2004), induces aromatase (Sanderson et al. 2000; Sanderson et al. 2001) and causes methemoglobin (Allran and Karasov 2001). With the above variety of effects, there may be additional mechanisms of atrazine action that contribute to the varied responses. In addition, these effects occur at different concentrations, some following a monotonic response and others not. With most mechanisms, there is a threshold above which a response is elicited. However, in other modes of action, particularly hormonal action, a window exists within which a response will be

seen; concentrations either below or above this window of response will not elicit an effect. This window of vulnerability exists because hormonal systems are already within an optimally responsive range and any change in hormone level results in a change in response. Hormones also exist within feedback systems. When a critical change in free hormone occurs, feedback loops act to correct the circulating levels by attempting to bring them back to homeostasis. These feedback mechanisms can overcompensate and cause an overshoot effect whereby an opposing response is observed over time. With these mechanisms, it is possible to have varying, even opposing, effects from a single chemical.

Environmental estrogen impact on human health

One indication of our results is that there is an increased input of estrogenic compounds to the environment. This input has implications for human health as well as wildlife. Moreover, varied and increased responses to low doses of estrogenic compounds have been previously documented. Male reproductive health in humans has been reported to be in decline over the last several decades and this deterioration is associated with exposure to hormonally active (mostly estrogenic, some androgenic) environmental chemicals (Jensen et al. 1995; Toppari et al. 1996; Sonnenschein and Soto 1998). The

effects include reduced sperm counts and increased male reproductive disorders like infertility (Sharpe and Skakkebaek 1993; Akingbemi and Hardy 2001; Takai et al. 2001; Honma et al. 2002). Bisphenol A (BPA) is 2,2-(4,4-dihydroxydephenyl) propane used in the production of polycarbonate plastics and epoxy resins (Staples et al. 1998). Human exposure occurs via BPA's presence in food can linings and dental sealants (Farabollini et al. 1999; Honma et al. 2002). BPA exposure has been found to have direct action on the male reproductive tract. After an eight-week exposure to orally administered 5-50 ppb BPA, testosterone levels in mice were depressed over 15-fold and histologic changes in the testes included multinucleated giant cells (Takao et al. 1999). Nagel et al. (1997) found 20 ppb BPA to increase prostate weight in mice compared to controls.

Reproductive effects due to BPA exposure are not limited to males. Rates of earlier onset of puberty in females and breast cancer have also increased over the last 50 years, matching the increased prevalence of male reproductive anomalies (Markey et al. 2001). Mouse exposure to 2.4 ppb BPA ***in utero*** resulted in an accelerated time course between vaginal opening and first estrus (Howdeshell et al. 1999). Female mice showed increased rates of mammary gland ductal migration resulting in a greater number of ducts, terminal ducts, terminal end buds and alveolar

buds following *in utero* exposure to 25 and 250 ppb BPA compared to controls (Markey et al. 2001).

These low estrogenic doses also may have carcinogenic activity. Prenatal exposure of rats to 25 and 250 ppb BPA increased the percentage of fibroblastic cells and decreased the number of androgen receptor-positive cells in the ventral prostate, which enhanced the invasive potential of nascent tumors (Ramos et al. 2001). An increase in tumor prevalence is an example of a later effect resulting from early changes. Female mice treated *in utero* were heavier than control mice at weaning, though they were of similar weights at birth (Howdeshell et al. 1999). Takai et al. (2001) also found similar delayed effects on mouse postnatal development following prenatal exposure to 1 nM BPA.

Although BPA's low dose effects have been documented, higher doses have failed to elicit similar responses (Nagao et al. 1999; Masutomi et al. 2004). Indeed, an inverted-U response was presented for developmental rate of mouse embryos cultured with 100 pM to 100 µM BPA (Takai et al. 2000). Sakaue et al. (2001) found a U response pattern for sperm production in adult male rats orally dosed with 2 ppt to 200 ppm BPA.

Of concern is that BPA exposure levels, as measured in blood samples from pregnant women and placental blood, are within the range found to be toxic to reproductive organs of offspring in animal studies. In addition, there are numerous contaminants in the environment, to which humans are regularly exposed, that have similar patterns of effect. These contaminants include diethylstilbestrol (Newbold 1995), safrole (Borchert et al. 1973), methoxychlor (Cummings 1997), maleic hydrazide (Epstein and Mantel 1968), genistein (Nejaty et al. 2001) and vinclozolin (Uzumcu et al. 2004). Effects in the environment from hormonally active chemicals may reflect changes occurring in human health, or serve as sentinel indicators of present and/or future threats.

Suggested Future Directions

Significance of secondary sex trait disruption

It is unknown whether the changes in male secondary sexual characteristics (eye-to-ear ratio and coloration of the throat) observed in our studies extend to other secondary sexual traits. These traits include male vocalizations (made possible by larger larynges, Sassoon and Kelley 1986), male maintenance and defense of breeding territories, female perception of male secondary sex traits and sex-specific breeding behaviors (the male must clasp the female in an embrace known as amplexus and apply pressure to release eggs for fertilization). A long-term comprehensive

monitoring study needs to be carried out where the secondary sexual traits, breeding success and habitat characteristics of different populations can be evaluated. The goals would be to determine whether different populations have different rates of maturity, determine the correlation of secondary sex characteristics and uncover primary sexual output (i.e., do secondary sex traits reflect degree of gonadal development), and which environmental factors are associated with changes in maturity rates. Not only may chemical contamination play a role in sexual maturity and ability, but predators, competition, food sources and other environmental factors may have an influence.

Much work has been done to understand the hormonal controls in laryngeal sexual dimorphism. Male larynges are larger than those of females due to more muscle fiber numbers of a uniform size, higher metabolic activity (increased oxidative capacity) and more extensive innervations, all under androgen control with thyroid hormone influence (Sassoon and Kelley 1986; Sassoon et al. 1987; Kelley and Dennison 1990; Robertson et al. 1994; Boyd et al. 1999). This understanding needs to extend to the other secondary sexual traits. Whether these dimorphisms arise from differences in response to external stimuli due to distinctive endocrine content or from sex-specific sensory, central nervous system or motor components that produce these traits is unknown,

but will aid in understanding potential disruptions therein (Kelley 1988).

Gonadal dysgenesis and function

We observed varying degrees of testicular dysgenesis; however, it is unknown whether these histological changes are functionally relevant. The degree of function that can be lost (either due to decreased spermatogenesis or testicular volume), before effects on reproduction are seen needs to be determined. In the laboratory, how much spermatogenetic activity can be lost before fertilization rates are affected can be studied with the use of progressive demasculinization. These dysfunctions may lead to population declines.

Monitoring studies may also reveal consequences of species-specific sensitivities. In an ecological community, one effect of environmental changes may be shifts in species dominance as one species succumbs to stressors not apparent in another.

Additional field studies

We initially had two additional sites (Longstreet I and II) that incurred heavy atrazine input, as both sites are surrounded by sweet corn upon which atrazine is used (see Figure 6.1 for site characteristics). These sites were eliminated from the study

due to the lack of usable frog populations. The reason for the lack of frogs is unknown, but it would be interesting to determine if frogs could become established at these sites. If frogs are successfully transplanted to these ponds and are able to sustain a population through breeding, then it would appear that lack of frog populations was due to opportunity and accessibility issues. This cause seems unlikely, especially since snapping turtles and other wildlife use these ponds as habitat. Another possibility is the site substrate is toxic to sub-adults, which try to establish themselves following dispersal events from other sites. Alternatively, frogs may be able to utilize this habitat, but cannot successfully breed. This is the apparent case at Rahilly. In the Rahilly pond, bullfrogs are present, but there is no active breeding. The single female green frog captured at this site and the single male green frog observed were the only two of their species observed at Rahilly over the three-year study period. Therefore, it would also be informative to see if green frogs could be established at this pond, especially since it incurs a larger input of metolachlor (92.1 ppb, Chapter 3) than Robson West (17.4 ppb), where green frog males experienced the greatest reduction in testicular weight. Would this larger input of the pesticide be enough to cause population collapse of a green frog population? Similarly, are high levels of atrazine at the

Longstreet sites sufficient to prevent individual establishment, possibly through lethal effects?

Mesocosm studies

Chemical contamination effects need to be studied in the context of the animal's habitat in order to reveal real-world consequences of exposures. Do the added stressors of predation, competition, food sources and other environmental characteristics affect an animal's response to chemical contamination and vice-versa? These ecological interactions can be best revealed in field mesocosm studies, where inputs to the system can be controlled and impacts studied under approximately natural conditions.

Normative data for background parasitism, malformation frequencies and hematological parameters need to be developed for both species in the mid-Atlantic region. Parasites utilizing amphibians as intermediate hosts need to be identified and an atlas generated to aid in identification of these parasites in tissue sections. In addition, studies need to be conducted to better understand environmental interactions and their impacts on both parasites and their hosts. Studies of pH effects on developmental rates have been carried out (Freda and Dunson 1984; Cassano et al. 2006). This body of research on low

pH should be expanded to include its potential role in gross malformations and organogenesis.

Repeat of laboratory study

Because our laboratory study with *X. laevis* only included one replicate and our results did not follow traditional monotonic patterns, we recommend that this study be repeated, with the considerations below, to confirm findings. Our studies only examined testosterone and corticosterone levels after metamorphosis. Since skeletal defects became apparent at onset of metamorphosis, testosterone and corticosterone levels following metamorphosis may bear little relevance to effects initiated at metamorphosis. Therefore, the time courses of the thyroid hormones (T_3 and T_4), testosterone, corticosterone and estradiol levels need to be determined prior, during and after metamorphosis. Marked changes may occur due to treatment in these hormones just prior and/or during metamorphosis with levels coming down to baseline afterwards. Although differences in testosterone and corticosterone levels were not significantly different after metamorphosis, the changes in hormone levels prior and during metamorphosis may be large enough to result in statistically significant differences between treatment groups. This sampling regime will also detect changes in estradiol and whether or not its levels increase in accordance with decreased

testosterone levels. Such a finding would support atrazine's proposed mechanism of action: induction of aromatase. Monitoring T_3 and T_4 levels may reveal the interactions between the hormones. In addition to testing in *Xenopus*, these studies could be carried out in *Hyla femoralis* (pine woods treefrog) or *H. squirella* (squirrel treefrog), which both have genetically identifiable sex; that is, their sex can be confirmed by cytology (Berset-Brändli et al. 2006). In this manner, hormone levels can be determined knowing the sex of the animal, allowing sex-specific comparisons to be performed.

Other laboratory-based studies

With a study utilizing *Xenopus*, it would be informative to perform polymerase chain reaction (PCR) and/or microarray analysis to determine changes in aromatase transcripts and/or induction of the aromatase gene. In doing so, it is important to account for different aromatase isoforms. The aromatase enzyme of the gonads may be different from that of the brain. Studies by Mills et al. (2006) have shown induction of the brain isoform of aromatase in response to atrazine treatment in the fish cunner (*Tautogolabrus adspersus*) with no change in gonadal aromatase activity.

Retarded testicular development was observed at our lowest atrazine concentration tested, 1 ppb. This study needs to be repeated in order to explore the even lower limits of atrazine toxicity. With this as the goal, sub-parts per billion concentrations would be utilized to determine the lowest observable adverse effect level for retarded testicular development due to atrazine exposure. The endocrine potential and mechanism(s) of action of metolachlor toxicity need to be explored under controlled conditions to determine if effects found in the field can be reproduced in the laboratory. Furthermore, the mechanism behind agro-contaminant interference with oxygen carrying capacity and formation of methemoglobin needs to be elucidated (Prasad et al. 1991; Mencoboni et al. 1992; Hussein et al. 1996; Allran and Karasov 2000).

Long-term effects. Few data exist for evaluating the long-term impacts of chemicals like atrazine, though exposure in early development could lead to permanent and irreversible effects. Such data would only be obtained from comprehensive full-life cycle experiments (Sohoni et al. 2001). Following exposure to 21 μg/L atrazine for 48 hours during sexual differentiation, *Xenopus laevis* tadpoles presented with impairment in both testicular and ovarian development (Tavera-Mendoza et al. 2002a, 2002b). In females, there was a 13% increase in secondary

oogonia, with a 30% decrease in primary oogonia over control animals, indicating delay in maturation of the ovary. In addition, atresia in both secondary and primary oogonia increased by 18% compared to controls (Tavera-Mendoza et al. 2002b). These atretic cells represent germ cells that failed to complete development, which were subsequently resorbed by the tissue. In males there was a 57% decrease observed in testicular volume, a 70% decline in primary spermatogonial cell nests, and a 74% decline in nursing cells compared to controls (Tavera-Mendoza et al. 2002a). Testicular resorption and/or aplastic development were detected in 80% of the atrazine-exposed larvae. Based on these data, Tavera-Mendoza et al. (2002a; 2002b) proposed that the effects observed in both sexes of *Xenopus laevis* following atrazine exposure of larvae were permanent because the primary sources of germ cells had been compromised for the life of the animal. This transformed state would significantly reduce the animal's capacity to reproduce. These individual effects can potentially impact population survival if animals fail to sexually mature normally. In contrast, however, Denver (1998) and Hayes et al. (2003) hypothesize that the plasticity displayed in the amphibian larval period gives amphibians the ability to metamorph early and remove themselves from unfavorable conditions. This plasticity would act as a form of behavioral resistance to

environmental contamination, allowing the animals to delay sexual differentiation until after metamorphosis when they are able to escape contaminated environments and encounter more favorable habitat.

To study the role of plasticity on the long-term effects of atrazine exposure, two sets of *Xenopus laevis* would be reared in the laboratory at an effective atrazine concentration for retarded gonadal development. One group would be exposed to atrazine during its entire larval period, while the second group would serve as control (follow Figure 6.2). At the end of the treatment period, one-third of the juveniles from each group would be sacrificed for analysis of endocrine disruption effects via histological examination and hormone analyses. The remaining frogs would then be placed into new treatment groups (one-third of original group) to allow development into adults. These secondary treatment groups would be generated in the following manner. The remaining amphibians from the control group would be divided so that half are placed into tanks with atrazine and half into tanks without atrazine. The same procedure would occur for the remaining amphibians exposed to atrazine during their larval period; half would continue to be exposed to atrazine, and half would no longer receive atrazine treatment. Following treatment, all animals would be analyzed

for reproductive effects. These endpoints could include gonadal structure, hormone levels, and reproductive output and behavior. In addition, effects on egg develop and viability with only ***in ovo*** exposures could be observed to test maternal transfer of effects.

Such a study could potentially answer several questions. From the animals exposed to atrazine only as larvae, we would be able to determine if amphibians are able to recover following cessation of exposure and to what extent. Can gonadal function recover and catch up following a delay in development or maturity once proper hormonal cues are restored, if they are restored, or is gonadal dysgenesis an irreversible permanent affect that persists into adulthood? There may also be carryover effects. In streamside salamanders (***Ambystoma barbouri***) exposed to atrazine as embryos and larvae, there was increased mortality due to enhance dessication eight months after treatment (Rohr et al. 2006). Conversely, if organisms exposed to atrazine as larvae are able to recover, even with continued exposure as adults, this would indicate that amphibians are solely susceptible to atrazine during the larval period and can recover after metamorphosis, possibly even in the continued presence of this chemical stressor. This would be an important finding suggesting that atrazine's effects are

transient. The herbicide may not have to encounter the postmetamorphic animal, in order to harm it. From the animals exposed only as adults, we can assess the animal's sensitivity to atrazine exposure after metamorphosis. According to Hayes et al. (2002b), adult frogs are still susceptible to atrazine. The researchers found that 25 ppb atrazine was sufficient to lower testosterone levels in adult male *Xenopus laevis*. However, in a study by Iguchi et al. (2001), effects in *Xenopus laevis* due to estradiol treatment (malformations, crooked vertebrae, depressed organogenesis, smaller heads and larger abdomens) were only observed if treatment occurred prior to Gosner larval stage 39 (onset of metamorphosis). These important questions need to be evaluated in order to address the environmental costs of atrazine use and managing strategies for population regulation.

Role of Aromatase. The mechanism of action leading to endocrine disruption by atrazine seems to be the induction of aromatase, leading to a disturbance in steroidogenesis (Hayes et al. 2002b). Elevated aromatase expression would increase local levels of estrogens (Sanderson et al. 2000), which have a variety of actions, including action as a sex hormone, modulation of transmembrane receptor function and affects on intracellular transduction cascades (Moosmann and Behl 1999). These elevated levels of estrogen are at the expense of androgen levels and may potentially cause estrogen-mediated pathologies as ratios become skewed. These pathologies include disruption of early cell interactions within the gonad and other tissues (Villalpando and Merchant-Larios 1990; Newbold 1995; Metcalfe et al. 2000; Schönfelder et al. 2002b). As a result, proper testicular differentiation may be prevented.

The ability of atrazine to induce aromatase needs to be studied. If atrazine acts through induction of aromatase, one would expect a similar profile of gonadal effects following the use of an aromatase inducer, like 8-bromo-cyclic adenosine monophosphate (B-cAMP). B-cAMP is an ideal aromatase inducer because it acts via the Protein Kinase A (PKA) pathway, the suspected mechanism of action for aromatase induction by atrazine (Sanderson et al. 2000; Sanderson et al. 2001). To further demonstrate atrazine's action through aromatase, an aromatase inhibitor can be utilized to block atrazine effects on the gonads. An aromatase inhibitor that acts as a suicide substrate is 4-hydroxy androstenedione (4-OHA), providing 95-98% irreversible inhibition (Sanderson et al. 2000; Kao et al. 2001). Parallel treatments of larval stage amphibians (*X. laevis*) could be set up in a 2x3 factorial design (Table 6.1): treatment (control or atrazine) and aromatase substrate (none, B-cAMP or 4-OHA).

The expected outcomes of these different treatments are as follows (Table 6.1). We do not expect to see any pathology in the gonads of animals in the control group and these animals would therefore serve as reference. The male tadpoles exposed to B-cAMP should experience decreased testosterone levels, with a concomitant increase in estradiol over control, possibly

leading to ovarian development, or an intersex state. The female tadpoles in this treatment may experience atresia of the ovaries or increased frequency of secondary oocytes with increased estradiol levels (Tavera-Mendoza et al. 2002b). This effect would reveal an estrogen-mediated pathology. Male tadpoles in the 4-OHA treatment may display over-development of testicular tissues or be relatively unaffected. The female tadpoles exposed should experience increased testosterone levels, with a concomitant decrease in estradiol; thereby developing testes (Yu et al. 1993; Crain et al. 1997), as treatment with an aromatase inhibitor can induce sex reversal to a male phenotype (Miyata and Kubo 2000). Treatment with an aromatase inhibitor may also lead to intersexed gonads if inhibition is incomplete, as occurred in leopard frogs (*Rana pipiens*) exposed to the aromatase inhibitor, flavone, where oocytes were found displaying onset of vitellogenic growth despite surrounding testicular tissues (Mackenzie et al. 2003).

The male tadpoles exposed to atrazine alone should experience decreased testosterone levels with a concomitant increase in estradiol and thereby develop ovaries (Reeder et al. 1998; Hayes et al. 2002b; Hayes et al. 2003). In addition, males may also display underdeveloped testes with reduced testicular volume, decreased primary spermatogonia as well as reduction of nursing

cells as observed in our studies and by others (Tavera-Mendoza et al. 2002a). The female counterparts may develop atresia of the ovaries due to the increased circulating estradiol (Tavera-Mendoza et al. 2002b), an estrogen-mediated pathology. If the outcome of this treatment is similar to that for B-cAMP alone, this provides evidence of atrazine inducing aromatase via the same pathway as B-cAMP; that is, the PKA pathway. In the case of atrazine plus B-cAMP treatment, the males should experience lower testosterone levels than atrazine alone and the effects should be similar but with potentially greater severity. Likewise, the females of this cohort may experience greater atresia of ovarian tissues than those in the atrazine or B-cAMP treatments alone. For the atrazine and 4-OHA treatments, one should see a balance of effects, much like the control treatment, which would indicate that atrazine induces aromatase via the same pathway as B-cAMP, the PKA pathway. This study may also provide evidence that atrazine does not directly, or at all, bind to aromatase but to an endogenous inducer or inhibitor. For instance, aromatase induction may be a general response to atrazine exposure and not solely occur in aromatase expressive tissues (Sanderson et al. 2000). This may be an indication that atrazine is able to affect gene expression. We may want to use a CYP 1A2 (the predominant enzyme in atrazine metabolism) blocker, like α-napthoflavone, so atrazine is not

metabolized, and therefore, resides in the animal longer, potentially producing greater effects (Fort et al. 2000).

The studies proposed above would serve to support our findings and others that atrazine has the ability to alter the reproduction and survivability of frogs through endocrine processes.

Figure 6.1. Longstreet I and II ponds
Satellite images of Longstreet I and II sites, located off of route 528 (Chesterfield-Jacobstown Road) between Chesterfield-Arneytown and Harrison Roads. Each pond is approximately 23 x 90.5 yards^2. All satellite pictures were obtained from Google Earth (image: ©2007 State of New Jersey; ©2007 Tele Atlas). Marker = 0.04 km.

Figure 6.2. Exposure paradigm for long-term study on persistent atrazine effects

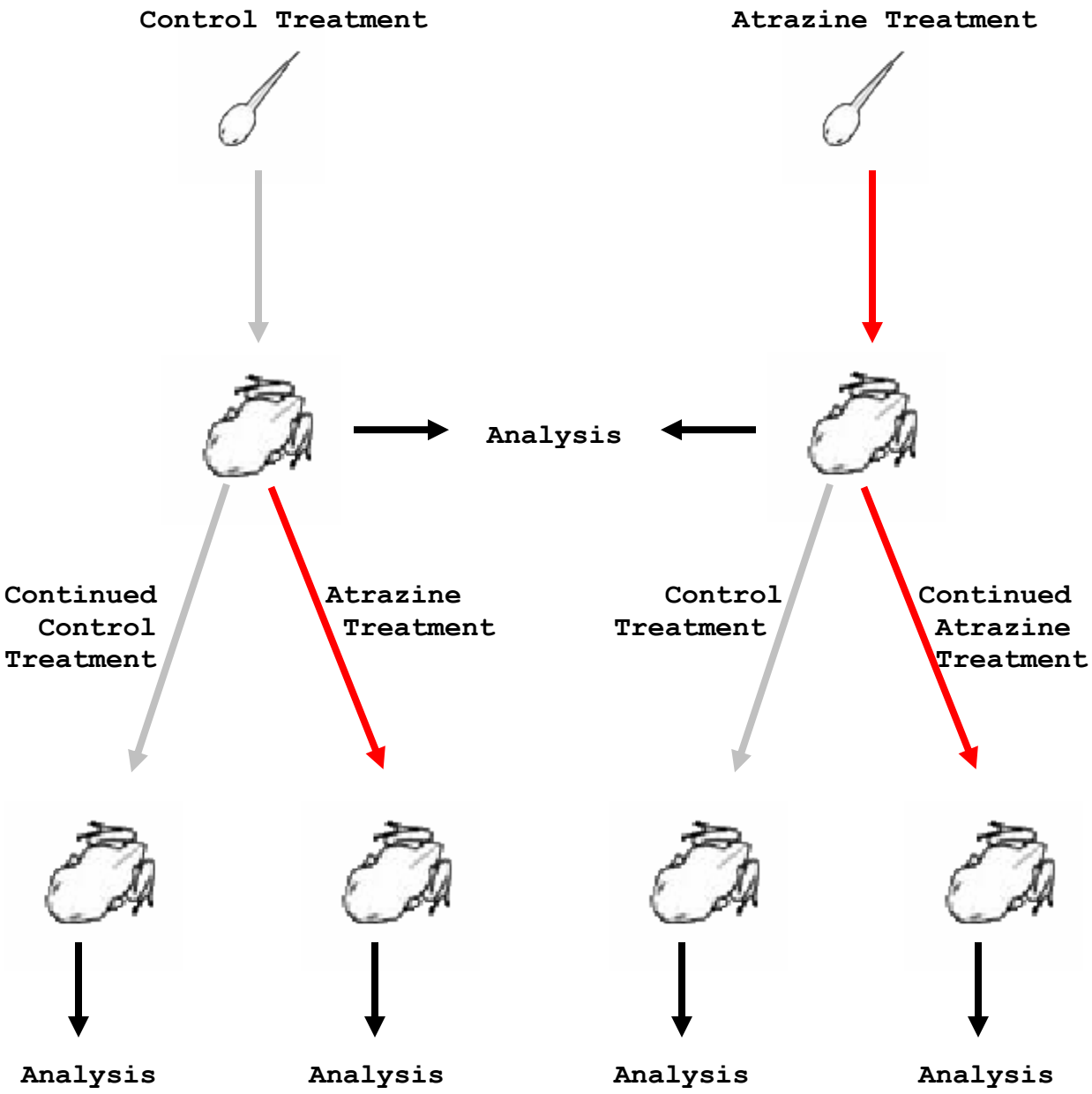


Table 6.1. Aromatase experiment 2x3 factorial design
Expected outcomes if atrazine induces aromatase through the PKA pathway.

		B-cAMP	**4-OHA**
Control			
Males	normal	T → E_2 ovarian tissue	↑ T
Females	normal	↑ E_2 ovarian atresia	T ¬ E_2 testicular tissue
Atrazine			
Males	T → E_2 ovarian tissue	T → E_2 ↑ ovarian tissue	normal
Females	↑ E_2 ovarian atresia	↑ E_2 ↑ ovarian atresia	normal

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Curriculum Vita

Marisol Maria Gutierrez

Education

1994 - 2000 B.A., Biology; University of Delaware, Newark, DE.

2000 – 2007 Ph.D., Environmental Toxicology; Rutgers University, New Brunswick, NJ.

Occupations and Positions

2000 – 2005 National Institute of Environmental Health Sciences Graduate Fellow, Joint Graduate Program in Toxicology; Rutgers University, NJ.

2001 – 2002 Supplemental Instruction Leader, General Biochemistry; Rutgers University, NJ.

2006 – 2007 Graduate Assistant, School of Pharmacy; Rutgers University, NJ.

Presentations

2007 Gutierrez, M. M., Ledoux, T., Cooper, K. R. and Robson, M. G. Frogs at risk: Evidence of endocrine disruption in wild New Jersey frogs captured from agricultural ponds. Poster presentation, 23rd Hudson-Delaware Chapter (HDC) of SETAC Annual Meeting, Stockton, NJ

2007 Gutierrez, M. M., Ledoux, T., Cooper, K. R. and Robson, M. G. Histological observations in native New Jersey frogs captured from agricultural ponds containing atrazine and other contaminants. Poster presentation, 46th SOT Annual Meeting, Charlotte, NC

2006 Gutierrez, M. M., Ledoux, T., Cooper, K. R. and Robson, M. G. Endocrine disruption of bullfrogs and green frogs by atrazine and other environmental contaminants in New Jersey. Poster presentation, 27th SETAC Annual Meeting, Montréal, Canada

2006 Gutierrez, M. M., Ledoux, T., Cooper, K. R. and Robson, M. G. Endocrine disruption by atrazine and other environmental contaminants on native New Jersey frogs. Poster presentation, 45th SOT Annual Meeting, San Diego, CA

2005 Gutierrez, M. M. and Robson, M. G. New Jersey frogs, atrazine and endocrine disruption: The initial findings. Platform presentation, 11th NAC/SETAC Annual Meeting, Burlington, VT

2005 Gutierrez, M. M., Ledoux, T., Cooper, K. R. and Robson, M. G. New Jersey frogs vs. atrazine: Round one. Poster presentation, 11th NAC/SETAC Annual Meeting, Burlington, VT