REPRODUCTIVE AND RENAL PATHOLOGIES IN THE GIANT TOAD (*Bufo marinus*) ARE ASSOCIATED WITH HUMAN LAND USE PRACTICES

By

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To Michael
Thank you for enduring the initial insanity and for celebrating the successes

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By

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Chair: Colette St. Mary
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Many chemicals used by humans (such as pesticides) are known to disrupt the endocrine system of wildlife and lead to reproductive abnormalities. Despite some notable laboratory studies, the potential effects of these chemicals under field conditions have not been well examined. Therefore I surveyed five populations of the giant toad (*Bufo marinus*) that varied in proximity to either agricultural or suburban areas, to evaluate whether this gradient of land use left a signature on their reproductive and renal biology. I demonstrate that male toads (*Bufo marinus*) from agricultural areas in south Florida are feminized (e.g., they have female color patterns), demasculinized (e.g., they have smaller forelimbs, and fewer nuptial pads—masculine traits), and are more likely to have intersex gonadal tissues (i.e. ovaries and testes in the same animal) than toads from less agricultural (more suburban) sites. Toads from agricultural habitats also had reduced spermatogenesis and testosterone levels relative to male toads from suburban habitats.

To develop hypotheses about the mechanisms that result in toads from agricultural areas having such dramatic reproductive abnormalities, I compared the expression levels of several genes in ovaries, testes, and intersex gonads using a cDNA array. Many ovarian genes were

expressed similarly between ovarian and intersex gonadal tissue, which is surprising because the intersex toads are most likely genetically male, and because on average intersex gonads were only 30% ovary by weight. I also developed potential biomarkers for the intersex condition and identified gene networks that could be important for normal (and abnormal) gonadal function.

Given the striking difference in gonadal abnormalities across sites, I also determined if toads from these sites had differences in toxin-induced renal disease. Renal pathologies associated with pollutant exposure occurred at all of the sites but tended to be increased at the most agricultural sites. Therefore, although both agricultural and suburban sites are polluted; the types of pollutants at agricultural sites are associated with severe reproductive abnormalities in addition to renal pathologies while those at suburban sites are not. My work has increased our understanding of how human-induced environmental alterations can harm wildlife.

CHAPTER 1
ENDOCRINE DISRUPTING CHEMICALS IN AMPHIBIANS

Introduction

Endocrine disrupting chemicals (EDCs) are ubiquitous pollutants and their presence in the environment has created an important and difficult issue in wildlife conservation (Colborn and Smolen, 1996). *Any* physiological, behavioural or developmental process that is controlled or influenced by the endocrine system is susceptible to modification by EDCs. In fact, EDCs are known to have dramatic effects on vertebrate development, physiology, and behaviour (Crews and McLachlan, 2006; Fox, 2001; Guillette and Iguchi, 2003, 2004; Hayes *et al.*, 2002 ; Milnes *et al.*, 2006). Therefore, EDCs can change the reproductive success of individuals, alter patterns of sexual selection, and reduce the long-term fitness and persistence of affected populations. Indeed, environmental contaminants, such as EDCs, could have further reaching and much more important effects on amphibians and other wildlife than is presently appreciated.

Amphibians are thought to be particularly susceptible to the effects of EDCs because they have permeable skin, often reproduce in areas that receive large amounts of run-off and can be in intimate contact with environmental contaminants in all stages of their life history. For example, recent studies have documented reproductive abnormalities in amphibians from agricultural areas where EDCs are used (Hayes *et al.*, 2002; Hayes *et al.*, 2003; Ouellet *et al.*, 1997; Russell *et al.*, 1995; see Chapter 5 this volume). In addition, because EDCs are distributed globally they have been hypothesized to contribute to the global decline of amphibian populations (Carey and Bryant, 1995; Hayes *et al.*, 2003). It is important to note, however, that relatively little is known concerning the role EDCs potentially play in amphibian population declines. This is an important question to be addressed via research at all levels of organization, including the molecular and cellular mechanistic levels, and individual, population, and community levels.

Although, to date, relatively little is known about the effects of EDCs on amphibians, there is substantial literature from other species, and a growing understanding of the endocrinology of amphibians from genes to individual physiological performance. Therefore, since many generalities have been observed, literature on these topics from other vertebrates and various ecological systems are used here to better understand the data currently available for amphibians specifically, and examples of endocrine disruption in amphibians are emphasized.

Brief Introduction to the Endocrine System

The endocrine system consists of ductless endocrine glands that secrete hormones directly into the bloodstream or into surrounding tissues. Endogenous hormones transmit information and regulate biological processes (Fig. 1-1). Lipophilic hormones, such as steroid hormones, are well suited for signaling because they diffuse readily from a source cell (or capillary) to a target cell, where they bind to specific protein receptors (Fig. 1-2). The binding of a steroid hormone to its receptor induces an allosteric transformation that enables the hormone-receptor complex to bind to high affinity sites in the DNA and modulate gene transcription (Mangelsdorf *et al.*, 1995; Yamamoto, 1985) (Figure 3). Steroid hormone receptors belong to a super-family of nuclear receptors that function as transcription factors, capable of altering the gene expression of thousands of genes (Terasaka *et al.*, 2004; Watanabe *et al.*, 2003). Therefore, hormones are secreted from an endocrine gland, and are transported through the body to regulate biological processes in 'distant' tissues by modulating gene expression. Lipophilic hormones, including the steroids, retinoids, and thyroid hormones, are important regulators of development, cellular differentiation and organ physiology in all vertebrates studied to date (Mangelsdorf *et al.*, 1995).

Indeed, most EDCs identified to date are known to influence gene expression by interacting with the steroid, thyroid, and retinoid super-family of receptors (Crews and McLachlan, 2006) (Fig. 1-2). In addition to interactions with hormone receptors, EDCs alter the

function of hormone degradation, carrier proteins, and key steroidogenic enzymes, all of which are crucial for normal gene expression and hormonal homeostasis (Guillette and Gunderson, 2001; Sanderson, 2006) (Fig. 1-1). Altered gene expression can lead to a variety of negative effects-- including abnormalities in the structure (via development and growth) and function of organs associated with hormonal signaling and regulation, or induction of cancers. EDCs are also known to influence gene expression epigenetically, which can induce transgenerational effects (Crews and McLachlan, 2006; Mukerjee, 2006).

Definition of Endocrine Disrupting Chemicals

Endocrine disrupting chemicals are defined differently by various entities; the nuances among definitions can have important implications for legislation and can influence the way in which research on EDCs is conducted. For example, the World Health Organization's (WHO) International Program on Chemical Safety (IPCS) recently organized and conducted a Global Assessment of the State-of-the-Science of Endocrine Disruptors (IPCS, 2002). The current definition of an endocrine disruptor according to that program is "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (IPCS, 2002; Sanderson, 2006). The European Commission's definition is similar to this but also includes a definition of a *potential* endocrine disruptor which acknowledges that consideration of a chemical's safety can be appropriate if that substance could *possibly* disrupt the endocrine system (European-Commission, 1997). However, the US EPA (http://www.epa.gov/scipoly/oscpendo/edsparchive/2-3attac.htm) defines an endocrine disruptor as "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development and/or behavior". The EPA explicitly states that the

agency "does not consider endocrine disruption to be an adverse effect per se, but rather to be a mode or mechanism of action potentially leading to other outcomes, for example carcinogenic, reproductive, or developmental effects…". These differences are important because the WHO's or European Commission's definition suggests that altered endocrine function can induce adverse health effects through any number of mechanisms, while the EPA's industry-friendly definition requires demonstration of a particular mechanism of toxicity that leads to adverse health effects beyond disruption of the endocrine system itself. Although it is clearly extremely informative to understand the exact mechanism of toxicity of a particular environmental pollutant and to identify relevant consequences of exposure, these details can, and do, take many years, and in some cases decades, to work out. One consequence is that many endocrine disrupting chemicals can remain in use for many years. In addition, this definition creates a situation where significant time, and research funding, is spent working out mechanisms of action, and less time is spent in the field understanding effects at the population level.

Endogenous hormones control a wide variety of living processes and are physiologically active at extremely low concentrations (in the pg/ml or ng/ml range). Consequently, pollutants that behave like endogenous hormones can induce effects at concentrations well below traditionally perceived 'toxic' levels. Traditional chemical evaluation and risk-assessment procedures were not designed to manage such an unexpected mode of action. As a result, current regulations on the use of EDCs were made mandatory only after significant environmental damage had already occurred (Matthiessen and Johnson, 2007).

For the purposes of this chapter, EDCs are defined by paraphrasing Crews and McLachlan, (2006) and Tabb and Bloomberg (2006). The resulting definition implies that there are multiple

mechanisms through which endocrine disruption can occur and a diversity of effects and consequences to exposure:

> Endocrine disrupting chemicals are a class of environmental pollutants that mimic or block transcriptional activation elicited by endogenous hormones such that they resemble (or alter) natural biological signals (hormones) and, thus, can be misinterpreted by the organism leading to abnormal gene expression and phenotype.

The Nature and Sources of Endocrine Disrupting Chemicals

Many endocrine disrupting chemicals are persistent either because they have a long half-life or because they are added to environmental systems at a rate faster than they break down or are metabolized. EDCs are also ubiquitous and many are distributed globally---even areas once believed to be pristine are affected by their run off or fallout. Once deposited, EDCs can biomagnify in food chains (Bjerregaard *et al.*, 2001; Goksoyr, 2006; Guillette *et al.*, 2006; Lie *et al.*, 2003; Nations and Hallberg, 1992; Skaare *et al.*, 2000; Thurman and Cromwell, 2000). For example, organochlorine pesticides (e.g. DDT) and industrial chemicals, such as polychlorinated biphenyls (PCBs), are found in high concentrations in arctic ecosystems. Skaare et al. (2000) showed that polar bears (*Ursus maritimus*) and glaucous gulls (*Larus hyperboreus*) from Svalbard, in the Arctic Ocean midway between Norway and the North Pole, contain high levels of PCBs. Polar bear PCB levels were greater than eight times higher than those found in ringed seals (*Phoca hispida*), an important polar bear prey (Severinsen *et al.*, 2000; Skaare *et al.*, 2000). Similarly, glaucous gull PCBs are up to three orders of magnitude higher than in crustaceans and fish which make up their major food items (Borga *et al.*, 2003; Skaare *et al.*, 2000).

Interestingly, there are sex differences in exposure to lipophilic environmental contaminants such as PCBs that are related to differences in excretory pathways. For example, egg yolk and milk are important, female-specific, excretory pathways for lipophilic compounds (Skaare *et al.*, 2000). The occurrence of high levels of PCBs and DDE (a metabolite of DDT) in

the eggs of the green frog (*Rana clamitans*) and spring peeper (*Pseudacris crucifer*) in southern Ontario, Canada, suggests that sex-specific exposure and excretory pathways occur in amphibians. Therefore, larvae are exposed to environmental as well as to maternally derived (via the yolk) concentrations of contaminants at critical developmental stages (Russell *et al.*, 1997; Russell *et al.*, 1995). These exposures could certainly be negatively affecting amphibian populations in many places, but the ecological implications have not been well studied (Russell *et al.*, 1997; Russell *et al.*, 1995).

Some EDCs enter the environment as "natural" sources such as hormones originating from humans, live stock, or plants (phytoestrogens). For example, some estrogenic hormones, such as 17beta-estradiol and estrone, are naturally synthesized *and* excreted by women. Several studies have shown that these chemicals, as well as 17 alpha –ethinylestradiol, the synthetic estrogen in contraceptives, can be found in aquatic environments associated with domestic waste water treatment plants at concentrations up to 5, 12, 47 and 7.5 ng/L respectively (e.g. Belfroid *et al.*, 1999). Although many waste water treatment sites contain concentrations below detection limits, 17 alpha -ethinylestradiol is known to induce the development of intersexed gonads in some gonochoristic fish at levels well below those detected by Belfroid (Jobling *et al.*, 2002 ; Länge *et al.*, 2001; Norris and Carr, 2006; Palace *et al.*, 2002; Pawlowski *et al.*, 2004; Woodling *et al.*, 2006). Furthermore, in the Ruhr district of Germany, 17 alpha –ethinylestradiol (used in birth control) has been detected in surface waters in concentrations between 1- 4 ng/L, which can induce intersexed gonads. The naturally synthesized estrogens, 17β-estradiol and estrone, however, were below the quantification limit of 1 ng/L (Lintelmann *et al.*, 2003).

Another source of "naturally derived" EDC pollution involves the hormones released from the waste of animals on feedlots or from waste used as fertilizer (reviewed by (Johnson *et al.*,

2006). These hormones are either naturally synthesized or "prescribed" as growth stimulants, or for other purposes. Intensive livestock rearing or agriculture leads to runoff of androgenic hormones at levels high enough to induce endocrine disruption in wild fathead minnows (*Pimephales promelas*) (Ankley *et al.*, 2003; Matthiessen *et al.*, 2006; Orlando *et al.*, 2004; Soto *et al.*, 2004). Steroid hormones, such as 17 beta -estradiol and testosterone, have even been found in spring water from mantled karst aquifers in agricultural areas (Peterson *et al.*, 2000). Therefore, hormone contamination from agricultural practices is not limited to surface waters and is likely distributed across long distances via ground water.

Phytoestrogens are endogenous compounds found in plants and are structurally and functionally similar to estradiol and other endogenous estrogens. They are released into the environment in a variety of ways, including sewage treatment plant effluent (Pawlowski *et al.*, 2003; Spengler *et al.*, 2001), runoff from agricultural areas treated with manure (Burnison *et al.*, 2003) and effluent from wood pulp mills (Clotfelter *et al.*, 2004; Mahmood-Khan and Hall, 2003). Although it is easy to understand how pollution containing natural or synthetic hormones can alter the endocrine systems of wildlife, there is an extraordinarily diverse array of man-made (synthetic) chemicals that also induce endocrine disruption. These chemicals include pesticides, flame retardants, plastics or plasticizers, heavy metals, industrial compounds (e.g. coolants or insulators), pharmaceuticals (e.g. antidepressants) and cosmetics (Goksoyr, 2006; Propper, 2005). Each of these broad categories contains many different chemicals that are known or suspected to induce endocrine disruption. Importantly, individual chemicals within and among these general categories (e.g. pesticides) can have very different chemical structures or be from different chemical classes, which complicates the ability to identify or predict chemicals that cause endocrine disruption.

Despite the large diversity of chemicals that can disrupt the endocrine system, they often have similar effects, albeit via different mechanisms of toxicity. For example, *most* known EDCs are estrogenic (induce estrogen-like effects), which can lead to feminization of males or development of intersexed gonads (Crews and McLachlan, 2006). However, a growing literature also has shown that aquatic pollutants potentially alter thyroid, androgen, progesterone and retinoic acid signaling (Guillette, 2006). In fact, many PCBs and polybrominated biphenyl ethers (PBDEs), used for decades as flame retardants, alter thyroid hormone function (Zoeller *et al.*, 2002). A growing list of fungicides and other pesticides have anti-androgenic activity (Gray *et al.*, 2006). Interestingly, no *synthetic* environmental compounds have been reported to have androgenic effects (McLachlan, 2001) except pharamaceutical agents (i.e. synthetic androgens) prescribed by, and released into, the environment as part of cattle feedlot operations (Durhan *et al.*, 2007). Plant sterols found in paper-mill effluent, however, can be metabolized by native aquatic bacteria to produce androgens, and have been shown to masculinize female fish (e.g. *Gambusia affinis holbrooki*) (Denton *et al.*, 1985; Parks *et al.*, 2001; Toft *et al.*, 2004). Thus, it is important to recognize that no single endocrine mechanism is at risk, but rather, EDCs can alter various endocrine signaling pathways via multiple mechanisms.

Modes of Action—How and Why?

The most commonly studied mode of action of EDCs is their action as a ligand (e.g. a hormone), thus inducing inappropriate activation or antagonism of nuclear hormone receptors (Fig. 1-2). However, hormone receptors can be influenced through several other mechanisms. EDCs can modulate receptor degradation, or alter receptor activity, such as by changing the activity or availability of co-factors essential for receptor function (Tabb and Blumberg, 2006). In addition, EDCs can modulate the degradation and clearance of endogenous hormones or alter synthesis of hormones, such that inappropriate amounts or types of steroid hormones are formed

(Guillette and Gunderson, 2001; Sanderson, 2006; Tabb and Blumberg, 2006). Finally, and possibly most importantly, some EDCs have been shown to alter genome-wide DNA methylation patterns. Methylation controls gene expression and if it occurs during specific developmental windows, gene expression patterns can be permanently altered, and these changes are inherited by offspring (Anway *et al.*, 2005; Edwards and Myers, 2007).

Nuclear Receptors

Nuclear receptors are protein receptors that bind to specific hormones (also called ligands) (Figs. 2, 3). This binding changes the conformation of the receptor such that it can bind with another receptor-ligand complex or with a coactivator-ligand complex. After this dimerization, the complex binds to specific DNA sequences in the regulatory region of target genes (called responsive elements) (Fig. 1-3), and triggers gene expression (Janosek *et al.*, 2006). There are 47 known nuclear receptors and, as a group, they constitute the nuclear receptor superfamily. These receptors are involved in modulating a wide range of physiological functions across eukaryotes including cellular growth and proliferation, development and differentiation, and maintenance of homeostasis (Blumberg and Evans, 1998; Janosek *et al.*, 2006). EDCs can bind to and activate a receptor and induce expression of responsive genes (McLachlan, 2001; Sanderson, 2006). Alternatively, other EDCs bind to the receptor and block the endogenous hormone from its receptor, which results in inappropriately low levels of ligand-responsive gene expression. Such influences on sex hormone receptors have been extensively studied over the past few decades (Tabb and Blumberg, 2006); for a review see (Janosek *et al.*, 2006). Some chemicals, such as ethanol, however, activate transcription of estrogen-controlled genes by influencing up-stream genes, not by binding the estrogen receptor (ER) (Janosek *et al.*, 2006).

Many members of the nuclear receptor superfamily are degraded via the ubiquitin-proteasome pathway. Ubiquitin labels old or damaged proteins for degradation, which is then

carried out by proteosomes (proteins that degrade other proteins). Receptor turnover is one mechanism whereby cells control gene expression and prevent over stimulation by endogenous hormones (Tabb and Blumberg, 2006). EDCs can modulate receptor degradation because they do not induce the same proteosome response that endogenous hormones elicit. For example, (Masuyama and Hiramatsu, 2004) showed that in the presence of estradiol both estrogen receptor alpha (ERalpha) and beta (ERbeta) interacted with active sites within the proteosome and were degraded. However, in the presence of Bisphenol A (a constituent of many plastics) which activates ER transcription, ERbeta degradation was blocked (Masuyama and Hiramatsu, 2004). Reduced degradation of ERbeta leads to a build up of these receptors in the cell, and thus is expected to lead to increased expression of ERbeta-responsive genes (Tabb and Blumberg, 2006). Other EDCs have been shown to alter ligand receptor activity (Jansen *et al.*, 2004) or the activity of co-activators essential for receptor function (Tabb and Blumberg, 2006).

Hormone Catabolism and Carrier Protein Binding

Altering hormone biotransformation (synthesis) and catabolism (break down) is yet another way EDCs can modulate hormone balance (Guillette and Gunderson, 2001; Tabb and Blumberg, 2006). For example, two nuclear receptors are important regulators of the catabolism of xenobiotics (a biologically active pollutant or drug), chemicals, and steroid hormones. These receptors are: (1) human steroid and xenobiotic receptor/rodent pregnane X receptor (SXR/PXR) and (2) constitutive androstane receptor (CAR) (Forman *et al.*, 1998; Kretschmer and Baldwin, 2005; Tabb and Blumberg, 2006; Xie *et al.*, 2000). Both of these receptors are highly expressed in the liver and intestine, and control induction of several key enzymes that orchestrate the biotransformation, catabolism, and transport of endogenous hormones and xenobiotics. Many EDCs have been found to activate these receptors and increase expression of their target genes (Kretschmer and Baldwin, 2005; Tabb and Blumberg, 2006). EDC-induced activation of

enzymes that degrade hormones and xenobiotics has a two-fold effect. First, endogenous hormones have a shorter half-life and thus are biologically active for less time. Second, in degrading xenobiotics, these enzymes increase levels of EDC metabolites, some of which are more toxic than the parent compounds.

Plasma-binding proteins sequester and control the bioavailability of hormones such that they cannot pass through the plasma membrane and bind to nuclear receptors (Nagel *et al.*, 1997). Many xenobiotic chemicals do not bind to plasma-binding proteins and, thus, are readily available to the cell and can induce endocrine disruptive actions (Arnold *et al.*, 1996; Crain *et al.*, 1998). In contrast, Danzo (1997) showed that several xenobiotics induced disassociation of endogenous ligands from their binding proteins and competed for binding proteins just as strongly as did natural ligands. Therefore, some xenobiotics can competitively bind to steroid-binding proteins, thereby increasing the bioavailablity of endogenous hormones because they are free to enter the cell (Danzo, 1997). In addition, several studies have shown that receptors for these binding proteins can be present on the plasma membranes of some cells. The hormone-binding protein complex binds to these receptors and initiates a signal transduction cascade (for a review see Danzo, 1998). Xenobiotics that bind in place of the endogenous hormones can inhibit or stimulate signal transduction (Danzo, 1998).

Steroid Biosynthesis

Steroid hormone synthesis involves a complex network of chemical reactions that convert a cholesterol-based substrate into a product (Fig. 1-4) through the activity of several substrate-specific cytochrome P450 enzymes, steroid dehydrogenases, and reductases (Guillette *et al.*, 2007; Sanderson, 2006). The cytochrome P450 enzymes are especially important because each one is responsible for specific chemical conversions that are essential for steroid biosynthesis (Miller, 1988). Importantly, the activity of many of these enzymes can be modulated by various

endocrine-disrupting chemicals, resulting in impaired development, growth, sexual differentiation, reproduction, and the development of particular cancers (for a review see Sanderson, 2006). For example, aromatase (CYP19) is responsible for controlling the rate-limiting step in the conversion of androgens to estrogens (e.g. testosterone to 17beta- estradiol), and its modulation by EDCs has been shown to interfere with the homeostasis and function of sex steroid hormones, resulting in feminization and demasculinazation (Crain and Guillette, 1997). For example, several triazine herbicides (atrazine, simazine, and propazine) and a number of their metabolites (atrazine desethyl and atrazine desisopropyl) induced aromatase activity and gene expression up to 2.5-fold in human H295R adrenocortical carcinoma cells (Fan *et al.*, 2007; Sanderson *et al.*, 2002; Sanderson *et al.*, 2000). Similar results were shown in alligator neonates (Crain and Guillette, 1997). This mechanism could explain why male amphibians exposed to artrazine in laboratory and field studies are feminized and have intersexed gonads (Hayes *et al.*, 2002; Hayes *et al.*, 2003). Alternatively, Ankley *et al.* (2005) demonstrated the fungicides prochloraz and fenarimol inhibit aromatase activity as well as bind to the androgen nuclear receptor in the fathead minnow (*Pimephales promelas*). Both fungicides caused significant anti-androgenic alterations in endocrine function of the fish, thereby decreasing reproductive success (Ankley *et al.*, 2005).

Epigenetics

Epigenetics refers to changes in gene function that are heritable (mitotically or meiotically) but do not involve changes in nucleotide sequence. For example, all the cells in the body of a multi-celled organism originate from the same single-celled zygote and thus share the same DNA sequences. Processes that lead to cellular differentiation and maintenance of specific cell functions involve epigenetic changes in genes rather than change in nucleotide sequence (e.g. mutation). In other words, gene function and expression among different cells is altered in a

mitotically heritable way, in this case without changing the DNA sequence. One mechanism through which this occurs is by methylation of DNA. This occurs when methyl groups are added to cytosines in the DNA. Methylation is inherited by daughter cells through DNA synthesis and cell division, and typically leads to suppression of gene expression, whereas de-methylation is associated with increased gene expression (Ballestar and Esteller, 2002; Crews and McLachlan, 2006; Edwards and Myers, 2007; Holliday and Pugh, 1975). For example, several of the inactive genes on female Barr bodies are methylated and the active and inactive states of these chromosomes are inherited from mother cell to daughter cells. Therefore, the pattern of DNA methylation among chromosomes (and alleles) of somatic cells is stably maintained, but germ cells and pre-implantation embryos can experience genome-wide re-programming of the methylation pattern that generates cells with new (meiotically) heritable developmental potential (Reik *et al.*, 2001; Reik and Walter, 2001a).

Epigenetic processes, via differential methylation, are also involved in gene imprinting which occurs when genes function differently depending on whether they are located on maternal or paternal chromosomes (Reik and Walter, 2001b). The DNA of eggs and sperm are methylated differently and inheritance of these differences leads to differential gene expression between the sexes (Zuccotti and Monk, 1995). In addition, during gonadal sex determination DNA in primordial germ cells is demethylated and remethylated in a sex-specific manner (Reik and Walter, 2001a). The methylation patterns that occur in the germ line can be permanent and inherited from parent to offspring.

The mechanisms controlling methylation during development are under intense investigation, and estrogens are thought to be involved in some cases (McLachlan, 2001). For example, estrogens, through interactions with their receptors, are known to increase the levels of

c-fos and c-jun in target cells (Kamiya *et al.*, 1996; McLachlan, 2001). C-fos and c-jun are transcription factors that are up-regulated in response to many physiological signals, and in turn up-regulate transcription of other genes involved in a diverse array of functions. Over-expression of c-fos leads to an-upregulation of (for example) cytosine methyltransferase, the enzyme involved in increasing DNA methylation (McLachlan, 2001). Estrogen's influence on methylation, however, is complex and can be tissue and gene specific. For example, 17beta estradiol exposure in male white Leghorn roosters is associated with removal of methyl groups at the estrogen response element (estradiol-receptor binding site) of a vitellogenin (yolk protein) gene, and this demethylation is associated with inappropriate transcription and translation of vitellogenin in males (Jost *et al.*, 1990; Saluz *et al.*, 1986). A similar mechanism of vitellogenin activation is known to occur in frogs (e.g. *Xenopus laevis*) (Andres *et al.*, 1984; Crews and McLachlan, 2006).

Hormone exposure early in life can alter methylation, and thus epigenetically alter the set point for later response to the same or different hormones (Saluz *et al.*, 1986). Given the potential role of hormones, such as estrogens, in controlling methylation, and the complexity of the mechanisms involved, one should expect EDCs that modulate plasma concentrations of estrogens, or influence their receptor expression, to induce changes in methylation patterns in ways that could be heritable. Indeed, it has recently been demonstrated that several EDCs can influence epigenetic programming through DNA methylation, and that when these changes occur at particular stages during development they are permanent and inherited by offspring (Crews and McLachlan, 2006; Tabb and Blumberg, 2006). For example, Anway *et al.* (2005) showed that rat pups (*Rattus rattus*) exposed to the fungicide vinclozilin (anti-androgenic) or the insecticide methoxyclor (estrogenic) during sexual differentiation experienced inappropriate

genome-wide methylation patterns in the male germ line. The altered methylation patterns were associated with altered sperm development and lower reproductive success (Anway *et al.*, 2005). This methylation pattern and reduction in fertility was transmitted through the male germ line to nearly all males in each generation across the four generations that were examined. The ability for endocrine disrupting chemicals to reprogram the germ line and induce trans-generational pathologies clearly has important implications for ecology, evolution, and conservation of exposed wildlife, including amphibians.

Phenotypic Responses

Examining the effects of EDCs is difficult and complex for a variety of reasons. There are multiple ways to define endocrine disruption, multiple physiological mechanisms through which disruption occurs, diverse abnormalities and pathologies that can be induced by EDCs, and multiple levels at which to study these effects. In addition, most toxicological studies have focused on identifying effects of high concentrations of contaminants (e.g. mg/L), often focusing on measuring end points such as mortality. Fewer studies have examined sublethal effects of chemicals, such as endocrine disruption, at ecologically, or physiologically, relevant concentrations (e.g. μg/L).

In particular, it is difficult to understand the effects of EDCs on amphibian populations because relatively little research on the effect of these chemicals has been conducted on this group of vertebrates, and almost all of it has been based on very few species under laboratory conditions. It is known, however, that several chemicals can modulate timing of metamorphosis (e.g.Freeman *et al.*, 2005), as well as drastically alter organ development. For example, Hayes *et al*. (2003) collected leopard frogs (*Rana pipiens*) that were intersexes (male and female gonads in the same gonochoristic individual) from sites across the United States that were contaminated with the widely used herbicide atrazine, as well as other agricultural chemicals. In addition, the

present authors (in preparation) have found a clear relationship between exposure to agricultural habitats where known EDCs, such as atrazine, are used, and intersexed primary and secondary sexual characteristics in *Bufo marinus*. They also observed that pollutants such as nitrate, largely from fertilizer use, a ubiquitous global pollutant of fresh water and near marine ecosystems, can alter the rate of development of tadpoles, presumably through an effect on thyroid hormone function, and can alter ovarian steroidogenesis (Barbeau *et al.*, 2007; Edwards *et al.*, 2006).

Thyroid

The thyroid system is highly conserved among vertebrate species, so thyroid hormone chemistry, its synthesis and delivery system, its receptors, and its regulation within the hypothalamic-pituitary-thyroid (HPT) axis are all comparable across vertebrates (Zoeller and Tan, 2007; Zoeller *et al.*, 2007). The thyroid gland and its hormones regulate growth, development, metabolism, and reproduction, so alterations of the thyroid system caused by EDCs are of concern (Opitz *et al.*, 2006a). The thyroid system involves many mechanisms that can be altered by EDCs (as discussed above), such as alterations in receptor binding, hormone synthesis, and clearance. These processes and mechanisms are affected in similar ways across diverse taxa, including mammals and amphibians (Fort *et al.*, 2007; Opitz *et al.*, 2006a). Several groups of chemicals are known, or are thought to, induce thyroid disruption (Boas *et al.*, 2006). For example, polychlorinated biphenyls (PCBs) (such as coolant/insulating fluids and flame retardants), dioxins (e.g. agent orange, chemicals found in some insecticides, and industrial byproducts from chlorination) and polybrominated biphenyl ethers (PBDEs) (such as some flame retardants) are known to cause insufficient synthesis of thyroid hormone (hypothyroidism) in exposed animals. Environmentally relevant doses appear to affect human thyroid function as well. Although data on the effects of flame retardants on human populations is limited, there is great concern as the concentrations of these chemicals have dramatically increased in mother's

milk over the past decade. These compounds appear to be similar in action to PCBs, which have been studied in detail for decades and which clearly influence thyroid function. Also of major concern are the phthalates, used as plasticizers and chemical stabilization agents (e.g. in personal care products), as they also affect thyroid function, but appear to be stimulatory (Boas *et al.*, 2006).

Structural and functional changes required for successful amphibian metamorphosis are dependent on the presence and appropriate concentrations of the thyroid hormones 3,5,3'-triiodothyronine (T_3), and 3,5,3',5'-tetraiodothyronine (T_4) (Crump *et al.*, 2002; Fort *et al.*, 2007; Sachs *et al.*, 2000). Thyroid hormones bind to nuclear thyroid receptors (TR), and this complex can activate, or inhibit, gene transcription in a tissue-specific manner. Particular tissues are proliferated after thyroid hormone exposure, whereas others undergo apoptosis (programmed cell death) (Crump *et al.*, 2002; Helbing *et al.*, 1996; Helbing *et al.*, 1992; Helbing and Atkinson, 1994; Sachs *et al.*, 2000). Blocking the natural synthesis of thyroid hormone inhibits metamorphosis whereas exposing tadpoles to exogenous thyroid hormone induces precocious metamorphosis (Sachs *et al.*, 2000). Therefore, thyroid hormones are essential for stimulating the complex and diverse genetic programs required for amphibians to successfully complete metamorphosis. The essential nature of thyroid hormones for amphibian metamorphosis, coupled with the fact that thyroid function is well conserved across vertebrates, has lead to the suggestion that tadpoles are an ideal bioassay system to identify thyroid function disruption by environmental contaminants (Crump *et al.*, 2002; Degitz *et al.*, 2005; Fort *et al.*, 2007; Opitz *et al.*, 2005; Zhang *et al.*, 2006). A chemical evaluation protocol referred to as the *Xenopus* metamorphosis assay (XEMA) has been developed and evaluated via a ring test where six laboratories participated to evaluate inter-laboratory variation in results (Opitz *et al.*, 2005).

Opitz *et al*. (2005) demonstrated that the XEMA test can provide a sensitive, robust, and practical approach for the detection and evaluation of chemicals that affect the thyroid system.

The herbicide acetochlor [2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl) acetamide] has been shown to accelerate metamorphosis in several amphibian species (Cheek *et al.*, 1999; Veldhoen and Helbing, 2001). Acetocholor, however, only accelerates metamorphosis in animals that are "primed" by previous exposure to thyroid hormone. Therefore, it was suggested that acetochlor did not bind to and activate the thyroid receptor directly but enhanced T_3 action by different receptor-mediated mechanisms (Cheek *et al.*, 1999). Veldhoen and Helbing (2001) showed that an environmentally relevant dose of acetochlor significantly enhanced TRbeta mRNA levels in T_3-primed *Rana catesbeiana* within 24 hours. Therefore, acetochlor does not bind to TRbeta but it does increase TRbeta expression, so that endogenous thyroid hormone can bind (Veldhoen and Helbing, 2001). Crump *et al*. (2002) investigated acetochlor's influence on gene expression in *Xenoupus leavis* by developing a 420-gene cDNA array from known frog genes. They found that 26 genes are modulated by thyroid hormone and acetochlor. Twenty-four genes were up-regulated whereas only two were inhibited. A detailed understanding of the physiological changes induced by altering this number of thyroid-dependent genes required for normal metamorphosis or the long-term effects on juvenile and adult metabolism and health are unknown. Given that there is a natural spike in thyroid hormones just before metamorphosis, metamorphic animals could be "naturally primed" by thyroid hormone. Under these conditions, acetochlor exposure could induce higher than usual TRbeta (and other) mRNA levels and cause the animals to proceed through metamorphosis faster than normal. The ecological implications of this acceleration are as of yet unknown, but could lead to higher mortality, smaller size at metamorphosis, and altered morphology.

Another approach to the investigation of EDCs is to study their effects at the tissue level. Changes in thyroid histology (morphology) are indicative of alterations in the physiology of the pituitary-thyroid axis. For example, inhibition of thyroid hormone secretion inhibits the negative feed-back on the pituitary which would normally stop secretion of thyroid stimulating hormone (TSH). This induces increased TSH that results in an increase of the size of thyroid gland follicles (hypertrophy of thyrocytes) and this effect can be observed and quantified histologically. For example, perchlorate, an oxidizer used in solid-fuel rockets, inhibits thyroid hormone synthesis by inhibiting the sodium-iodide symporter that acts as an ion pump transporting iodide into thyroid epithelial cells so that they can synthesize thyroid hormones (Opitz *et al.*, 2006b; Tietge *et al.*, 2005). The effects of perchlorate on cricket frogs (*Acris crepitans*) from contaminated streams in central Texas were evaluated through histological analyses. Individuals living in streams with the greatest mean water perchlorate concentrations (~ 25 microg/L) showed significantly greater follicle cell hypertrophy. In addition, a significant positive correlation was found between thyroid follicle cell height and mean water perchlorate concentrations across all sites (Theodorakis *et al.*, 2006). Similar results were found for *Campostoma anomalum*, a species of fish, from the same habitats, which suggests that perchlorate affects thyroid function across many taxa through a similar mechanism. Perchlorate reduced thyroid hormone, and the thyroid follicles experienced prolonged stimulation by TSH.

Since the mechanism whereby perchlorate effects thyroid function is known, it is an appropriate chemical to use to help understand how effects induced by EDCs at the tissue level can scale up to influence processes, such as metamorphosis, at the individual level. For example, African clawed frogs (*Xenopus laevis*) exposed to ecologically relevant concentrations of ammonium perchlorate (59 and 140 microg/L) experienced significant hypertrophy of the

thyroid follicular epithelium, as well as inhibition of forelimb emergence, tail resorption, and hindlimb development relative to control animals (Goleman *et al.*, 2002). All these findings suggest that perchlorate alters thyroid function (as expected), but they also demonstrate that many thyroid-hormone-dependant developmental programs required for metamorphosis are negatively affected. Interestingly, only the highest concentration of perchlorate reduced whole-body thyroxine concentrations relative to the control treatment. This finding highlights the importance of measuring multiple endpoints; if thyroid hormone concentrations were measured without consideration of other morphological endpoints, the conclusion would have been drawn that perchlorate does not influence thyroid function in this species. In addition to alterations in metamorphic development, Goleman *et al*. (2002) found that perchlorate induced a skewed sex ratio such that significantly fewer males were recorded at metamorphosis. They concluded that ammonium perchlorate altered thyroid activity and gonadal differentiation in developing *X. laevis*, which suggests that animals that proceeded through seemingly normal metamorphosis could suffer lower reproductive success as adults. Importantly, the concentrations of perchlorate used in this study were below concentrations reported in surface waters contaminated with ammonium perchlorate, so perchlorate contamination likely poses a threat to normal development in natural amphibian populations (Goleman *et al.*, 2002). In a similar study, Tietge *et al*. (2005) exposed *X. laevis* tadpoles to sodium perchlorate during metamorphosis, and measured thyroid histology as well as metamorphic timing. Histological effects on the thyroid were found at levels as low as 16 microg/L and metamorphosis was retarded significantly by perchlorate concentrations as low as125 microg/L. Histological effects occurred at concentrations below those required to induce delays in metamorphosis which suggests that thyroid histology is a more sensitive biomarker for detecting thyroid-disruption induced by

perchlorate (Tietge *et al.*, 2005). Importantly, it is unclear how such alterations in the thyroid during development influences later life stages (e.g. adult metabolism) independent of whether metamorphosis was delayed.

Development—Homeobox Gene Expression

A significant amount of the early work on EDCs focused on developmental endpoints as the developing embryo is sensitive to very small concentrations of hormones required for normal development of many organs and organ systems (Bern, 1992). It is recognize today that the developing embryo has sensitive windows of responsiveness when endocrine signals, at appropriate concentrations, induce organizational changes that fundamentally modify cellular differentiation and endocrine responsiveness later in life (Guillette *et al.*, 1995). Many diverse genes, including the Homeobox (Hox) genes, have been shown to be responsive to endocrine signaling, making them targets for EDCs.

Hox genes are evolutionarily conserved transcription factors that are essential for orchestrating embryonic development as well as cellular differentiation in adults. The expression of particular collections of Hox genes in specific places within the embryo creates a collinear expression continuum that regulates development in a segment-specific pattern and determines the anterior-posterior axis in the developing embryo (Daftary and Taylor, 2006). Hox genes are also essential in the adult where they mediate cellular differentiation and control the developmental plasticity required to specify the function of new cells. Although Hox genes have been studied, and their role in cell fate determination is well known, the mechanisms by which their expression is controlled are not well understood. Recent work has demonstrated that nuclear receptors and their corresponding ligands (hormones) can regulate Hox gene expression in the embryo and adult (reviewed by (Daftary and Taylor, 2006). For example, in mice (*Mus musculus*) estrogens are necessary for the normal Hox gene expression required during

embryonic development of the female reproductive tract (Block *et al.*, 2000; Daftary and Taylor, 2006). Exposure to diethylstilbestrol (DES), a synthetic non-steroidal estrogen, results in altered endogenous estrogen signaling and a change in the spatial expression of Hoxa9, Hoxa10, and Hoxa11, such that each is expressed more caudally throughout the developing female reproductive tract leading to alterations in its morphology (Block *et al.*, 2000; Daftary and Taylor, 2006). Human females exposed to DES during development exhibit reproductive tract abnormalities with phenotypes that can be explained by a similar shift in Hox gene expression (Block *et al.*, 2000; Daftary and Taylor, 2006). For example, glandular tissue normally present in the uterus and cervix is observed in the vagina in women exposed *in-utero* to DES, which is consistent with caudal displacement of HoxA10 and HoxA11 expression (Block *et al.*, 2000; Daftary and Taylor, 2006). This work demonstrates that endocrine regulation of Hox genes by estrogens during embryogenesis is necessary across a variety of taxa. Estrogens are also thought to control functional differentiation in the adult reproductive tract (Daftary and Taylor, 2006).

Given the critical role of hormones such as the estrogens in modulating Hox gene expression, any perturbation to these endogenous signals by EDCs is expected to induce developmental abnormalities (Iguchi *et al.*, 2001). Indeed, methoxychlor (a replacement insecticide for DDT) reduces estrogen binding to the estrogen receptor (ER), and disrupts the ability of the 17beta-estradiol-ER complex to bind to the estrogen response element (ERE) for the HoxA10 gene in endometrial cells, resulting in a decrease in HoxA10 gene expression (Fei *et al.*, 2005). Therefore, methoxychlor functions as an endocrine disruptor by affecting 17beta - estradiol signaling in endometrial cells. In mice and rats, these changes inhibit modification of the endometrium (to create the dicidua), which blocks implantation of the embryo, and has deleterious effects on fertility. Importantly, in mice, this reduction of HoxA10 gene expression

is permanent and continues in adults even if they were only exposed as neonates (Fei *et al.*, 2005). The mechanisms inducing continued repression of Hox gene expression in the absence of continued methoxychlor exposure are not known, but likely result from epigenetic modification, possibly via increased methylation (Fei *et al.*, 2005; McLachlan, 2001). Indeed, Wu *et al*. (2005) clearly demonstrated that differences in methylation of HoxA10, and the subsequent decline in HoxA10 gene expression, is significantly associated with increased endometiosis in woman, *and* they argue that enodmetriosis is an epigenetic disease.

Unfortunately, to our knowledge no work has explicitly evaluated the occurrence and importance of EDC exposure on Hox gene expression in amphibians. It is likely that similar effects are operating in amphibians because Hox genes, and their roles in embryonic development and cellular differentiation in adults, are highly conserved. Important aspects of amphibian development and metamorphosis are controlled by Hox genes. For example, the number, position, and type of limbs in amphibians depend on the proper expression of specific Hox genes. Retinoic acid regulates Hox gene expression and altering the quantity of retinoids during development results in serious developmental abnormalities across various taxa (Gardiner *et al.*, 2003; Koussoulakos, 2004). Application of retinoic acid to chicken limb buds alters homoeobox gene expression and induces duplication of limbs (Ogura and Evans, 1995).

Indeed, one of the most compelling explanations for the skeletal dysplasias observed in frogs from Minnesota, and elsewhere, is that the severely malformed limbs are due to exposure to environmental contaminants that function as retinoid (e.g. Vitamin A and retinoic acid) mimics (Gardiner *et al.*, 2003). Gardiner *et al* (1999) analyzed skeletal abnormalities in the Minnesota frogs and identified two major classes of abnormalities (Gardiner and Hoppe, 1999). First, the initiation of limb development was altered, which induced absent or supernumerary

limbs. Second, limb growth and pattern formation were modified such that primary and supernumerary limbs both had particular skeletal abnormalities, including truncation and dwarfism. Skeletal elements were also folded back on themselves, forming characteristic "bony triangles" (Gardiner *et al.*, 2003; Gardiner and Hoppe, 1999). Retinoid (e.g., retinoic acid) exposure during limb bud development can induce the formation of boney triangles in frogs, chickens, and mice (Gardiner *et al.*, 2003). In addition, retinoic acid can induce supernumerary limbs, incomplete or missing limbs, complete limb duplications, as well as duplicated pelvic girdles across diverse taxa, including several frog species (Gardiner *et al.*, 2003). Most of these abnormalities have been observed in field-collected malformed specimens. In addition, Gardiner *et al*. (2003) extracted hydrophobic substances from water samples collected from sites where malformed frogs were routinely found, and tested them for their ability to activate the retinoic acid receptor (RAR). Biologically active retinoids were found in water samples from severely affected sites. Therefore, it is possible that a single pollutant that functions as a retinoid mimic could be responsible for all the malformed phenotypes that have been observed (Gardiner *et al.*, 2003). Interestingly, a recent survey of 5,264 hylid and ranid metamorphs in 42 Vermont wetlands demonstrated that proximity to agricultural land was associated with an increase in limb malformations (Taylor *et al.*, 2005). No analysis of the mechanisms underlying these limb abnormalities have yet been carried out (Gardiner *et al.*, 2003). It seems likely, however, that retinoic acid mimics could modulate Hox gene expression and induce a variety of abnormalities, depending on the developmental stage and length of time of exposure.

Reproduction

Reproductive organs produce a variety of hormones including steroids such as testosterone, estrogen, and progesterone, as well as peptide hormones such as inhibin, activin, and Müllerian inhibiting hormone, so they function as endocrine glands and they appear to be a

major target for EDCs. Several environmental chemicals alter the development and function of the reproductive system, thereby altering endocrine system function. These affects have been documented across all classes of vertebrates including humans (reviewed by (Milnes *et al.*, 2006). Although the mechanisms that drive sex determination and gonadal development appear to vary widely among species, the underlying genetic and endocrine control of gonadal development, growth, and function, as well as the roles of hormones on secondary sexual characteristics, are highly conserved among vertebrates. Most EDCs studied to date exhibit estrogenic or anti-androgenic activity and can influence sex ratio, sexual maturation, gonadal morphology and function, as well as spermatogenesis, fertility, hormone levels (steroidogenesis, metabolism), secondary sexual characteristics and reproductive behaviour (Milnes *et al.*, 2006). Clearly, all of these characteristics are important for maintaining healthy and persistent populations, especially for taxa that are known to be declining globally, such as amphibians.

The effects of EDCs on the development and functioning of the reproductive system of amphibians are not well understood relative to other wild life such as fish, alligators, and mammals. Amphibians, however, could be especially sensitive to EDCs because gonadal differentiation in this taxon is highly sensitive to sex hormones and occurs over an extended period of time, sometimes beginning prior to metamorphosis and lasting for several months (Hayes, 1998; Qin *et al.*, 2003). In genetic males, estrogen can induce a complete and permanent sex reversal or induce development of intersexed gonads (ovarian tissue and testicular tissue in the same gonad). Intersexed gonads typically occur in two forms: oocytes can be scattered throughout the testes forming an ovo-testis, or the ovary can be distinct and well delineated from the testicular tissue (Milnes *et al.*, 2006). These different phenotypes likely occur through

different molecular mechanisms and/or timing of exposure, but these details have not yet been fully investigated.

Although the data on the effects of EDCs on the reproductive system of amphibians are relatively limited, it has been clearly demonstrated that EDCs can induce female-skewed sex ratios and alter gonadal development and concentrations of hormones. Tavera-Mendoza *et al*. (2002a,b) exposed *Xenopus laevis* tadpoles to ecologically relevant levels (21 microg/L) of atrazine for 48 hours during sexual differentiation. Histological analysis of the ovaries showed that atrazine-exposed females experienced a 20% reduction in primary germ cells compared to 2% in controls. Atrazine-exposed males had a 57% reduction in testicular volume, and primary spermatogonial cell nests were reduced by 70%. Furthermore, Sertoli (nurse) cells, which provide nutritive support for the developing germ cells, declined by 74% and testicular resorption occurred in 70% of the males, while 10% failed to fully develop testes. These findings are significant because primary germ cells are thought to comprise the total number of germ cells for the entire reproductive life of the organism, and such a remarkable reduction in only 48 hours suggests that atrazine, even in pulsed exposures, could severely negatively affect reproductive success of both females and males (Tavera-Mendoza *et al.*, 2002a, b).

As mentioned above, African clawed frogs (*Xenopus laevis*) exposed to environmentally relevant doses of ammonium perchlorate during metamorphosis had female-skewed sex ratios (Goleman *et al.*, 2002). Several other chemicals, however, have been demonstrated to induce female-biased sex ratios. For example, Kloas *et al*. (1999) exposed *X. laevis* tadpoles to estradiol (positive control), nonylphenol (used in surfactants, detergents, and pesticides), bisphenol A (plastic or plasticizer), octylphenol (surfactant) and butylhydroxyanisol (pharmaceutical for treating retroviruses). Each chemical induced a significantly higher number of females

compared to controls. These results demonstrate that a diverse array of chemicals can, and do, alter sex ratios making them female-biased. There are two ways in which this type of bias can occur. Males could, in general, be less resistant to chemicals and suffer higher mortality than females. Alternatively, the genotypic males in these experiments may undergo sex reversal so that they appear female.

Many chemicals have been shown to induce feminization of male gonadal tissue. Qin *et al*. (2003) exposed *X. laevis* to Aroclor 1242 and Aroclor 1254 (aroclors are mixtures of PCBs) and recorded both gross morphological and histological differences relative to the vehicle control. Control animals had normal ovaries or testes from the standpoint of gross morphology whereas PCB-exposed animals had abnormally sized and asymmetrical testes as well as ovotestes. Ovotestes, in that study, were characterized by ovaries in the cranial and/or caudal portion of the gonad with testes located medially. PCB exposure did not alter the proportion of females across treatments but the portion of males with morphologically normal testes was reduced. Histological examinations revealed that testes that were classified as morphologically normal, as well as those classified as abnormal upon gross evaluation, had oocytes interspersed throughout the tissue. In addition, testes of PCB-exposed animals were more loosely organized and had fewer seminiferous tubules, spermatogonia, and spermatozoa than was the case for controls. Qin *et al*. (2003) argued that Aroclor 1242 and Aroclor 1254 feminize gonadal differentiation in *X. laevis* and that *X. laevis* could be especially sensitive to endocrine disruption. Thus, this species is an appropriate model for studying endocrine disruption.

Hayes *et al*. (2002) showed that very low ecologically relevant levels of the common herbicide atrazine can induce *X. laevis* tadpoles to develop intersexed gonads and exhibit decreased larynx size (demasculinized), and they suggested that these changes could reduce

fertilization success and alter breeding call characteristics. Indeed, *X. laevis,* collected from agricultural areas in South Africa where atrazine and other chemicals are used, suffer decreased testosterone levels (Hecker *et al.*, 2004). Importantly, atrazine is a widespread compound and the concentrations of atrazine used in that study are realistic levels (e.g. "acceptable" for drinking water according to the EPA) to which many amphibian species are exposed in the wild, putting them at risk of impaired sexual development.

Indeed, much work has been conducted on *X. laevis* and this species has proven to be an important model since it is easy to obtain, breed, and rear in the laboratory. Several studies, however, have documented feminization in amphibian species other than *X. laevis*. For example, leopard frogs (*Rana pipiens*) exposed to ecologically relevant concentrations of atrazine (>0.1 ppb) had retarded gonadal development (gonadal dysgenesis) and testicular oogenesis (intersexed). Males that developed slowly and were thus exposed to atrazine for longer periods of time, also experienced oocyte growth (oogenesis and vitellogenesis) (Hayes *et al.*, 2003). Hayes et al. (2003) also conducted field surveys of sites that varied in atrazine exposure across the United States and observed gonadal dysgenesis and intersexuality in animals from sites contaminated with atrazine. They concluded that these integrated laboratory and field studies on leopard frogs, coupled with similar findings in *X. laevis*, demonstrate the effects of atrazine on amphibians in general and suggest that atrazine and other endocrine-disrupting pesticides could be playing a major role in amphibian declines around the world.

Another important group of contaminants that is commonly found in the environment due to its importance to humans is plastic that contains bisphenol A or plasticizers (various phthalates) that give plastic its pliability. Bisphenol A has been shown to induce female skewed sex ratios in *X. laevis* (Mosconi *et al.*, 2002). Ohtani *et al*. (2000) exposed genetically male

tadpoles of *Rana rugosa* to dilute solutions of the plasticizer dibutyl phthalate and three 17beta -estradiol-positive controls for four days during gonadal sexual differentiation (days 19-23). At day 40, the gonads of the tadpoles were examined histologically. Positive control and dibutylphthalate-treated, genetically male tadpoles had complete ovaries or were intersexed in a dose-dependent manner. Therefore, dibutyl phthalate was able to induce intersexuality, but it was approximately 1000-fold less potent than 17beta -estradiol (Ohtani *et al.*, 2000). Even considering the reduced potency, Ohtani *et al*. (2000) concluded that dibutyl phthalate is an estrogen-like hormone that alters testicular differentiation, and thus is a dangerous environmental contaminant.

It is known that gonadal development in many amphibian species can be altered by exposure to estrogens or to estrogenic contaminants (Hayes, 1998). Therefore one would expect larvae living in environments containing sewage effluent that is contaminated with human birth-control chemicals to have altered gonadal development. To test this hypothesis, Park and Kidd (2005) added low concentrations of 17alpha-ethinylestradiol (EE_2) to an experimental lake in northwestern Ontario, Canada. A target concentration of 5 ng/L was maintained by adding 17alpha-ethinylestradiol to the treatment lake three times a week during the open-water seasons (May to October) for three years (2001, 2002, and 2003). Egg masses were reared in cages at the EE_2 lake and in two reference lakes. In the EE_2 lake, hatching success was reduced significantly in green frogs (*Rana clamitans*) but not in mink frogs (*Rana septentrionalis*), relative to each species in the reference lakes. Ethinylestradiol had no effect on sex ratios of either species and no intersex gonads were observed in tadpoles of either species at the reference sites. Although no green frog tadpoles were intersexed at the EE_2 site, approximately 6% and 13% of the caged mink frog tadpoles were intersexed in 2001 and 2002 respectively. Therefore, these two species

responded differently to EE_2 exposure. Green frogs suffered higher mortality at hatching but survivors did not have intersexed gonads, whereas mink frogs did not have high hatchling mortality but did experience altered gonadal development. This highlights the fact that different species respond differently to the same environmental contaminant. Un-caged mink frog tadpoles were also studied at each of the sites, and EE_2 had no effect on sex ratios, but by the third year 28.6% of wild EE_2-exposed first-year tadpoles had intersexed gonads whereas none were intersexed in the reference lakes. These results indicate that concentrations of 17alpha-ethinylestradiol, comparable to those found in sewage treatment effluents and some surface waters, can affect hatching success as well as gonadal development in native amphibians (Park and Kidd, 2005).

In addition to intersexed or completely feminized gonads, alterations in circulating steroid concentrations have been reported in amphibians exposed to EDCs. For example, Hayes *et al*. (2002) exposed adult African clawed frogs to 25 ppb atrazine (level acceptable in drinking water according to the EPA) and found that they exhibited a 10-fold decrease in testosterone concentrations relative to animals housed in 0 ppb. They hypothesized that atrazine increased aromatase, the enzyme that converts testosterone to estrogen, and caused the decrease in testosterone. As discussed above, this mechanism is known to occur in other vertebrate species (Fan *et al.*, 2007; Sanderson, 2006; Sanderson *et al.*, 2002; Sanderson *et al.*, 2000).

Altered gonadal development and hormone concentrations associated with EDC exposure induce other abnormal physiological responses. One important example of this is the induction of the normally female-specific protein vitellogenin (yolk protein) in males. Typically males do not express the vitellogenin gene but when they are exposed to estrogen or estrogenic chemicals this gene is upregulated. Therefore, plasma vitellogenin in males is a biomarker for estrogenic

xenobiotics. For example, adult male red-eared slider turtles (*Trachemys scripta*) and African clawed frogs given injections of estradiol, the synthetic estrogen diethylstilbestrol (DES), or the insecticide DDT experienced an induction of vitellogenin. In both species, estradiol and DES treatments induced more vitellogenin than did DDT, which demonstrates that DDT is estrogenic, but as expected from previous studies investigating its estrogenicity, it does not elicit as strong a response as do actual estrogens (Palmer and Palmer, 1995). Since that study, and many others establishing vitellogenin as a reliable biomarker, researchers have begun to use vitellogenin to detect estrogen exposure in amphibians (Palmer *et al.*, 1998); methodology reviewed by (Wheeler *et al.*, 2005). Mosconi *et al*. (2002) reported a dose-dependent induction of vitellogenin in male European frogs (*Rana esculenta*) and crested newts (*Triturus carnifex*) exposed to the surfactant 4-nonylphenol. They also demonstrated that 4-nonylphenol inhibited gonadotropin release from the hypothalamus and prolactin secretion by the pituitary, but increased plasma androgen concentrations. Importantly, the metabolic cost associated with production and elimination of vitellogenin and its impacts on reproductive success of males have not been considered or investigated in detail (Milnes *et al.*, 2006).

Other physiological responses that can be altered when EDCs are misinterpreted as hormonal signals involve secondary sexual traits. These include many sexually dimorphic characteristics such as differences in forelimb size, or colouration, or the presence of nuptial pads. For example, female colouration in the reed frog (*Hyperolius argus*) is estrogen dependent, so Noriega and Hayes (2000) used early induction of female colour pattern as a biomarker for estrogenic activity. *H. argus* tadpoles were exposed to the insecticide o,p-DDT and six of its congeners and *in vivo* colour changes were compared among treatments. Estradiol, o,p-DDT, o,p'-DDE and o,p'-DDD prematurely induced adult female colouration in juvenile

animals, whilst p,p'-DDT, p,p'-DDE and p,p'-DDD did not (Noriega and Hayes, 2000). This concept that estrogen-dependent colouration could be used as a biomarker of estrogenic chemicals was utilized by McCoy *et al*. (unpublished data) during field surveys of giant toads (*Bufo marinus*) living in sugarcane agricultural areas in South Florida. It was found that as many as 40% of the males (defined as having testes and nuptial pads) were intersexed (also had ovaries) and many males, including individuals with morphologically normal testes, exhibited female colouration, whereas those from reference sites were not intersexed, and exhibited the sexually dimorphic male colour pattern.

Behavior

The endocrine system and the central nervous system (CNS) are integrated such that the proper functioning of one is dependent upon the proper functioning of the other. For example, particular regions of the brain are known to be sexually dimorphic and these differences occur in response to different hormonal influences during development. Having the proper hormonal milieu at crucial stages in brain organization is essential for future (adult) sex-specific behavioural responses (Moore *et al.*, 2005; Palanza *et al.*, 2002). Altering this hormonal milieu during fetal development can permanently change adult behaviour.

The hypothalamus links the nervous and endocrine systems by releasing stimulating or inhibiting hormones through blood vessels to the anterior pituitary which in turn signals endocrine glands such as the thyroid, adrenal, or gonad via tropic (releasing) hormones (Fig. 1-1). These endocrine glands can then synthesize and secrete specific hormones that induce various physiological effects and provide feedback that alters pituitary and hypothalamic (CNS) function. Since these systems are integrated, hormonal effects on behaviour (CNS) can be direct, or indirect (e.g. altering thyroid hormones can influence metabolism that in turn influences behaviour) (Zala and Penn, 2004).

The endocrine and nervous (neuroendocrine) systems are so highly integrated that chemicals which alter the endocrine system also influence the central nervous system and therefore behaviour. There are numerous diverse examples of endocrine disruption leading to alteration of behaviour; thus, Zala and Penn (2004) suggested that endocrine disrupting chemicals be renamed as "neuroendocrine disrupters". Indeed, behaviour is becoming a more common endpoint in standard toxicological assays. Many researchers suggest that behaviour is a useful bioindicator of endocrine disruption because it is an easily measured, integrated physiological response that is related to alterations of specific neural pathways and is dependent on environmental context (Clotfelter *et al.*, 2004; Crews *et al.*, 2007; Panzica *et al.*, 2005). An animal's behaviour is indicative (a surrogate or sentinel) of its health and the tools needed to evaluate behaviour are relatively inexpensive and easy to implement. Many of the physiological mechanisms that control behaviour, however, are not understood in detail, so it can be difficult to determine the mechanism(s) through which specific EDCs modulate behaviour. Thus, there is some debate about the usefulness of behaviour as a measure of endocrine disruption (Clotfelter *et al.*, 2004). If one cannot ascribe an endocrine mechanism, how can one know that disruption of the endocrine system is leading to the altered behaviour? Understanding how EDCs alter the mechanisms controlling and modulating behaviour is an extremely important and open field of exploration.

Although the mechanisms of endocrine or neuroendocrine disruption leading to altered behaviour are not always clear, there are numerous examples of known EDCs that induce alterations in behaviour. Recent reviews by Zala and Penn (2005) and Clofelter *et al*. (2005) demonstrated that locomotion, balance, feeding, antipredator behaviour, communication, aggression, various aspects of learning, and reproductive behavior (including courtship, mate

choice, mating, nesting, and parental care) can all be altered by chemicals that are known to disrupt the endocrine system.

Many aspects of amphibian behaviour are altered by chemical pollutants including locomotion, activity, exploratory behavior, response to cues emanating from predators, communication, learning, and mating. The following discussion is restricted to examples describing how known EDCs alter behaviour in amphibians. PCB 126 (insulating fluid for transformers and capacitors) has been shown to reduce swimming speed in tadpoles of *Rana clamitans* and *R. pipiens*, which suggests that both feeding and predator escape could be affected in natural systems; these ecological consequences, however, were not tested (Rosenshield *et al.*, 1999).

Bridges (1999) explicitly tested whether responses by tadpoles to predators were altered in the presence of the insecticide carbaryl. Tadpoles of *Hyla versicolor* were exposed to two ecologically relevant levels of carbaryl (1.25 and 2.50 mg/L), the vehicle control, and a negative control (acetone solvent and water respectively) for only 24 hours. After the exposure, tadpole behaviour was examined in the presence and absence of adult red-spotted newts (*Notophthalmus viridescens*) housed in a mesh container. The containers allowed visual, tactile, and chemical cues to be transmitted but did not allow predatory attack. Tadpole activity, measured as percentage of time spent swimming, resting, and feeding and time spent in refugia, was recorded every three minutes for one hour (Bridges, 1999). Control tadpoles spent 20% of their time in refugia in the presence of a predator, but did not enter refugia when no predator was present; instead they actively fed. Animals in the low-carbaryl treatment spent roughly equal amounts of time in refugia whether a predator was present or not, whereas those in high-carbaryl treatment spent less time in refugia when the predator was present than when it was absent. In addition,

tadpoles exposed to high levels of carbaryl were, on average, less active than were those in control treatments but they spent more time foraging in the presence of a predator than in its absence. Control tadpoles in Bridges' (1999) experiment were able to effectively detect predators, hide in their presence, and actively forage in their absence. In other words, they behaved adaptively. The tadpoles exposed to carbaryl, however, did not show these adaptive behaviours, especially at the high carbaryl concentration. This study clearly demonstrates that EDCs can alter behaviour in unexpected ways.

In a similar study, salamander larvae (*Ambystoma macrodactylum*) exposed to methoxychlor (replacement insecticide for DDT) exhibited a reduced startle response and traveled shorter distances in response to disturbance; they also experienced increased predation by dragonflies (Eroschenko *et al.*, 2002; Verrell, 2000). Importantly, this was not the first time that exposure to a known EDC induced behaviour that increased predation. Three decades earlier, Cooke (1970, 1971) showed that common frog tadpoles (*Rana temporaria*) exposed to DDT were uncoordinated and hyperactive and were preferentially preyed upon by warty newts (*Triturus cristatus*). In addition to the increased predation that prey experience after chemical exposure, predators could suffer increased bioaccumulation if they preferentially eat exposed individuals (Cooke, 1970; Cooke, 1971).

The occurrence of maladaptive behaviour in the presence of EDCs suggests that learning could be altered by exposure to such chemicals. To examine this hypothesis, Steele *et al* (1999) exposed tadpoles of *Rana clamitans* and *R. catesbeiana* to 0 or 750 microg Pb/L for 5-6 days; then, animals were conditioned to associate a light source (conditioned stimulus) with a mild shock (unconditioned stimulus). Tadpoles exposed to lead had a higher mean response time and

showed less avoidance. It was concluded that sublethal exposure to lead adversely affects acquisition learning and retention in tadpoles (Steele *et al.*, 1999).

In addition to maladaptive antipredator responses, feeding, and learning behaviour, exposure to EDCs also has been associated with altered communication and reproductive behaviour in amphibians. For example, Park and Propper (2002) exposed male red spotted newts (*Notophthalmus viridescens*) to ecologically relevant levels of the insecticide endosulfan or to an acetone carrier control for four days. After the exposure period, olfactory response tests were run in a Y-maze in which each arm contained a female used to attract males. Individuals exposed to the low concentration of endosulfan took longer to respond to the presence of females. Morphological measurements of pheromone glands demonstrated that endosulfan decreased the alveolar and luminal area. Park and Popper (2002) also studied the mating success of individuals from endosulfan treatments by placing males with a female and recording the presence of spermatophores after 24 hours. They found that males from endosulfan treatments had dramatically lower mating success and concluded that environmental chemicals may lead to population declines in amphibians by disrupting chemical communication and mating behaviour (Park and Propper, 2002).

Implications

Conservation and Ecology

Contaminants are distributed globally; even areas once believed to be pristine are affected by run off, wind blown contaminants, or fallout through precipitation (Davidson and Knapp, 2007; Sparling *et al.*, 2001; Thurman and Cromwell, 2000). Importantly, many amphibians are very sensitive to contaminants and are exposed throughout their life history. For instance, they have highly permeable skin as adults and larvae. Many reproduce in aquatic environments affected by runoff or precipitation, so adults can be exposed throughout their lives, in transit to

breeding sites, and while breeding. Their larvae experience critical hormone-regulated developmental stages while in these potentially polluted environments, making them particularly susceptible to endocrine disruption. Living in, and around, contaminated areas and undergoing critical stages of development while exposed to endocrine-disrupting chemicals is expected to negatively affect individuals as well as populations (Hayes *et al*. 2006b; but see Glennemeier and Begnoche 2002).

The link between pollutants and amphibian declines has, in some cases, been weak, occurring as a *post hoc* hypothesis after declines have occurred. For example, the last remaining natural populations of Wyoming toads were known to be exposed to fenthion, an organophosphate insecticide used for mosquito control (Lewis *et al.*, 1985). Importantly, fenthion is known to block androgen receptors (in mammals) and androgen-dependent physiological processes (Tamura *et al.*, 2001). Carey and Bryant (1995) speculated that this exposure could have been involved in the ultimate extinction of wild Wyoming toads (*Bufo hemiophrys baxteri*). Male toads in the last population showed reduced clasping behaviour during breeding, which is likely an androgen-dependent behaviour, and hatchability of the few fertilized clutches was low (Carey and Bryant, 1995). Appropriate studies to evaluate the effects of fenthion on Wyoming toad populations were not conducted because the connection between the contaminant and the population extinction was made too late. Although Wyoming Toads have been reintroduced, they are not currently self sustaining, and research on these "wild" populations is hampered by the scarcity of the toads (Dreitz, 2006).

Importantly, however, several strong lines of evidence suggest that pollution induces population declines and extinctions of amphibians. For example, population declines of several amphibian species are associated with wind-borne agricultural chemicals (Davidson and Knapp,

2007; Sparling *et al.*, 2001). In an important and creative study, Sparling *et al*. (2001) used altered cholinesterase (ChE) activity to demonstrate that exposure to wind-borne pesticide was associated with reduced population status in the Pacific Tree Frog (*Hyla regilla*). Cholinesterase (ChE) is an enzyme that rapidly clears the neurotransmitter acetylcholine from neuronal synapses. The clearance of acetylcholine modulates neuron-to-neuron communication and is essential for proper muscle function. Many insecticides and some herbicides that are typically sprayed in agricultural areas contain pyrethoids and organophosphates. These chemicals are known to inhibit ChE activity and reduced ChE activity is a biomarker of exposure to these pesticides.

Sparling *et al*. (2001) found that cholinesterase (ChE) activity in *H. regilla* tadpoles from mountainous areas downwind of the highly agricultural Central Valley, where the most severe amphibian population declines in California have occurred, was depressed compared to those from sites on the coast or north of the valley where declines are less precipitous (Sparling *et al.*, 2001). In addition, as many as 86% of the populations in areas with reduced ChE had detectable levels of endosulfan, up to 50% had measurable organophosphorus levels, and 40% carried DDT residues. This study clearly establishes an association between pesticide exposure, physiological response to that exposure, and poor population status. Although a cause-and-effect relationship is difficult to establish with field studies that use such epidemiological approaches, these data are highly suggestive of such a relationship.

An alternative hypothesis that could explain population declines in these areas of California is that introduced non-native fish have increased mortality of tadpoles and driven populations to decline. Therefore, Davidson and Knapp (2007) studied the relative effect of introduced fish and pesticides on the mountain yellow-legged frog (*Rana muscosa*). They

conducted an enormous set of field surveys that included 6,831 sites studied over a seven-year period (1995-2002). At each site, they quantified habitat characteristics and presence or absence of *R. muscosa* and fish. They calculated windborne pesticide exposure for each site from pesticide application records and predominant wind directions. The probability of *R. muscosa* presence was significantly reduced by the presence of fish *and* pesticides. However, the effect of pesticides at the landscape scale was much stronger than the effects induced by fish (Davidson and Knapp, 2007). Importantly, the degree of protection from windborne pesticides was also a significant predictor of *R. muscosa* presence. These results demonstrate that windborne pesticides are contributing to amphibian declines in pristine locations (Davidson and Knapp, 2007). Many agricultural contaminants are known to function as endocrine disrupting chemicals that induce effects at very low concentrations. California's amphibian populations could be suffering declines not because they are exposed to high quantities or lethal concentrations of contaminants, but because they are exposed to very low concentrations of hormonally active agents that alter thyroid function, development, reproduction, and behaviour.

These contaminant-associated declines are not surprising given what is known about EDCs, but it is important to note that few studies have investigated the population-level effects of chronic exposure to pollution, nor have any, to our knowledge, explicitly modeled the way in which EDCs could be involved in the global amphibian decline. Chemical pollutants, including EDCs, are expected to play an important role in modulating population dynamics because they can directly induce mortality and, at sublethal levels, they influence growth and development, decrease the ability of larvae to avoid predators, and alter reproductive success (Carey and Bryant, 1995). In addition, because the immune system interacts directly with the endocrine

system, EDCs can affect susceptibility to pathogens (Carey and Bryant, 1995; Hayes *et al.*, 2006b).

Importantly, EDCs could contribute to recent outbreaks of infectious disease in amphibians, especially when they occur in complex mixtures as is typical of contaminated environments. For example, Hayes *et al* (2006) studied the effects of nine different agricultural pesticides used on cornfields in the Midwestern United States by exposing tadpoles of *Rana pipiens* to very low concentrations (0.1ppb) of each chemical, separately or in various combinations, and measuring differences in growth, development, gonadal differentiation, and immune function. Many of the single chemical treatments led to differences in larval growth and development but the pesticide mixtures had more severe effects. Pesticide mixtures damaged the thymus which was associated with immunosuppression and the contraction of flavobacteria meningitis (gram negative bacteria). This disease left the animals debilitated; they were in poor condition and unable to hold themselves upright. Interestingly, the control animals tested positive for the presence of this bacteria but did not show signs of the disease (Hayes *et al.* 2006). This type of interaction between contaminants and disease agents, making the disease more pathogenic, has been hypothesized for many years (Carey and Bryant, 1995).

Another recent study has investigated the influence of EDCs on disease susceptibility by exposing tiger salamanders (*Ambystoma tigrinum*) to atrazine and measuring peripheral leukocyte levels and susceptibility to *Ambystoma tigrinum* virus (ATV), a pathogen implicated in some amphibian die-offs (Forson and Storfer, 2006). Atrazine significantly decreased peripheral leukocyte levels and increased susceptibility of the larvae to ATV infection, illustrating that atrazine influences the immune system. Interestingly, there were familial differences in infection rates, making particular genotypes more (or less) sensitive. Over all, this study shows that in

addition to its effects on testicular development, ecologically relevant concentrations of atrazine can have immunosuppressive effects that could contribute to ATV epizootics (Forson and Storfer, 2006).

Evolution

Another important consequence of EDC exposure is that it can alter the evolutionary trajectory of a species. A classic example of pollutant-induced evolutionary change is the darkening of the peppered moth (*Biston betularia*) in response to the darkening of trees by soot in England during the industrial revolution. There are also many taxa that have evolved pesticide resistance. Pollutants can induce strong selective forces by increasing mortality and altering genetic variability. Sublethal levels of pollutants can also influence evolutionary processes. For example, intergenerational transfer of contaminants (through egg yolk or mother's milk) can alter development, reproductive physiology, metabolism, and behaviour in maladaptive ways (Fox, 1995). In addition, many recent experiments have demonstrated that gene imprinting and induction of epigenetic modifications through methylation is controlled, at least is some cases, by estrogen or other hormones and can be modulated by EDCs such as methoxyclor. Therefore EDCs can induce persistent, heritable phenotypic changes, independent of mutagenesis. These phenotypic variants will be subject to selection and the evolutionary trajectory of affected species is expected to be altered. Evolutionary changes are in many cases irreversible and they impart an environmental legacy that is broader than that of the pollutant itself (Leblanc, 1994).

What Needs to Be Known

Hayes *et al*. (2006) reported that fewer than 30 published laboratory and field studies have addressed low concentration, endocrine-disrupting effects on amphibians. A few studies have examined the effects of contaminant exposure on wild amphibian populations (Davidson and Knapp, 2007; Ouellet *et al.*, 1997; Sparling *et al.*, 2001) but establishing cause and effect in such

studies is difficult, and describing physiological alterations inducing effects at the population level is daunting. Regardless of these difficulties, understanding the population-level effects of contaminants on amphibians and the role of EDCs in global amphibian decline is extremely important, albeit understudied. For example, although several studies have demonstrated that some chemicals can induce feminization of males and intersexuality, little work has focused on the population-level effects of male feminization; this problem is not restricted to amphibians (Hayes *et al.*, 2006a).

Many studies on the effects of contaminants such as EDCs have been conducted in controlled laboratory settings. Although these studies are extremely important, and necessary, for determining the mechanisms of toxicity, they do not even attempt to replicate natural systems. For example, many studies investigate the effects of chemicals at concentrations much higher than individuals experience in the wild. Reported effects from these studies do not help explain whether exposed populations will suffer similar effects because the mechanisms of toxicity of EDCs can depend on concentration. A number of studies have documented inverted U-shaped dose responses to EDCs; that is, low to intermediate concentrations can produce larger effects than high doses due to the integrated nature of physiological responses, and the presence of negative feedback mechanisms. For example, survival was significantly lower for four different amphibian species when they were exposed to 3 ppb of atrazine compared with either 30 or 100 ppb and these mortality patterns depended on tadpole stage (Storrs and Kiesecker, 2004). Therefore, the deficiency in studies on the effects of lower, ecologically relevant concentrations of EDCs in amphibians has likely resulted in an underestimation of the impacts of these pesticides (Carey and Bryant, 1995; Hayes *et al.*, 2006a; Hayes *et al.*, 2002). In addition, wild amphibian populations are exposed to complex mixtures of contaminants and other

stressors, such as UV radiation, but most studies have investigated the effects of single contaminants without considering other environmental variables (Blaustein *et al.*, 2003; Hayes *et al.*, 2002; Sullivan and Spence, 2003). Contaminant concentrations also fluctuate drastically in natural systems, and these fluctuations can induce unexpected and unpredictable effects, but few studies have investigated fluctuating contaminant exposures (Edwards *et al.*, 2006). Furthermore, most studies focus on effects of contaminants during, but not after, exposure. This can lead to underestimations of the effects of contaminant because survival of later life-history stages (e.g. adults) could be negatively affected whereas earlier, exposed, stages are not (Harris *et al.*, 2000; Rohr and Palmer, 2005; Rohr *et al.*, 2006b). More realistic exposures that replicate wild systems, and investigate effects across amphibian life history will help describe the effects that contaminants have on amphibian populations.

Questions that attempt to link individual-level effects induced by a contaminant in the laboratory to effects in the field are difficult and require integration across broad fields that have traditionally been isolated from one another. Although recent studies have begun to address these issues, it is not clear how the mechanisms of toxicity or the effects of chemical pollutants change relative to competition, predation, alterations in animal density, food quantity, or the presence of pathogens (Forson and Storfer, 2006; Relyea and Mills, 2001; Rohr *et al.*, 2004; Rohr *et al.*, 2006b; Romansic *et al.*, 2006). Knowing how ecological factors influence an organism's response to chemicals will help predict the effects of pollutants in the wild, and will advance an understanding of how to restore polluted habitats (Rohr *et al.*, 2006a).

The phenomenon of endocrine disruption has been focused on documenting estrogenic, androgenic, antiandrogenic, and antithyroidal actions of specific chemicals, and these pathways of toxicity have now been established in numerous vertebrate species (Guillette, 2006). Many

potential mechanisms of endocrine disruption, however, have not been studied. For example, little work has been conducted on the effects of EDCs on other steroid pathways, such as those including the progestins (e.g. progesterone) and glucocorticoids (e.g. the stress hormone corticosterone) or on the retinoids (Berube *et al.*, 2005; Guillette, 2006; Hinson and Raven, 2006; Leiva-Presa and Jenssen, 2006). Importantly, it is known that progesterone is important for oocyte maturation in amphibians and that EDCs, such as methoxychlor, disrupt progesterone-induced oocyte maturation in *Xenopus leavis* (Guillette, 2006; Pickford and Morris, 1999, 2000; Pickford and Morris, 2003). In addition, the adrenal is thought to be the most common toxicological target organ for EDCs (Harvey *et al.*, 2007) but relatively little work has focused on understanding the effects of EDCs on the stress axis (hypothalamus-pituitary-adrenals) (Pottinger, 2003). Evaluation of the adrenals are also neglected in EDC screening and testing for regulatory purposes (Harvey *et al.*, 2007). Finally, retinoid imbalances are associated with multiple effects, including changes in secondary sexual characteristics, inhibition of spermatogenesis, and decreased embryo survival. Importantly, retinoid homeostasis is affected by several contaminants (Berube *et al.*, 2005). Future studies on endocrine disruption must broaden current approaches. The mechanisms and endocrine end points examined need to be expanded, and studies that allow evaluation of how organisms are affected in wild habitats need to be conducted (Guillette, 2006).

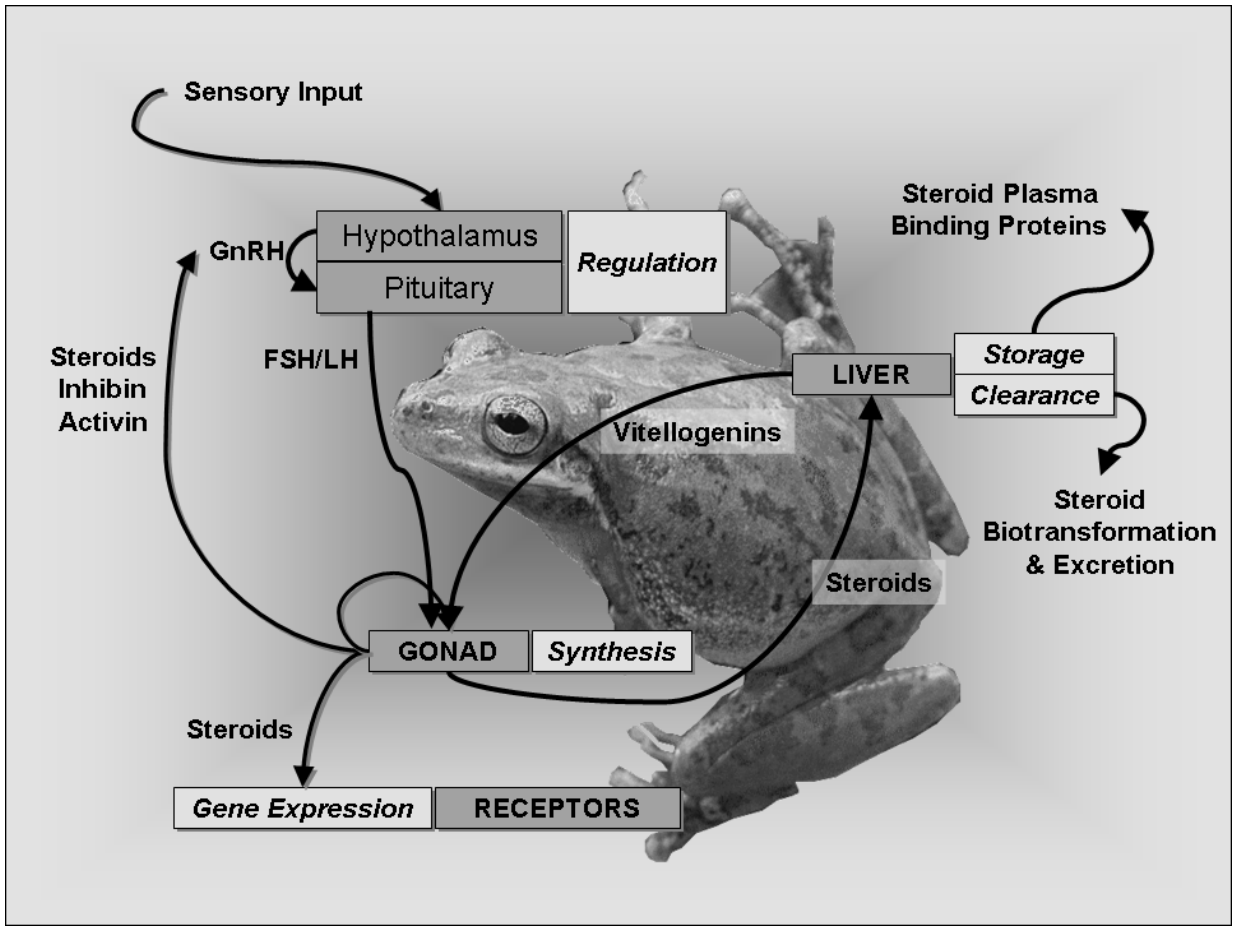

Figure 1-1 The endocrine system regulates physiological processes by integrating signals from the external environment via the sensory system and internal signals that are conveyed by endogenous (or internal) hormones (e.g. GnRH, FSH, LH, steroids, inhibin, activin). These hormones are secreted by various organs (dark gray boxes), and regulate specific functions (light gray boxes) within target organs. Any physiological, behavioral or developmental process that is controlled or influenced by the endocrine system is susceptible to modification by EDCs. For example, EDCs alter the synthesis of steroid hormones (e.g. within the gonad), by altering key steroidogenic enzymes. Altered steroid production affects feedback mechanisms to the brain which changes endocrine regulation. Altered steroid production also changes gene expression within target cells and organs. EDCs can also directly interfere with gene expression by binding to nuclear steroid receptors. At the liver, EDCs can interfere with hormone degradation and clearance, and alter the function of steroid plasma binding proteins. All of these processes (light gray boxes) are crucial for hormonal homeostasis.

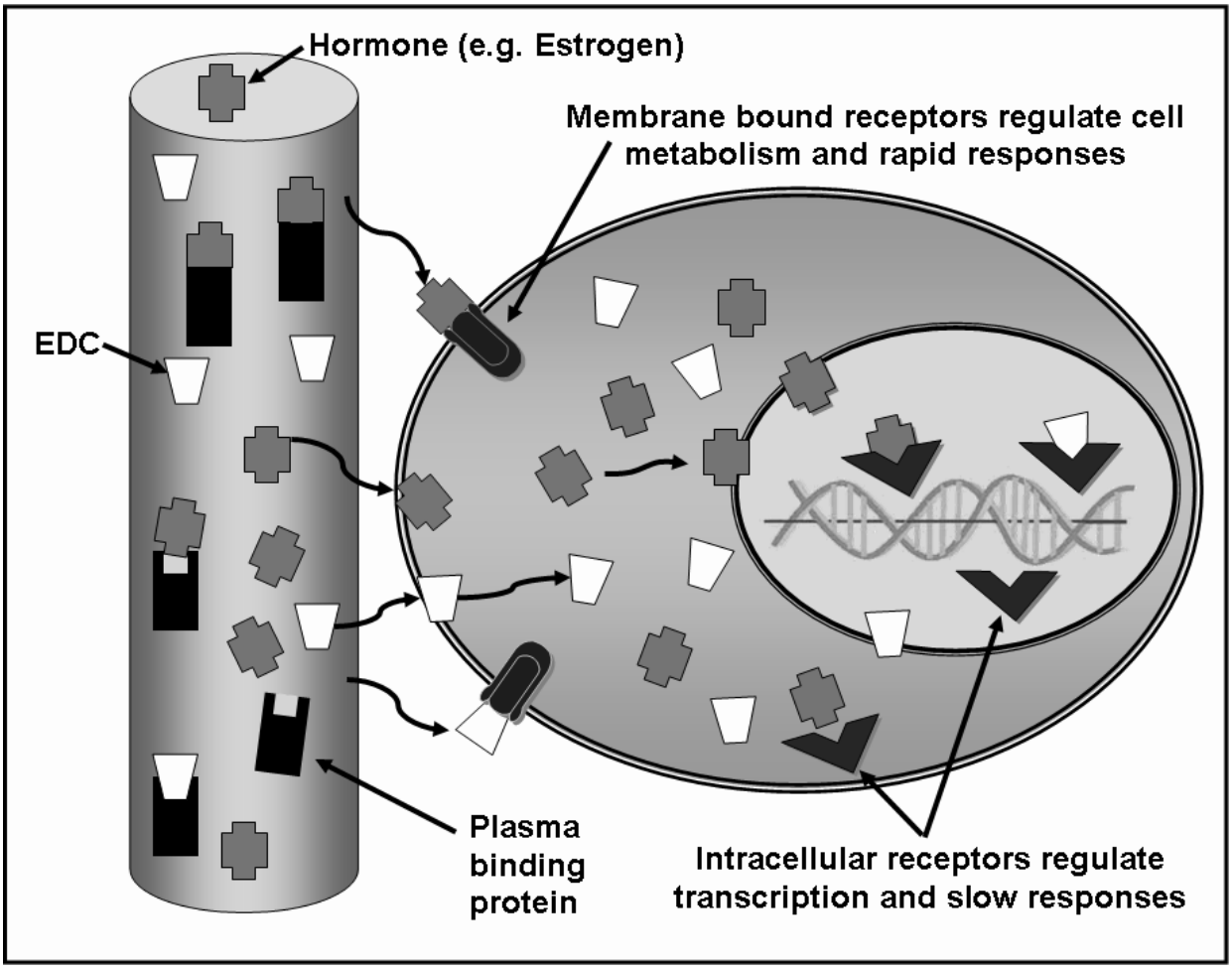

Figure 1-2 Lipophilic hormones (grey crosses) easily diffuse from a capillary into a target cell and bind to specific intracellular protein receptors (Black Vs). This complex (receptors and ligand) binds to the DNA and induces gene expression that modulates a wide range of physiological functions such as cellular growth and proliferation, development and differentiation, and maintenance of homeostasis (slow responses). Most EDCs (white trapezoids) identified to date influence gene expression by interacting with the steroid, thyroid, and retinoid receptors found within the cell (Black Vs). Some EDCs can bind to and activate a ligand receptor which induces gene expression of responsive genes, whereas others block gene expression by competing for receptors without mimicking biological function. Recent evidence suggests that EDCs can also bind to membrane receptors and influence rapid cellular responses such as metabolism.

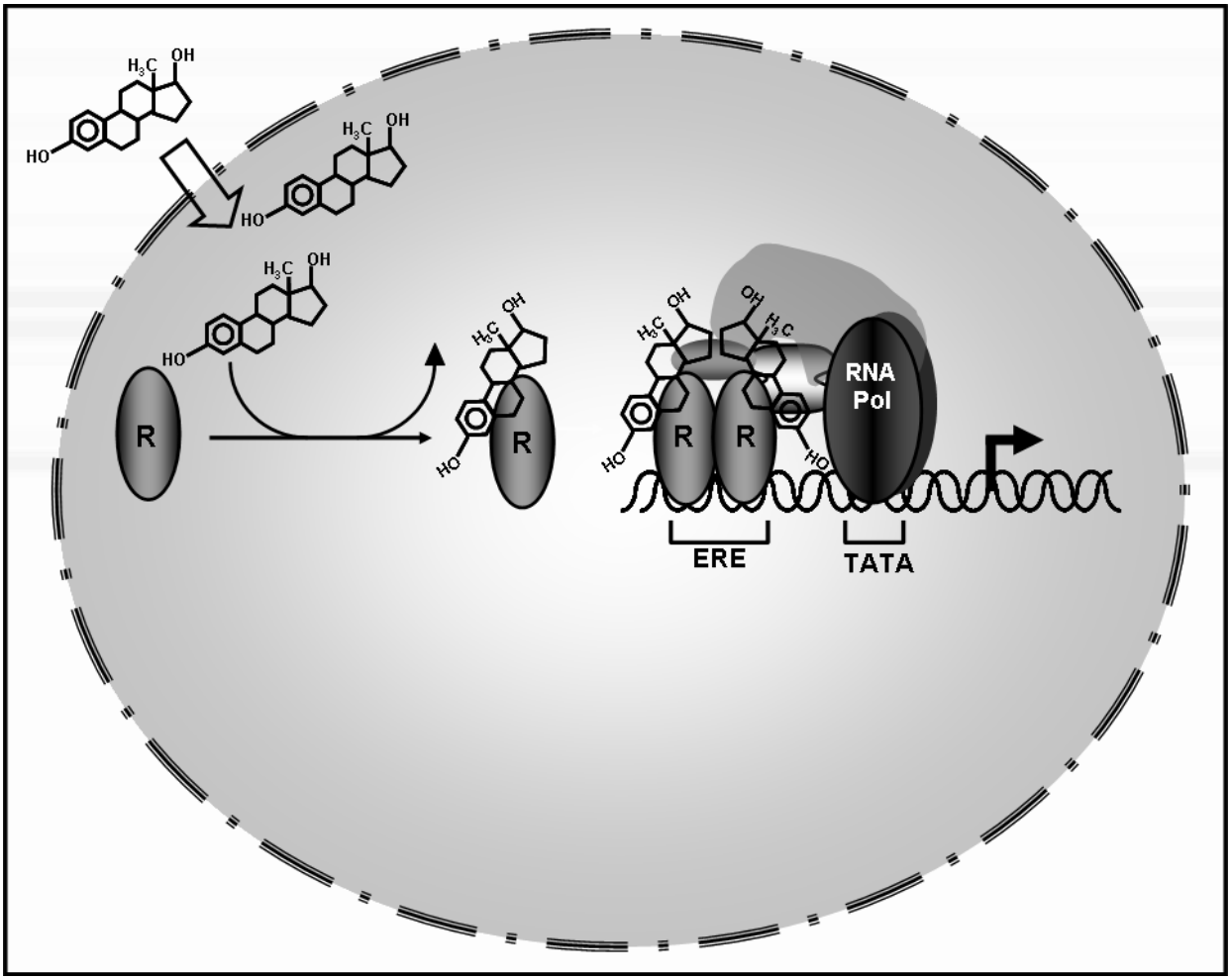

Figure 1-3 Steroid, retinoid, and thyroid hormones are lipophilic substances that can cross the nuclear membrane (dashed circle). These hormones bind specific receptors (grey oval labeled "R") that induce allosteric transformation and enable the hormone-receptor complex to bind to high affinity sites in the DNA, called response elements (REs). This binding modulates gene transcription. EDCs can interfere with receptor binding, modulate receptor degradation, alter receptor activity, or the activity and availability of co-factors (ovals shown above and between the receptor complex and RNA polymerase). All of these processes are essential for normal receptor function and gene expression. Altered gene expression can lead to a variety of negative effects including abnormalities in the structure and function of organs, or induction of cancers.

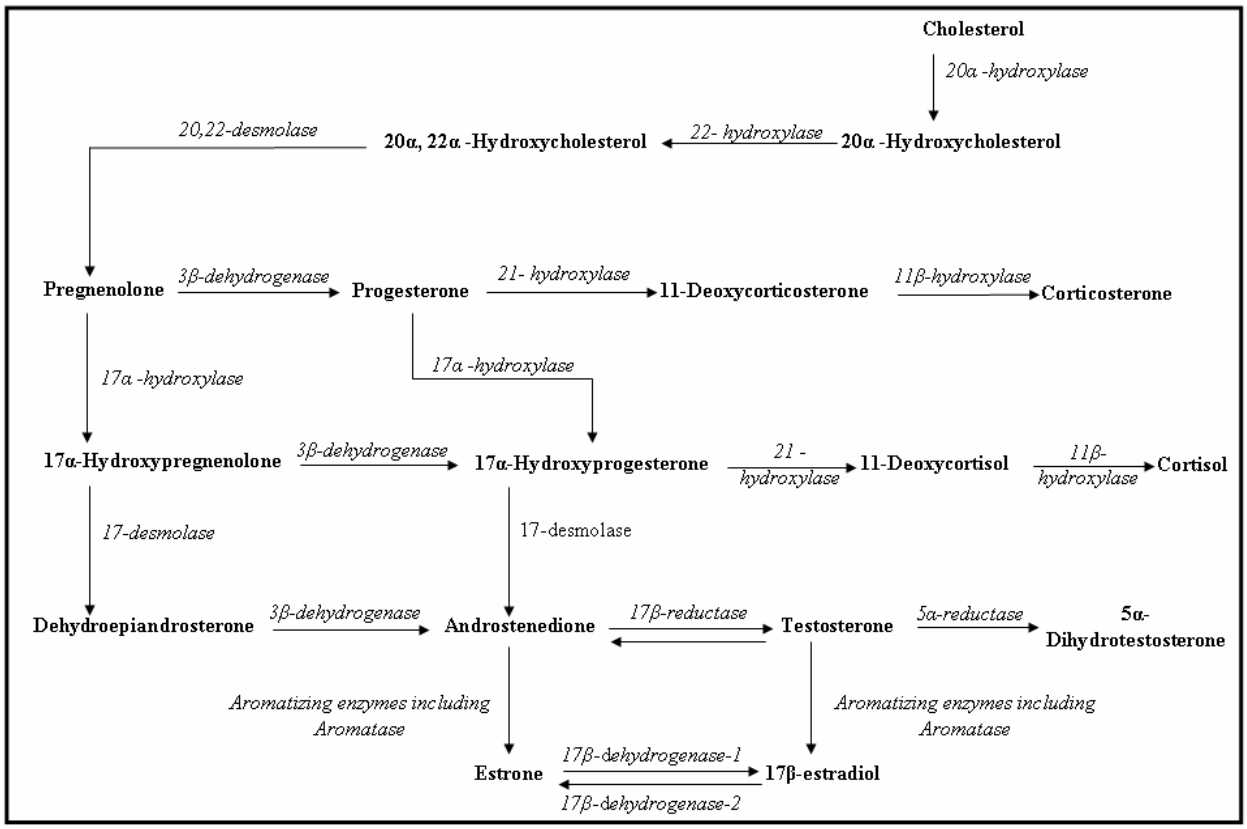

Figure 1-4 Steroid hormone synthesis involves a complex network of chemical reactions that convert a cholesterol based substrate into a product (e.g. hormones) through the activity of several substrate-specific cytochrome P450 enzymes, steroid dehydrogenases, and reductases. The activity of many of these enzymes can be modulated by various endocrine disrupting chemicals resulting in impaired development, growth, sexual differentiation, reproduction, and the development of particular cancers.

CHAPTER 2
GROSS GONADAL ABNORMALITIES AND ALTERED SECONDARY SEXUAL TRAITS ARE ASSOCIATED WITH AGRICULTURE

Introduction

Endocrine signaling regulates reproductive system development and plays an important role in organizing embryonic tissues to respond to hormonal signals later in life (Guillette *et al.*, 1995). Therefore, exposure to endocrine disrupting chemicals that alter normal hormonal signaling during embryonic development can permanently change reproductive system morphology and function of adults (Guillette *et al.*, 1995; McLachlan, 2001). Many of studies that have documented endocrine disrupting effects and associated reproductive abnormalities in wildlife populations have focused on the effects of sewage or pulp mill effluent on fish (Jobling *et al.*, 2002a; Jobling *et al.*, 2006; McMaster, 1995; McMaster *et al.*, 1991; Munkittrick *et al.*, 1994; Tyler & Routledge, 1998; van Aerle *et al.*, 2001; Woodling *et al.*, 2006). However, many of the chemicals used in agricultural practices are known endocrine disruptors that influence the thyroid and reproductive systems of non-target wildlife. For example, cattle feedlot effluent contains androgens from hormone implants that negatively affect the endocrine and reproductive systems of wild fathead minnows (*Pimephales promelas*) (Ankley *et al.*, 2005; Ankley *et al.*, 2001; Ankley *et al.*, 2003; Orlando *et al.*, 2004). Many pesticides disrupt the endocrine system of non-target wildlife and induce diverse reproductive abnormalities. For example, pesticides from farming practices negatively affect steroidogenesis, circulating hormone levels, hepatic transformation of androgens, and gonadal and thyroid morphology of American alligators (*Alligator mississippiensis*) (Guillette, 2001; Guillette *et al.*, 1994; Guillette & Iguchi, 2003; Hewitt *et al.*, 2002). Pesticides such as the fungicide vinclozolin negatively affect fish and mammal reproduction, and alter germ cell DNA methylation patterns making these reproductive effects heritable which could lead to population declines (Ankley *et al.*, 2005; Crews *et al.*, 2007;

Gray, Ostby & Kelce, 1994; Gray, Ostby & Marshall, 1993). Recent studies have suggested that agricultural contaminants are associated with amphibian reproductive abnormalities and population declines (Davidson & Knapp, 2007; Ouellet *et al.*, 1997; Sparling, Fellers & McConnell, 2001). Amphibians are declining on a global scale and the role that pollutants play in these declines is relatively unclear.

Amphibians make appropriate models for studying the effects of EDCs on wildlife, in general, and might serve as bio-indicators of EDC exposure. Their gonadal differentiation and secondary sexual characteristics are highly sensitive to sex hormones, and chemicals that alter sex hormone function (Hayes, 1998; Qin *et al.*, 2003). Alterations of normal gonadal function and hormone concentrations can generate identifiable changes in secondary sexual traits that are indicative of endocrine disruption and may be used as visual tags or biomarkers for such disruption (Hayes & Menendez, 1999; Noriega & Hayes, 2000; van Wyk, Pool & Leslie, 2003).

The objectives for this study were to survey *Bufo marinus*, across a gradient of agricultural intensities to investigate whether reproductive abnormalities in gonadal morphology are associated with agriculture, and to determine if gonadal abnormalities are associated with altered gonadal function. One of the primary functions of gonads is to produce hormones that control the expression of secondary sexual traits. Therefore, I compared the secondary sexual characteristics of toads with morphologically normal and abnormal gonads to determine whether gonadal function was compromised in toads with abnormal gonadal morphology. I also investigated whether specific secondary sexual characteristics vary as a function of abnormal gonadal morphology in predictable ways that promote the development endocrine disruption biomarkers. I hypothesized that toads collected from sites with greater agricultural activity, where known EDCs are routinely used (Appendix 1), would have a higher incidence of

reproductive abnormalities than toads collected from suburban sites with little to no agriculture nearby. In addition I hypothesized that toads with gonadal abnormalities would display predictable and easily measured abnormalities (biomarkers) in their secondary sexual traits.

The proportion of agriculture at each collection site in this study is inversely related to the degree of urbanization, and its associated stressors. Suburban sites are not pristine habitats; they are typically polluted with polyaromatic hydrocarbons (PAHs) associated with road runoff (Crosbie & Chow-Fraser, 1999; Maltby *et al.*, 1995). PAH concentrations have been used to estimate the proportion of urbanized land among different watersheds (Standley, Kaplan & Smith, 2000). Agricultural areas, however, are typically polluted with pesticides used to control weed, insect, and rodent populations (Crosbie *et al.*, 1999). Although, many people living in suburban areas use pesticides on their lawns urbanized and agricultural habitats are polluted with different milieus of chemicals that are expected to induce different suites of pathologies. I find that reproductive abnormalities indicative of endocrine disruption increase with agricultural intensity and are correlated with agricultural pollutants.

Methods

Study System

Female *Bufo marinus* are multi-colored (with spots ranging from white to dark brown or black, Figure1), whereas males are a solid toffee color (Lee, 1996; Zug, 1979). The dark coloration found on females is caused by high concentrations of melanin, which is thought to be modulated by estrogen (Costin & Hearing, 2007; Shah & Maibach, 2001). Therefore, populations of males that have higher endogenous levels of estrogen, or have been exposed to estrogenic chemicals are predicted to have darker skin and more mottling typical of females.

Male *Bufo marinus* have larger forelimb bones, and hypertrophied musculature relative to similarly sized females (Lee & Correales, 2002) (Figure 2-1). Forelimb musculature in males is

androgen dependant, and is believed to aid males during amplexus (Lee *et al.*, 2002). Reductions in androgens are likely to reduce forelimb size, and potentially negatively affect male reproductive success.

Male *Bufo marinus* also have nuptial pads on their first through third phalanges that function in mating (Figure 2-1) (Epstein & Blackburn, 1997; van Wyk *et al.*, 2003). The presence of epidermal hooks, secretory activity, and the number of nuptial pads are androgen dependent and occur only when animals are reproductively active (Epstein *et al.*, 1997; van Wyk *et al.*, 2003). Thus, nuptial pad activity and morphology during the breeding season is an index of both androgen and reproductive status (Epstein *et al.*, 1997; van Wyk *et al.*, 2003).

Another key characteristic of B*ufo marinus* that makes them an especially useful model in studies of endocrine disruption is that both male and female bufonids possess a Bidder's organ, which has been characterized as a "non-functional" or vestigial ovary (Farias, Carvalho-e-Silva & do Brito-Gitirana, 2002). Although it contains oocytes at various stages of oogenesis, there are several clear differences between ovaries and the Bidder's organ. Morphologically, an ovarian pocket does not form in the Bidder's organ (Falconi, Dalpiaz & Zaccanti, 2004; Falconi, Dalpiaz & Zaccanti, 2007; Witschi, 1933). In addition, the oocytes of the Bidder's organ do not accumulate vitellogenins, which are the female-specific egg yolk protein precursors (lipophosphoglycoproteins) produced in the liver under estrogen stimulation, and transported to maturing oocytes (Brown, del Pino & Krohne, 2002; Falconi *et al.*, 2004; Falconi *et al.*, 2007; Farias *et al.*, 2002). Although the functional significance of the Bidder's organ is unknown, this organ does not undergo vitellogenesis (oocytes are not yolked) in normal individuals (males or females); oogenesis in the Bidder's organ normally ends with degeneration of large previtellogenetic oocytes (Brown *et al.*, 2002; Falconi *et al.*, 2004; Falconi *et al.*, 2007; Farias *et*

al., 2002). Even when high levels of gonadotropins are administered, oogenesis of the Bidder's organ remains inhibited (Pancak-Roessler & Norris, 1991). Therefore, bufonids are not hermaphroditic, and under normal conditions the Bidder's organ does not function as an ovary in males (Hayes, 1998). Experimental castration, however, induces the oocytes in the Bidder's organ to become morphologically identical to ovarian oocytes, and they can then undergo vitellogenesis (Brown *et al.*, 2002). In addition, reducing the level of dehydrotestosterone in developing bufonids increases development and growth of Bidder's organ oocytes (Zaccanti *et al.*, 1994). Therefore, testes inhibit vitellogenesis in the Bidder's organ of males, and this inhibition is likely controlled, in part, by testicular androgens (Pancak-Roessler *et al.*, 1991; Zaccanti *et al.*, 1994)

Study Sites and Collection Techniques

I surveyed five sites in south Florida that varied in habitat type from completely agricultural to suburban (Figure 2-2), to investigate the association between intensity of agriculture and reproductive abnormalities in *B. marinus*. I calculated the total amount of agricultural land within a 5.6 km^2 area around each collection site, to determine the intensity of agriculture. The home range of *B. marinus* is ~2km^2 (Zug, Lindgren & Pippet, 1975), so this grid includes the area likely to be experienced by a toad from each local site.

I imported Google Earth digital satellite images and scale into Image Pro image analysis program (on August 20, 2007), and centered a 5.6 km^2 grid over each collection site. Each of the 9 cells within the grid represented approximately 622 m^2. Using these quadrats I estimated the percentage of agricultural area (vegetable and sugarcane farming, no livestock) within each cell, and averaged these values for each site. The five study sites ranged in percentage of agricultural land from 0 to 97%. Two sites (Wellington and Lake Worth) were completely suburban and contained zero agricultural land, but they varied in proximity to agriculture. Therefore, to

distinguish these two sites I calculated distance from each site to the closest agricultural area. Wellington (WT) was closer to farm land (5.18 km away) than Lake Worth (LW) (22 km away).

The sites were then assigned an "agricultural intensity" score of one through five that was based on the percentage of agriculture in the 5.6 km2 area around the site or its proximity to agriculture when there was no agriculture nearby (Figure 2-2). Lake Worth (LW) did not contain any agriculture, was completely suburban, and it was farthest from an agricultural area; therefore it was assigned a rank of one. Wellington (WT) was assigned a rank of two. Homestead (HS) contained 34% agricultural land and was ranked as three. Canal Point (CP-51.2%) and Belle Glade (BG-97%) contained the most agricultural land and were ranked 4 and 5 respectively.

At least twenty sexually mature adult individuals (>91mm, (Cohen & Alford, 1993) were collected from each site. Collections occurred at BG, CP, and WT during the summers of 2005 and 2006, HS toads were collected in summer 2005 and JP animals were collected in summer 2006. There was no effect of year on the percentage or types of abnormalities documented at sites where collections occurred in more than one year.

Toads from all locations were collected, while feeding, under streetlights. Each individual was euthanized with an overdose of the anesthetic MS222 (0.3% Tricaine Methanesulfonate, pH 7), photographed (Konica Minolta Dimage Z10 with 3.2 mega pixels), and measured for mass (Ohaus Scott 2) and snout vent length (Spi digital caliper). Gonads were removed and one gonad from each individual was fixed in neutral buffered formalin for gross morphological analysis.

Analysis of Primary Sexual Characteristics

Individuals were classified into four broad categories based on their gonad morphology to determine whether gonadal abnormalities increased with agriculture intensity: 1) normal males, 2) abnormal males, 3) intersex individuals, and 4) females. Normal males had testes and non-vitellogenic Bidder's organs, and no ovaries or oviducts. Abnormal males had testes and

vitellogenic Bidder's organs, but no ovaries or oviducts. Intersex individuals had testes, and ovaries or oviducts (the Bidder's organ was not considered for this grouping). Females had ovaries, oviducts, non-vitellogenic Bidder's organs, and no testes.

Normal, abnormal, and intersex individuals were ranked (1,2, and, 3 respectively) and differences in the mean rank across sites were evaluated with a Kruskal-Wallis one way analysis of variance to test whether sex group rank varied across sites. Kendall's Tau rank correlation was used to determine if the change in the sex group rank corresponded to agricultural intensity (test of association).

I created a scoring index to categorize individuals based on the type and total number of gonadal abnormalities each toad possessed. No obvious gonadal abnormalities were seen in females. Therefore, I focused on gonadal abnormalities in normal and abnormal males and intersex individuals. Scores were assigned based on abnormalities in each of the following three tissue types: 1) testes, 2) Bidder's organ, and 3) ovaries/oviducts (in the presence of testes and a Bidder's organ). Abnormalities involving testes included multiple and misshaped testes. Bidder's organ abnormalities included vitellogenic Bidder's, or multiple Bidder's organs. Abnormalities involving ovaries/oviducts included the presence of regressed ovaries, vitellogenic ovaries, multiple ovaries, or oviducts (in the presence of testes and Bidder's organs). As an example of my scoring protocol, an individual that had misshaped testes (1 abnormality in testes category), a vitellogenic Bidder's organ (1 abnormality in Bidder's organ category), and ovaries that were also vitellogenic (2 abnormalities in ovary/oviduct category) received a score of 4. The number of individuals with each abnormality type was evaluated across sites with a Kruskal-Wallis on way analysis of variance (SPSS 15.0) to determine if the occurrence of specific abnormalities differed across sites, and Kendall's Tau rank correlation was used to test whether

individual abnormalities were associated with agricultural intensity. In addition, the total number of abnormalities were summed and similarly evaluated to determine if more gonadal abnormalities occurred at specific sites and if the number of abnormalities corresponded to increased agricultural intensity. I was interested in evaluating whether the frequency of abnormalities corresponded with increasing agricultural intensity therefore I used a one-tailed test for all Kendall's Tau rank correlations (Siegel & Castellan, 1988).

Analysis of Secondary Sexual Characteristics

Therefore, I used photographs to compare number of nuptial pads, body color pattern, and forelimb size in relation to sex group (female, normal male, abnormal male, and intersex) to determine if secondary sexual traits varied among normal, abnormal, and intersex individuals.

Nuptial pads

I calculated the mean number of nuptial pads per forelimb for each intersexed, abnormal, and male individual. No individuals scored as female had nuptial pads. Univariate one way analysis of variance (ANOVA, SPSS 15.0) was used to determine if the number of nuptial pads was different among groups with different degrees of reproductive abnormalities (normal, abnormal and intersexed).

Color pattern

I characterized the amount of mottling across an individual's body (all sexes included) by drawing a transect line from the left eye to the vent directly onto the image of each individual using Image Pro Plus analysis program, and counted the number of color changes that occurred across this transect (Figure 2-1). Mottling scores of sex groups were compared using a one way ANOVA. I analyzed male and female mottling scores across sites using a two-way ANOVA (SPSS 15.0) to test for differences in color dimorphism (by sex) across sites (sex*site).

Forelimb size

I measured the width of the forelimb across the radioulna distal to the humerus (elbow), and perpendicular to the arm axis using Image Pro Plus analysis software (Figure 2-1) to test for differences among sex groups. I used ANCOVA with $\log_{10}$ elbow width as the dependent factor, $\log_{10}$ (body length) as the covariate, and gonadal abnormality group (male, abnormal, or intersexed) as the fixed effect to account for the influence of body size. Slopes were not significantly different across sex groups (interaction not significant), so the interaction was removed from the analysis, and the simplified ANCOVA (SPSS 15.0) was used to estimate marginal means for the elbow width of each group (+/- 95% confidence interval).

Results

Analysis of Primary Sexual Characteristics

Sex group rank (males=1, abnormal=2, intersex=3) increased as a function of agriculture (KW = 25.16, df = 4; $p<0.001$; Kendall's Tau = 0.8, p=0.008; Figure 2-3a). More intersex individuals occurred at the two most agricultural sites relative to the other sites. The total number of gonadal abnormalities also differed among sites and increased with increasing agriculture (KW = 21.2, df = 4; $p<0.001$; Figure2-3b, Kendall's Tau =0.8, p=0.008). Testicular abnormalities were relatively common but did not vary across sites or increase as a function of agricultural intensity (KW = 5.148, df = 4; p=0.272; Kendall's Tau =0.3, $0.24>p<0.41$, data not shown). Bidder's organ abnormalities varied marginally across sites (KW = 9.04, df = 4; p=0.06; Figure 2-3c) and was highest at the agricultural sites (Figure 2-3c), but did not statically correspond to increasing agriculture (Kendall's Tau =0.4, p=0.24). The rank of female tissue abnormalities was significantly different among sites and increased with increasing agricultural intensity (KW = 17.3, df = 4; p=0.002; Figure 2-3d, Kendall's Tau =0.8, p=0.008).

Secondary Sexual Characteristics

Nuptial pads

The mean number of nuptial pads was greatest for males (mean = 2.3) and was lower for abnormal males (mean = 2.1) and intersexed individuals (mean = 1.7) ($F = 3.5$, $df = 2,93$; $p=0.035$; Figure 2-4). Fifty percent of the normal males had three nuptial pads per forelimb (one on each digit), whereas only 33% of the abnormal and 29% of intersexed individuals had three nuptial pads (Figure 2-4). Forty-six percent of intersexed individuals had zero or one nuptial pad per forelimb compared with <20% for either abnormal or male individuals.

Mottling

Toads differed in mottling score as a function of sex category ($F=22.9$, $df= 3, 150$; $p<0.001$). Females were more mottled (mean mottling score 9.6) than males (mean mottling score 5.4), and intersex individuals were intermediate (mean mottling score 7.6) between and significantly different from both females and normal males (Figure 2-5). Abnormal males had a mean mottling score similar to intersex individuals (7.4), but showed such high variation that they were not distinguishable from any sex groups (Figure 2-5).

There was a significant decrease in mottling sexual dimorphism when males and females were compared by site, which was indicated by the significant interaction between sex and site ($F=2.7$, $df= 4, 103$; $p<0.033$). Although female mottling did not change as a function of agriculture, the sexual dimorphism in mottling became less pronounced as agricultural intensity increased (Figure 2-6).

Forelimb size

Forelimb width was also significantly different among sex groups ($F = 65.48$, $df 3, 89$; $p<0.001$) compared at the grand mean of SVL=2.08). Females had significantly smaller forelimbs than any other group (raw mean = 1.15cm), and as with color pattern intersex

individuals were intermediate (raw mean = 1.37cm) to and significantly different from both males (raw mean 1.51cm) and females (Figure 2-7). Abnormal males (raw mean 1.47cm) had larger forelimbs than females, and their forelimbs were intermediate in size between intersexed or normal males.

Discussion

Wildlife species living in agricultural areas, or areas polluted by agricultural runoff, are known to have various reproductive abnormalities, and controlled laboratory experiments have demonstrated that chemicals used at these sites can alter endocrine system function (Guillette *et al.*, 1994; Hayes *et al.*, 2003; Orlando *et al.*, 2004). This study shows that reproductive abnormalities in primary sexual traits of *Bufo marinus* are associated with agriculture. Intersexed individuals occur more commonly at agricultural sites than at less agricultural sites. The number and developmental stage of female gonadal tissues found in the presence of testes in this study is considered a measure of the severity of the intersexed condition. This index of intersexuality increased with agricultural intensity in a dose dependent manner (Figure 2-3 d). This dose response relationship is suggestive of a causal link between agricultural contaminants and development of intersexed condition. The exact cause for these abnormalities can not be identified by this study. However, nitrate (fertilizer), and some of the pesticides (e.g.s atrazine, glyphosate) used at the sites in this study are known endocrine disruptors, and are likely inducing the documented abnormalities.

Toads from agricultural sites had a variety of gonadal deformities that alter gonadal function and might reduce reproductive success. For example, vitellogenesis of the Bidder's organ in males is only known to occur after castration, which has lead to the conclusion that the testes are necessary in males to suppress vitellogenesis within the Bidder's organ (Brown *et al.*, 2002; Pancak-Roessler *et al.*, 1991; Zaccanti *et al.*, 1994) However, we found that

approximately 20% of the individuals (with testes) at the agricultural sites had vitellogenic oocytes within their Bidder's organs, which suggests that the testes of these toads do not function normally to suppress the Bidder's organ.

My data demonstrates that secondary sexual characteristics were altered in toads with abnormal and intersexed gonads, which suggests that testicular function was compromised in toads with these abnormalities. Secondary sexual characteristics are maintained via endocrine signaling, and are typically modulated via sex steroids produced in the gonads (Hayes *et al.*, 1999). For example, nuptial pads are androgen dependent breeding glands found on the digits of the forelimbs of several anuran (frog and toad) species, and function in mating (Emerson *et al.*, 1999; Epstein *et al.*, 1997; Lynch & Blackburn, 1995; Rastogi & Chieffi, 1971; Thomas & Licht, 1993; Thomas, Tsang & Light, 1993; van Wyk *et al.*, 2003; Wetzel & Kelley, 1983). In this study, the proportion of individuals with maximal nuptial pad development (e.g. three nuptial pads per forelimb) was lower in intersexed individuals suggesting that androgen status was compromised. Nuptial pad development is seasonal, and non-reproductive toads do not possess well developed nuptial pads. Therefore, decreased nuptial pad development in intersexed animals suggests that they might not be as reproductively active relative to normal males.

Forearm width, another androgen dependent sexually dimorphic secondary sexual characteristic, was altered in intersex individuals relative to males suggesting that they did not build the hypertrophied musculature typical normal levels of androgens, and thus properly functioning testes (Lee *et al.*, 2002). The dimorphism in nuptial pads and forearm width aids the male during amplexus, and is under sexual selection (Lee *et al.*, 2002). Therefore, abnormalities found in this study might adversely influence reproductive fitness and alter sexual selection.

The occurrence of intersex individuals could arise through feminization of males or masculinization of females. If females were masculinized I would have found individuals with relatively complete female reproductive tracts, and some combination of male traits (e.g., nuptial pads, decreased mottling, wider arms, small testes). Individuals did not have morphologies that appeared to be incrementally more masculine. No toads with relatively normal looking ovaries and small or partially developed testes were collected. Instead, intersex individuals typically had normal sized testes, but varied in ovary size and stage of oogenesis. In addition, no females (ovaries and oviducts, but no testes) were collected that had nuptial pads, or solid coloration. Females did not differ in color pattern across sites, but males categorized as normal were more mottled in agricultural areas, and the typical sexual dimorphism in mottling was not observed at the most agriculture site (Figure 2-6).

The sexually dimorphic external morphological traits of *B. marinus* make them an ideal model species for studying the effects of EDCs because these traits function as a visual "tag" for determining whether an individual is experiencing endocrine disruption. Female coloration in the reed frog (*Hyperolius argus*) is estrogen dependent, and early induction of female color pattern has been used as a biomarker for estrogenic activity (Noriega & Hayes, 1999; Noriega *et al.*, 2000). The dark skin coloration typical of female *B. marinus* is due to dense melanin concentrations that are thought to be modulated, at least in part, by estrogen and may, therefore, function as a biomarker of estrogen exposure as well (McLeod, Ranson & Mason, 1994; Shah *et al.*, 2001; Slominski *et al.*, 2004; Thorton, 2002).

The gross morphology of the Bidder's organ is an ideal biomarker for androgen status and testis function because it is known to develop into a vitellogenic ovary after experimental castration. Thus, animals exposed to antiandrogenic chemicals are expected to have vitellogenic

Bidder's organs. However, only bilateral orchidectomy has resulted in Bidder's organ oocyte growth and increased oogenesis, so the presence of one testis is sufficient to inhibit Bidder's organ development (Pancak-Roessler *et al.*, 1991). Therefore, the presence of abnormal Bidder's organs in toads investigated in this study suggests that testicular function was severely altered in agricultural toads.

In addition to having diagnostic external and internal biomarkers of endocrine disruption, *Bufo marinus* are widely distributed and could be used to study the relationship between various agricultural practices and the types and severities of reproductive abnormalities. A global study would provide important information about the types of pesticides that are associated with most abnormalities. In addition, *B. marinus* could be used to study the global distribution of EDCs and their effects across diverse habitats.

Although this study was not designed to determine the specific cause of the reproductive effects observed, a few of the pesticides used at these agricultural sites cause endocrine disruption and have well studied mechanisms of toxicity. For example, glyphosate (Round Up™) and atrazine are used at both sites and are known to disrupt steroidogenesis (Walsh *et al.*, 2000(Oliveira *et al.*, 2007; Soso, Barcellos & Ranzani-Paiva, 2007). Glyphosate disrupts steroidogenic acute regulatory (StAR) protein expression which modulates the initial step in the steroidogenic pathway (Walsh *et al.*, 2000). Such disruption leads to reductions in steroid hormone production, including both testosterone and estrogen, across diverse taxonomic groups. Glyphosate exposure leads to reductions in androgen and estrogen dependent traits. Although this study suggests that androgen dependent traits are reduced (demasculinization), possibly due to reduced androgens, I also found that estrogen mediated traits such as melanin are increased (feminization). Research conducted across several vertebrate classes have demonstrated that

atrazine exposure inhibits androgen-mediated development, *and* could produce estrogen-like effects by increasing aromatase transcription (Crain *et al.*, 1997; Fan *et al.*, 2007; Sanderson *et al.*, 2002). Therefore, the mechanisms of toxicity of atrazine are consistent with the patterns of demasculinization and feminization that we observed in this study, but other studies have not found strong relationships between agricultural use of atrazine and reproductive abnormalities in amphibians (Murphy *et al.*, 2006). The timing, amount, and fluctuation of contaminant exposure are important factors that determine the phenotypic response to EDCs, so these factors could explain the differences among studies (Edwards *et al.*, 2006; Guillette *et al.*, 1995; Milnes *et al.*, 2006).

Many of the observed gonadal abnormalities associated with agriculture in this study are likely reproductive deformities that occur during development and are maintained throughout the toads' adult lives. Intersexed gonads in this study are distinct ovarian and testicular tissues suggesting that reproductive development is fundamentally altered in toads living in these areas. The morphology of the intersexed gonads of these toads is dramatically different from the ovotestes that have been found in many fish species around the world after exposure to synthetic estrogens (Jobling *et al.*, 2002a; Jobling *et al.*, 2002b; Jobling *et al.*, 1998; Jobling *et al.*, 1996; Jobling *et al.*, 2006; Woodling *et al.*, 2006). Ovotestes occur when oocytes are found within the testes suggesting that endocrine signals leading to the differentiation of particular cells are disrupted. However, the abnormalities that we find in *B. marinus* from agricultural areas suggest that entire developmental cascades leading to the differentiation of the gonad are altered such that *both* male and female reproductive organs form. Both types of intersex phenotypes (distinct organs vs. ovotestes) alter gonadal function leading to altered secondary sexual traits, and could

decrease reproductive success. However, the developmental mechanisms leading to these different intersexed phenotypes are likely distinct and induced via different pathways.

Because, EDCs are distributed globally they have been hypothesized to be a factor in the global decline of amphibian populations (Carey & Bryant, 1995; Davidson *et al.*, 2007; Hayes *et al.*, 2002; Sparling *et al.*, 2001). However, it is important to note, that relatively little is known about the role EDCs play in amphibian population declines (McCoy and Guillette in press). I believe that *B. marinus* could be developed into a bioindicator of EDC exposure to facilitate molecular, cellular, and population level studies. With some creativity this toad which is often detrimental to wildlife species where it has been introduced, could be valuable for identifying, understanding, and mitigating the effects of EDCs on wildlife in general.

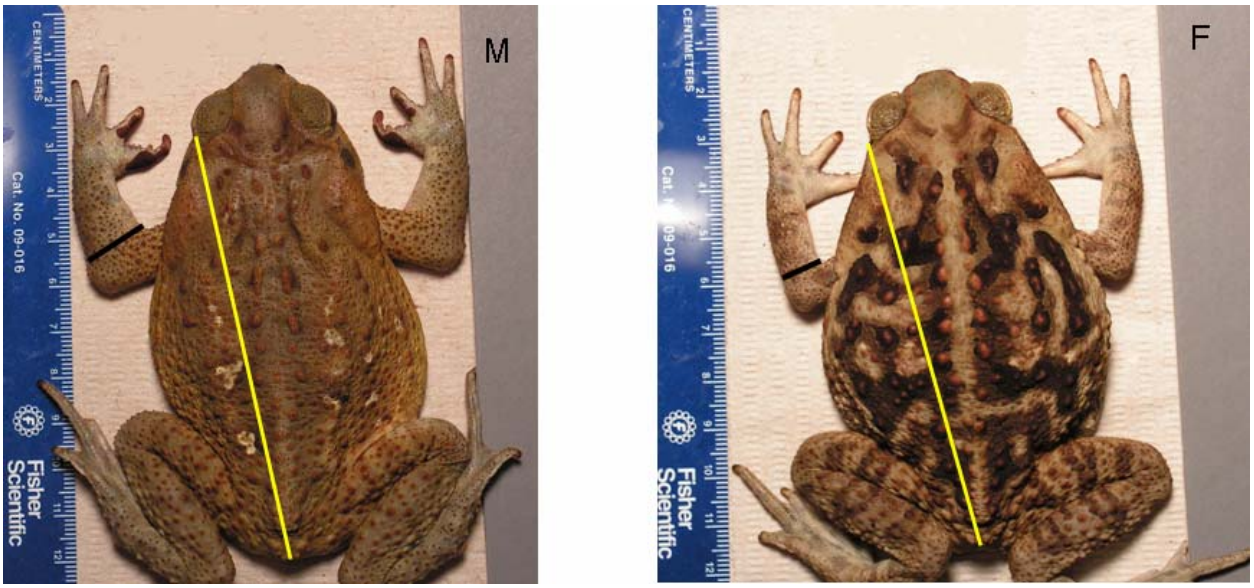

Figure 2-1 Male (M) and female (F) *Bufo marinus* have several sexually dimorphic characters. Males (M) are less mottled than females (F). Mottling scores were enumerated by counting the number of color changes that occurred across the yellow transect running down the back of the toad from the eye to the vent. Males have larger arms than females of equivalent size. The portion of the forelimb that was measured and compared among the groups is depicted as a black line. Males also can have nuptial pads on the first three digits (this male was scored as having two nuptial pads).

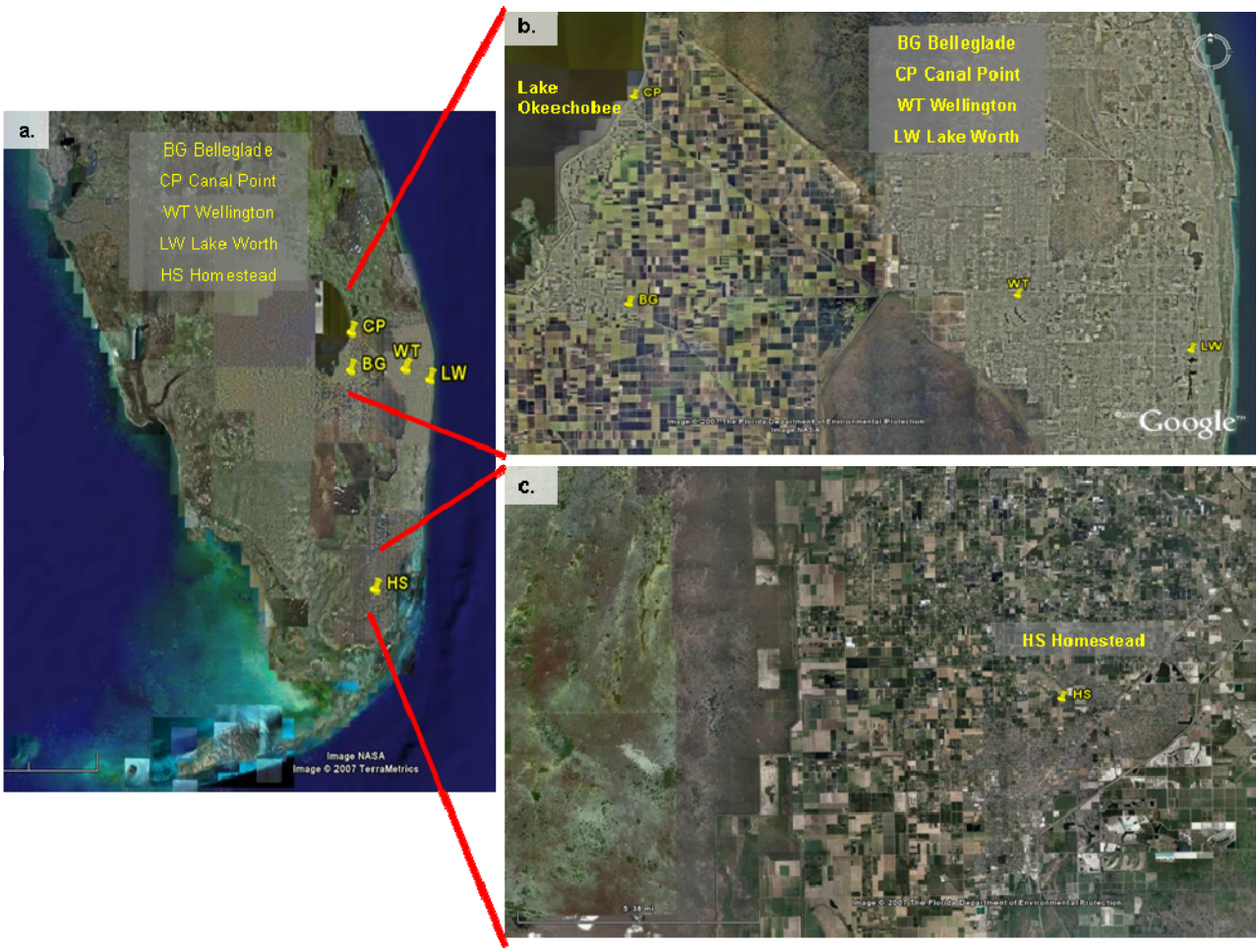

Figure 2-2 Field Sites. a. Southern Florida (USA) showing each collection site. b. Close up view of the four most northern collection sites. The area around Belle Glade (BG) and Canal Point (CP) is a patchwork of agricultural fields. The area around Wellington (WT) and Lake Worth (LW) are suburban developments. c. Close up view of the most southern collection site. The area around Homestead (HS) is a mixture of agricultural and non-agricultural land.

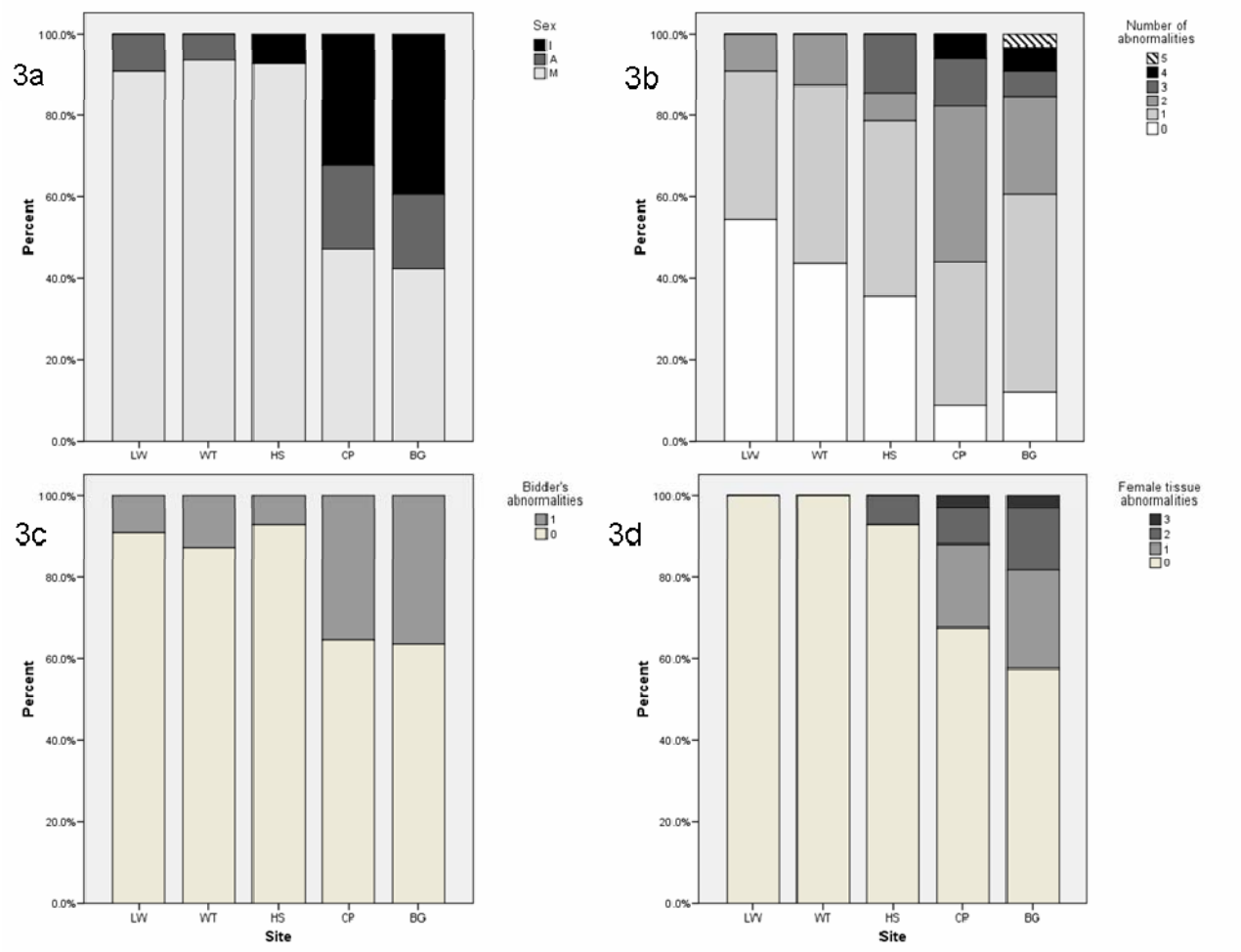

Figure 2-3 Individuals (LW=11; WT=16, HS=14, CP=34, BG=33) that had both testes and ovaries or oviducts were categorized as intersexed (I), those with vitellogenic Bidder's organs but no ovaries or oviducts were abnormal males (A), and males (M) had testes, a non-vitellogenic Bidder's organ, and no ovaries or oviducts. a. Percentage of individuals that were classified as intersexed, abnormal, and male at each site. b. Total number of testicular, Bidder's organ, and female tissue abnormalities were enumerated and the percentage of individuals having a specific frequency of abnormalities are plotted for each collection site. c. Percentage of toads with or with out Bidder's organ abnormalities at each collection site. d. Percentage of individuals with or without a specific number of female tissues or advanced stage of reproductive development. Aall of these individuals also had testicular testes. For example, intersexed toads that had ovaries (1) and oviducts (1), or that had ovaries (1) that were vitellogenic (1) were both scored as having two abnormalities. Agricultural intensity increases along the X-axis from left to right for each graph.

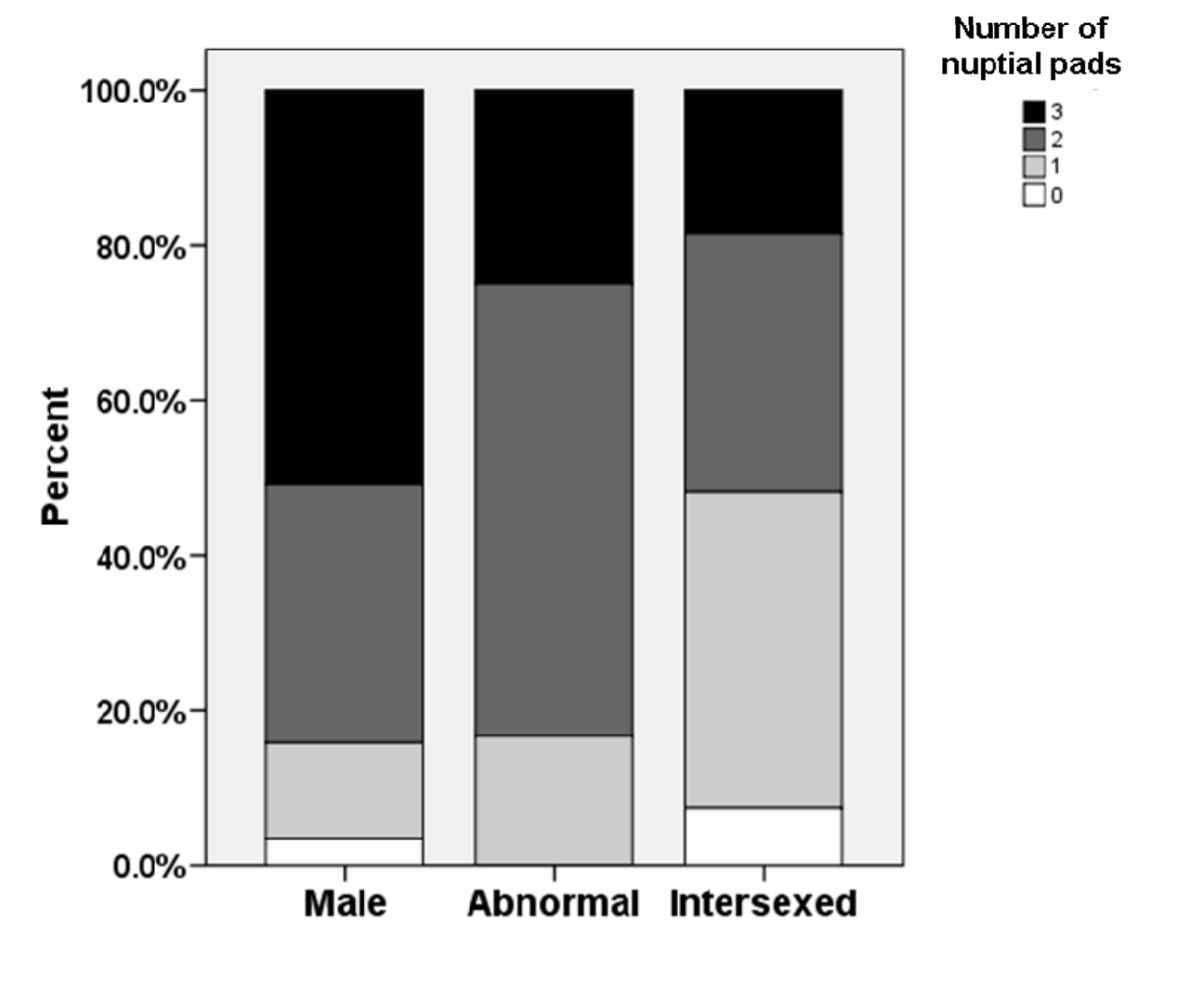

Figure 2-4 Percentages of male (N=59), abnormal (N=12), and intersexed (N=25) toads with zero through three nuptial pads per forelimb. No individuals scored as female had nuptial pads.

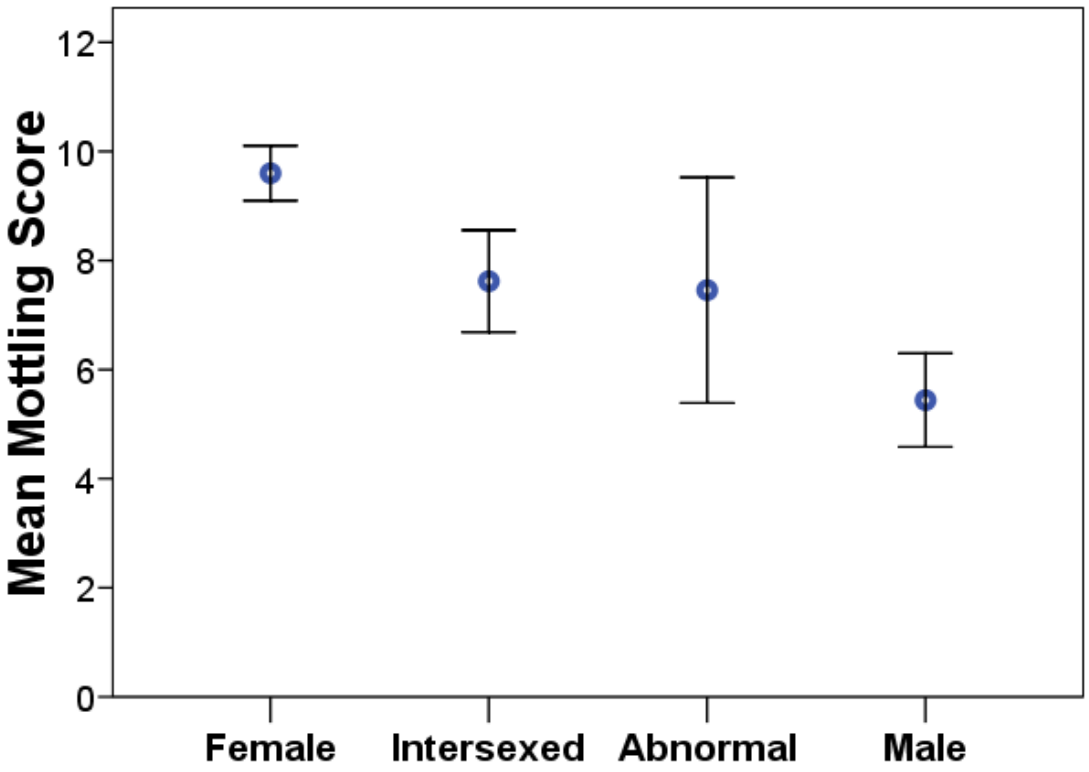

Figure 2-5 Mean mottling score across an individual's body for each sex. Mottling was evaluated by drawing a transect line from the left eye to the vent directly onto the image of each individual and counting the number of color changes (see Figure 1). Samples sizes were: females = 55, intersex = 29, abnormal = 11, and male = 59.

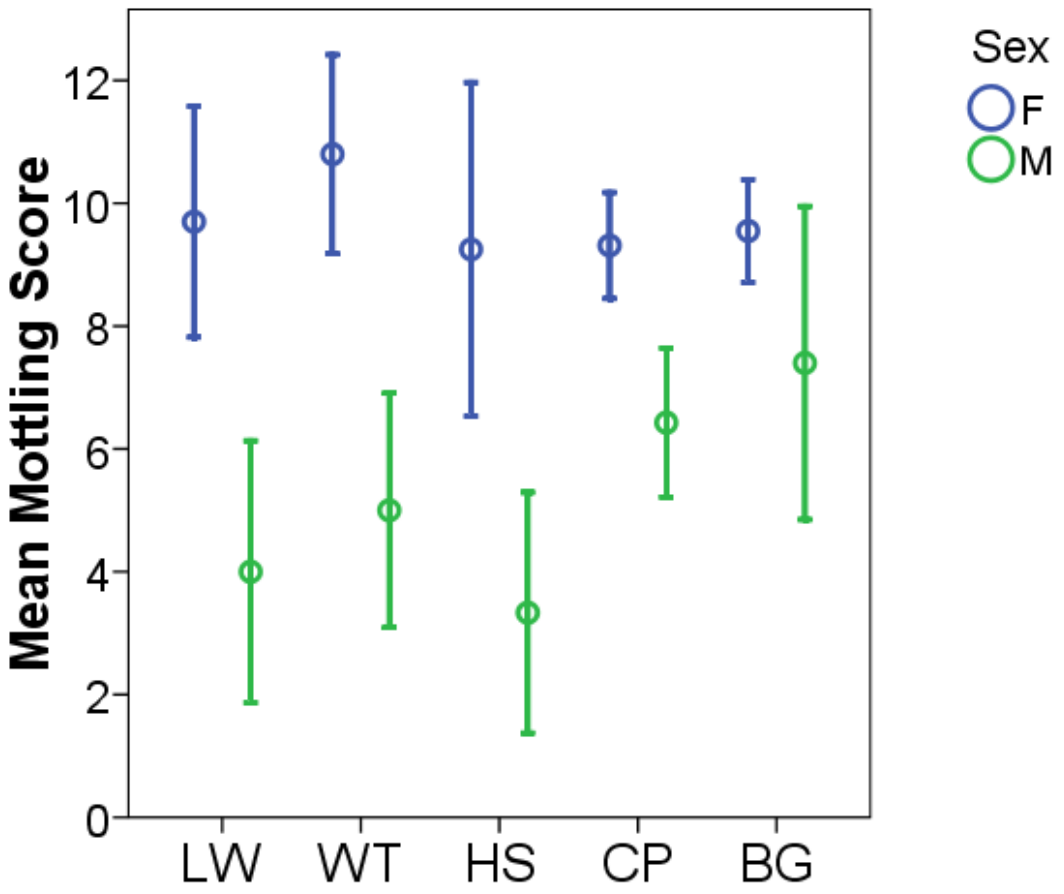

Figure 2-6 Mean mottling score for females and males across sites. Agricultural intensity increases along the X-axis from left to right for each graph. Sample sizes were: Females- LW=10, WT= 5, HS=4, CP= 16, BG =20; Males - LW=10, WT= 15, HS=10, CP= 10, BG =10

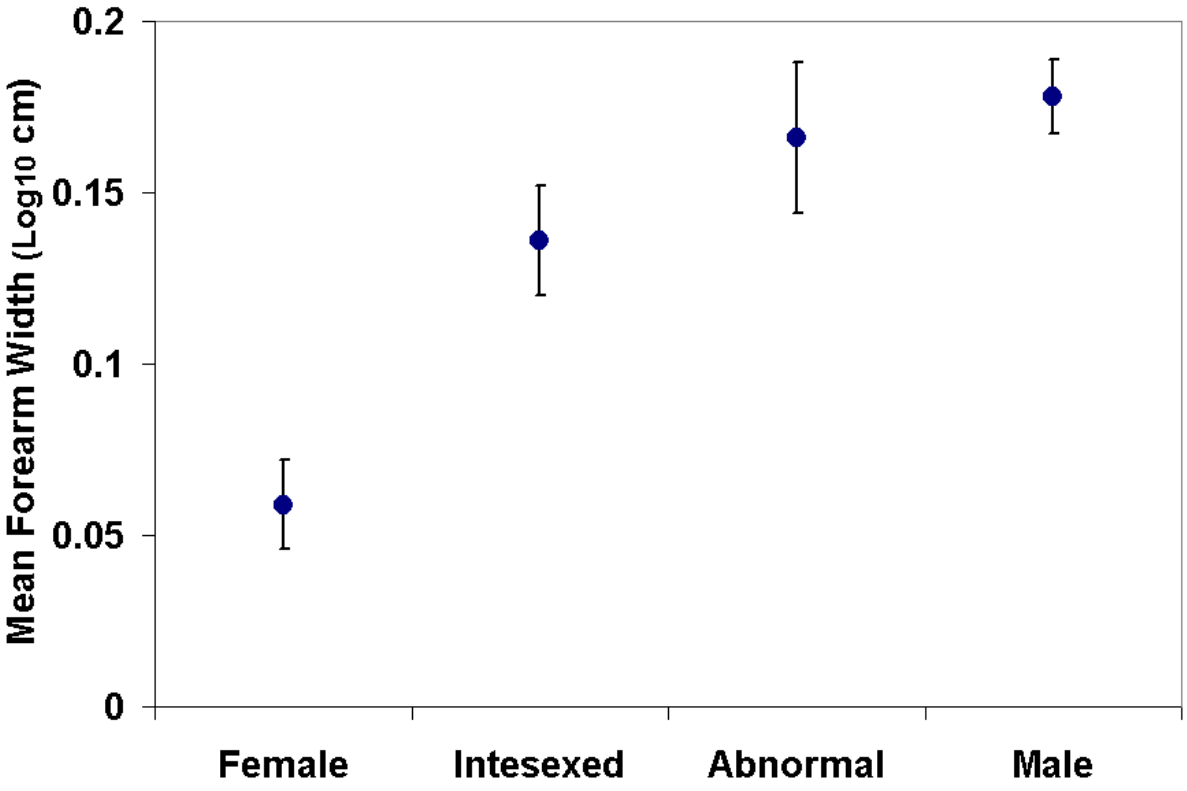

Figure 2-7 Mean forelimb width for each sex or abnormality group measured across the radioulna distal to the humerus (elbow), and perpendicular to the arm axis. Mean values are corrected for body size and represent estimated marginal means from the ANCOVA with $\log_{10}$ elbow width as the dependent factor, $\log_{10}$ (body length) as the covariate, and gonadal abnormality group (male, abnormal, or intersexed) as the fixed effect. Sexes are compared at a $\log_{10}$ SVL of 2.08. Bars are 95% Confidence intervals. Sample sizes were: females = 28, intersex = 17, abnormal = 9, and male = 41.

CHAPTER 3
MARINE TOADS LIVING IN AGRICULTURAL AREAS HAVE ALTERED GONADAL FUNCTION

Introduction

In adult vertebrates, gonads perform two primary functions. They are the location of germ cell production (e.g. spermatogenesis in males), and they function as important endocrine glands producing a variety of steroid and peptide hormones. As a result, effects of EDCs on gonadal development can affect spermatogenesis and endocrine hormone production. The gonads of animals that are exposed to endocrine disrupting chemicals (EDCs) during critical periods of development or sexual differentiation can be permanently altered so that they can not function normally in adults (Guillette *et al.*, 1995; Guillette *et al.*, 1994). Such permanent alterations that occur during embryonic development are referred to as organizational abnormalities.

Although the effects of endocrine disrupting chemicals (EDCs) have been documented extensively in vertebrates (Edwards, Moore & Guillette, 2005; Milnes *et al.*, 2006), amphibians are hypothesized to be especially sensitive to their effects (McCoy and Guillette, 2008 in press; Chapter 1). Amphibians have highly permeable skin and many live in habitats that receive runoff or precipitation that contains EDCs. Their larvae experience critical hormone-regulated developmental stages, especially during metamorphosis, making them particularly susceptible to permanent (organizational) endocrine disruption in polluted environments (e.g., Hayes *et al*. 2002).

I previously demonstrated a relationship between exposure to agricultural habitats where endocrine disrupting chemicals (EDCs) are used and abnormal and intersex gonads in *Bufo marinus* (Chapter 2). Gonad abnormalities were associated with demasculinized male secondary sexual characteristics and reductions in sexually dimorphic coloration (Figure 1). This association suggested that gonadal function was compromised since gonadally-derived steroid

hormones modulate secondary sexual characteristics. Here, I investigate whether two additional measures of gonadal function, spermatogenisis and sex hormone concentrations, are altered in *B. marinus* that have gonadal abnormalities resulting from inhabiting areas in close proximity with agriculture. Specifically, I tested the hypotheses that 1) spermatogenesis is altered in toads living in agricultural areas where EDCs are applied relative to suburban areas, 2) spermatogenesis is increased in males from non-agricultural areas relative to males, abnormal males, and intersexed toads from agricultural areas, 3) estradiol-17β and testosterone concentrations are altered in toads living in agricultural areas where EDCs are applied relative to suburban areas, and 4) estradiol-17β and testosterone concentrations are different among sex groups. Plasma estradiol-17β concentrations are expected to be greater in toads (excluding females) from more agricultural sites than from suburban areas, and testosterone is expected to show the opposite pattern. Females are expected to have higher estradiol-17β concentrations relative to all other sex groups, whereas males from non-agricultural areas are hypothesized to have lower levels than male, abnormal, and intersexed toads from agricultural areas. Testosterone concentrations are expected to be lower in females than in all other groups, whereas males from non-agricultural areas are expected to have higher concentrations relative to male, abnormal, and intersexed toads from agricultural areas.

Methods

I surveyed five sites in south Florida that varied in habitat type from completely agricultural to suburban to investigate the association between intensity of agriculture and reproductive abnormalities in *Bufo marinus*. At least 20 sexually mature adult individuals (> 91mm) (Cohen & Alford, 1993) were collected from each site during the summers of 2005 and 2006. Each individual was euthanized with an overdose of the anesthetic MS222 (0.3% Tricaine

Methanesulfonate, pH 7), and 3 to 4 ml of whole blood were collected via cardiac puncture. Whole blood was centrifuged, and plasma was collected for hormone assays. Plasma samples were frozen at -20°C immediately, transported back to the laboratory on dry ice, and then placed at -80°C until hormone assays were conducted. Gonads were removed and fixed in neutral buffered formalin for gross morphological (see Chapter 1) and histological analyses (reported here).

Histological Preparations

Fixed tissues were cut in half, dehydrated in alcohol, embedded in paraffin wax, and section so that the middle portion of each individual's testis was cross-sectioned and analyzed. Sections were 8 μm thick, and were stained using standard Hemotoxylin and Eosin (Presnell & Schreibman, 1997).

Quantification of Spermatogenesis

Five slides per animal were examined using an Olympus BX50 microscope,, and one section was evaluated per slide. Sections examined were approximately 180μm apart so that the same cells were not evaluated in different sections. Spermatogenic cells in five lobules per section were quantified, yielding a total of 25 lobules for each individual. For each lobule the number of spermatocysts containing either primary spermatocytes, secondary spermatocytes, or spermatids (both round and elongate) were quantified. The number of spermatozoa (loose and organized) per lobule was categorized as 0 (approximately 0-25% of the total area of the lobule), 1 (25-50%), 2 (50-75%), or 3 (75-100%).

Hormone Assays

Estradiol-17β (E_2) and testosterone (T) concentrations were analyzed with a validated radioimmunoassay using the 96-well FlashPlate PLUS system (PerkinElmer, City). Each flash plate was coated with 100μL of antibody (1:30,000 E_2, or 1:15,000 T) purchased from Fitzgerald

Industries International, Inc. (20-ER06 Estradiol-17 beta- 6 CMO lot#P410311520-TR05 Testosterone-19, Lot # P4033111), covered with aluminum foil to prevent evaporation, and incubated at room temperature for at least two hours. Standard curves (either E_2 or T) were prepared with the following concentrations: 1800, 600, 200, 66.7, 22.2, 7.4, and 2.5 pg/100 µL for each assay plate. Samples were extracted with diethyl ether and reconstituted with phosphate buffered saline gelatin (PBSG; 0.1M, pH 7.0) buffer buffer prior to being loaded into the assay plate. After antibody incubation each well was washed two times with 150 µL of PBSG buffer and each well was loaded with 100 µL of standard hormone or unknown sample, and the appropriate radiolabeled hormone (either E_2 or T). Plates were incubated at room temperature for three hours and counts per minute of radioactivity were analyzed using a Microbeta 1450 Trilux counter (PerkinElmer). Prior to performing hormone assays, these procedures were validated by ensuring a reconstituted plasma spiked standard curve was linear with the standard curve (internal standard) 2) and that dilution curves of extracted and reconstituted plasma were linear.

Statistical Tests

Statistical analyses were carried out using SPSS version 15.0. The composition of spermatogenic cell types was evaluated by site and sex group using separate analysis of variance (ANOVA) tests. Post Hoc comparisons to assess the affects of site and sex on spermatogenic cell numbers where conducted using Tukey HSD tests. Medians of ranked sperm abundance were evaluated among sites and sexes using Kruskal-Wallis one way analysis of variance tests. When 3 or more groups are evaluated in a Kruskal-Wallis test and when the number of observations within groups exceeds 5 the distribution of KW approximates the Chi square distribution, thus the chi square statistic is reported here (Siegel & Castellan, 1988).

Hormone concentrations were valuated using a linear mixed model where assay and assay by dependent factor (site or sex) interaction were identified as the random effects. Estradiol 17β to testosterone concentration ratios were Log 10 transformed and evaluated using a one way analysis of variance.

Site Comparisons

Spermatogenesis and hormone concentrations were compared among non-agricultural males, and agricultural male, abnormal, and intersex toads collected from different sites that varied in agricultural intensity. Agricultural intensity of each site was ranked from 1 to 5 based on the total % of agricultural land within a 5.6 km^2 area of each site, and distance to closest agricultural area (full methodological explanation in Chapter 2). In brief, Belleglade (BG-97%, rank of 5) and Canal Point (CP-51.2%, rank of 4) were the most agricultural; Homestead (HS-34%, rank of 3) was intermediate, and Wellington (WT),and Lake Worth (LW) were non-agricultural (suburban sites with no agriculture in the vicinity). Wellington was ranked 2 because it was close to agricultural areas (intensive agriculture 14 km), and Lake Worth, ranked as 1, was farther from agriculture (intensive agriculture 37 km).

Sex Comparisons

Toads were grouped into different sex categories based on gross gonadal morphology and their occurrence in non-agricultural (WT, LW) or agricultural (BG and CP) sites: 1) non-agricultural males (NAgM), 2) agricultural males (AgM), 3) abnormal agricultural males (A), 4) intersexed agricultural males (I), and 5) females (for sex hormone concentration comparisons only). Toads defined as "Males" had normal Bidder's organs and lacked female tissue. "Abnormal males" had apparently normal testes but abnormal Bidder's organs and no ovaries or oviducts, whereas "intersex toads" had testes along with ovaries or oviducts. Toads from the intermediate agricultural intensity site (HS) were not included in sex group analyses because it

was unclear whether they should be considered as non-agricultural males or as agricultural males (e.g. several HS toads were collected near a tree farm).

The number of spermatogenic cells were compared among non-agricultural males (NAgM), 2) agricultural males (AgM), 3) abnormal agricultural males (A), 4) intersexed agricultural males (I). Females were not evaluated for spermatogenesis.

Previous work found no differences among females across sites (see Chapter 2). Therefore, hormone concentrations among females were statistically compared to determine if it was appropriate to pool females into one group that could be efficiently compared to the other sex groups. Females did not vary across sites in either hormone (Estradiol 17β: df=4,59.08; F=1.05, p=0.39; Testosterone: df=4, 69.26 F=1.67 p=0.17). Therefore, females were pooled into a single group for among sex group hormone comparisons.

The ratio of each hormone concentration (E to T) was evaluated by sex group, with females excluded, to test whether the balance between estradiol 17β and testosterone differed among non-agricultural males and agricultural toads.

Results

Spermatogenesis – Site Comparisons

Spermatogenesis was reduced in toads living in agricultural areas. The number of primary spermatocytes (df=4, 91; F=8.59, p<0.001) was significantly lower at Belle Glade and Canal Point (most agricultural sites) relative to lake Worth (most suburban). Secondary spermatocytes (df=4,91; F=7.13, p<0.001) and spermatids (df=4,91; F=8.90, p<0.001) were significantly reduced in toads from the Belle Glade and Canal Point (most agricultural sites) compared to the more suburban sites Lake Worth, Wellington, and Homestead. Median sperm rank also varied across sites (df=4, Chi- square=14.978, p=0.005). Lake Worth and Wellington, non-agricultural

sites, had a higher percentage of individuals with median sperm ranks two and three whereas agricultural sites commonly had sperm ranks of one (Figure 3).

Spermatogenesis – Sex Comparisons

The number of primary spermatocytes ($df=3,77$, $F=6.88$, $p<0.001$), secondary spermatocytes ($df=3$, $F=7.41$, $p<0.001$), and spermatids ($df=3, 77$; $F=9.28$, $p<0.001$) were significantly higher in males from non-agricultural areas relative to abnormal, intersexed and male toads from agricultural sites (Figure 4). Median sperm rank also significantly varied by sex ($df=3$, Chi-square=9.739, $p=0.021$, Figure 5) with the lowest sperm rank (1) occurring more frequently in abnormal, intersexed and male toads from agricultural sites than in male toads from non-agricultural sites. However, a similar percentage of non-agricultural males and intersex toads had the highest sperm ranks.

Hormone Concentrations – Site Comparisons

Plasma estradiol-17β (E_2) among toads from different sites were not different ($df=4, 11.58$, $F=1.63$, $p=0.233$), whereas testosterone levels were significantly different across sites ($df=4$, 9.36, $F=5.50$, $p=0.015$, Figure 6).

Hormone Concentrations – Sex Comparisons

There were significant differences in estradiol-17β (E_2) concentrations among sex groups ($df=4, 20.94$; $F=11.09$, $p<0.001$), but these differences were driven entirely by the high E_2 concentrations in females. Testosterone concentrations were significantly different among sex groups ($df=4, 160.4$; $F=17.59$, $p<0.001$). Males from non-agricultural areas had the highest testosterone concentrations and females and intersexed frogs had the lowest (Figure 7B).

The ratio of estradiol 17β to testosterone concentration was significantly different among sex groups ($df=3, 90$; $F=4.242$, $p<0.007$). Intersex toads had the greatest estradiol to testosterone ratio (Figure 8).

Discussion

The underlying genetic and endocrine control of gonadal development, growth, and function, and the roles of gonadally-derived hormones on secondary sexual characteristics, are highly conserved among vertebrates. Nevertheless, these physiological mechanisms can be altered by exogenous exposure to sub-lethal concentrations of endocrine disrupting chemicals (EDCs) causing impaired development, growth, sexual differentiation, and reproduction (Sanderson, 2006). For example, aromatase (CYP19) is responsible for controlling the rate-limiting step in the conversion of androgens to estrogens (e.g. testosterone to 17β estradiol), and its modulation by EDCs (e.g. tamoxifen and atrazine) interferes with homeostasis and the function of sex steroid hormones resulting in feminization, demasculinization, and even sex reversal (Crain & Guillette, 1997).

In Chapter two of this volume, I presented results that demonstrated that both primary (e.g. gonadal morphology) and secondary sexual traits of toads are altered in agricultural areas where chemicals with endocrine disruptive potential are used. In addition, I observed that toads that had gonadal abnormalities also had feminized and demasculinized secondary sexual traits. One of the primary functions of gonads is to produce hormones that control the expression of secondary sexual traits, thus my results suggested that testicular function (e.g. spermatogenesis and hormone production) was reduced in toads living in agricultural areas and in toads with gonadal morphological abnormalities. Estrogens were hypothesized to to increase and testosterone to decrease (due to increased aromatase) in toads from agricultural sites and in toads with gonadal abnormalities generating the combination of feminized estrogen-dependent and demasculinized androgen-dependent traits.

In this study, testicular function was reduced in toads living in agricultural areas. Spermatogenic cell types were reduced in toads living in agricultural habitats relative to those

that did not. The reduction in testicular function was associated with abnormal gonadal morphology. For example, intersex toads had reduced spermatogenic cell numbers relative to normal males from non-agricultural areas. The physiological mechanism(s) through which spermatogenesis is altered in toads living in agricultural areas, or in those with intersex gonadal tissue is unknown. Production of primary and secondary spermatocytes is initiated by follicle stimulating hormone (FSH), whereas differentiation of spermatids is controlled by androgens produced by Sertoli cells induced by lutenizing hormone (LH). Both FSH and LH are secreted by the anterior pituitary after stimulation from the hypothalamus (Sriraman et al 2004; Rastogi et al 2005; Minucci et al 2005). Therefore, the reduction of the number of spermatogenic cells suggests that the hypothalamus or pituitary could be altered in toads living in agricultural areas as has been reported in another study of frogs (Sower, Reed & Babbitt, 2000). Alternatively, the Sertoli cells could be altered such that they can not respond to pituitary hormones appropriately. Regardless of the mechanisms, reduced spermatogenesis is likely associated with reduced fertility and could have important implications for the long term persistence affected populations.

Each of these hypothesized mechanisms could be a result of organizational alterations that occurred because toads were exposed to EDCs during embryonic development (Guillette *et al.*, 1995; Sower *et al.*, 2000). Such embryonic exposures permanently alters developing tissues such that they never function properly in adults. In addition, current body burdens and daily exposure to EDCs can exacerbate organizationally derived abnormalities as well as induce "activational" pathologies. Intersexed individuals have experienced organizational alterations of gonadal morphology, and it is notworthy that the number of primary and secondary spermatocytes and sprematocytes did not differ between intersexed and "normal" males from agricultural areas; males and intersexed individuals from agricultural areas show the same

reduction in spermatogenesis relative to males from non-agricultural sites. This result suggests that current exposure could be reducing spermatogenesis independent of gonadal morphology. However, the developmental stage at which exposures occur, as well as the concentration and length of time of exposure, and differences in the toxicant milieu can induce different abnormality phenotypes. Therefore, toads at agricultural areas might have different degrees or severities of abnormalities. For example, reduced spermatogenesis could be a result of organizational abnormalities induced by larval exposures to pulsed sublethal concentrations of pesticides, whereas toads that were exposed for longer time periods, or to particular concentrations or milieus of pesticides had additional gross morphological abnormalities. The mechanisms through which these gross morphological and functional abnormalities occur are unknown, and further research is required before any of these hypotheses can be explicitly evaluated.

Although non-agricultural males had higher sperm numbers than individuals from agricultural sites, a greater percentage of intersexed individuals had sperm ranks of three than normal agricultural males. Although unexpected, there are plausible explanations for this result. First, intersexed individuals might not be reproductively active. If they do not display breeding behavior, breed as frequently, or attract as many mates as normal males, then spermatozoa could accumulate relative to normal males. Second, estrogen increases the number of spermatogonia, the spermatogenic stem cells, in *Rana esculenta* (Minucci *et al.*, 1997). Although circulating estradiol 17β levels were not significantly higher in intersexed individuals, the ratio between circulating estradiol 17β and testosterone was significantly higher. In addition, estrogens from the ovarian tissue of intersexed individuals could act locally on the testes and increase

spermatogonia there by increasing spermatogenesis and eventually the production of spermatozoa (relative to agricultural males).

Although female estrodiol 17β concentrations were significantly greater than in all other sex groups, there were no differences across normal non-agricultural males and any of the agricultural toads. These patterns are contrary to expectations because toads from agricultural sites, and especially those with gonadal abnormalities, have altered secondary sexual traits that are sex hormone dependent. However, the lack of significant differences between intersexed toads and the other sex groups is influenced by the inherent variation in estradiol 17 β concentration with intersexed individuals. These toads varied in stage of oogenesis, and thus were expected to vary in estradiol 17 β concentrations. In addition, endogenous hormones are activated at extremely low concentrations, so small differences in estradiol 17β concentrations could induce large effects (Welshons *et al.*, 2003). Furthermore, the physiological responses that result from endogenous hormones are not simply a function of having enough of one hormone; they are also a function of the relative quantities of different types of hormones. Importantly, the ratio between estradiol 17β and testosterone concentrations was significantly greater in intersexed toads relative to all other sex groups, which could feminize and demasculinize secondary sexual traits.

There are several ways in which secondary sexual traits could be modulated without changes in circulating hormone levels. I predicted that circulating levels of estrogens would increase in agricultural toads and especially in intersexed individuals because atrazine (the herbicide used at the agricultural sites) increases aromatase expression and thus could lead to the feminized colorations patterns observed (see Figure 1). However, these color patterns could be caused by increased aromatase expression within the skin cells which would lead to the

feminized phenotype without requiring higher circulating estrogen concentrations (Thorton, 2002).

Synthesis of circulating androgens occurs in the Leydig cell of the testes, and is induced by lutenizing hormone. Circulating testosterone levels were lower in intersexed, abnormal, and "normal" male toads from agricultural sites relative to non-agricultural males which suggest that androgen synthesis could be reduced generally, or that the toads are clearing androgens more readily by liver biotransformation. Although testosterone concentrations were not different among sex groups within agricultural sites, these groups varied in secondary sexual characteristics such as arm size and nuptial pad quantity (Chapter 2). However, these demasculinized traits could be induced through several mechanisms. The relative ratios between estradiol 17β and testosterone could be important in the activation of masculine traits. In addition, the expression of 5α reductase, the enzyme that converts testosterone to the more potent androgen 5α dihydrotestosterone (DHT) could be lower. DHT influences secondary sexual traits and its concentrations could be altered across agricultural sites and in animals with gonadal abnormalities even when testosterone concentrations are not reduced. Binding proteins could also be modulated by EDCs, and if they become more numerous they will bind more testosterone or DHT and there will be less endogenous hormone free to exert physiological effects. Therefore total hormone concentrations would be similar among the sex groups in the agricultural areas, but the percentage that was free to exert masculinizing effects would be lower in the intersexed toads. Androgen receptor gene expression could also be reduced by EDCs which would make androgen responsive cells "blind" and unable to detect circulating androgens. The particular mechanisms through which these abnormalities are occurring need to be studied in more detail.

However, my results demonstrate that gonadal function is altered in animals living in agricultural areas.

Living in or near contaminated areas and undergoing critical stages of development while exposed to endocrine-disrupting chemicals is expected to negatively affect both individuals and populations (Hayes *et al*. 2006 but see Glennemeier and Begnoche 2002). Most EDCs studied to date have estrogenic or anti-androgenic activity and can influence sex ratio, sexual maturation, gonadal morphology and function, and spermatogenesis, fertility, hormone levels (steroidogenesis, metabolism), secondary sexual characteristics and reproductive behavior (Milnes *et al.*, 2006). All of these characteristics are important for maintaining ecologically functioning populations, especially for taxa that are known to be declining globally, such as many amphibian species.

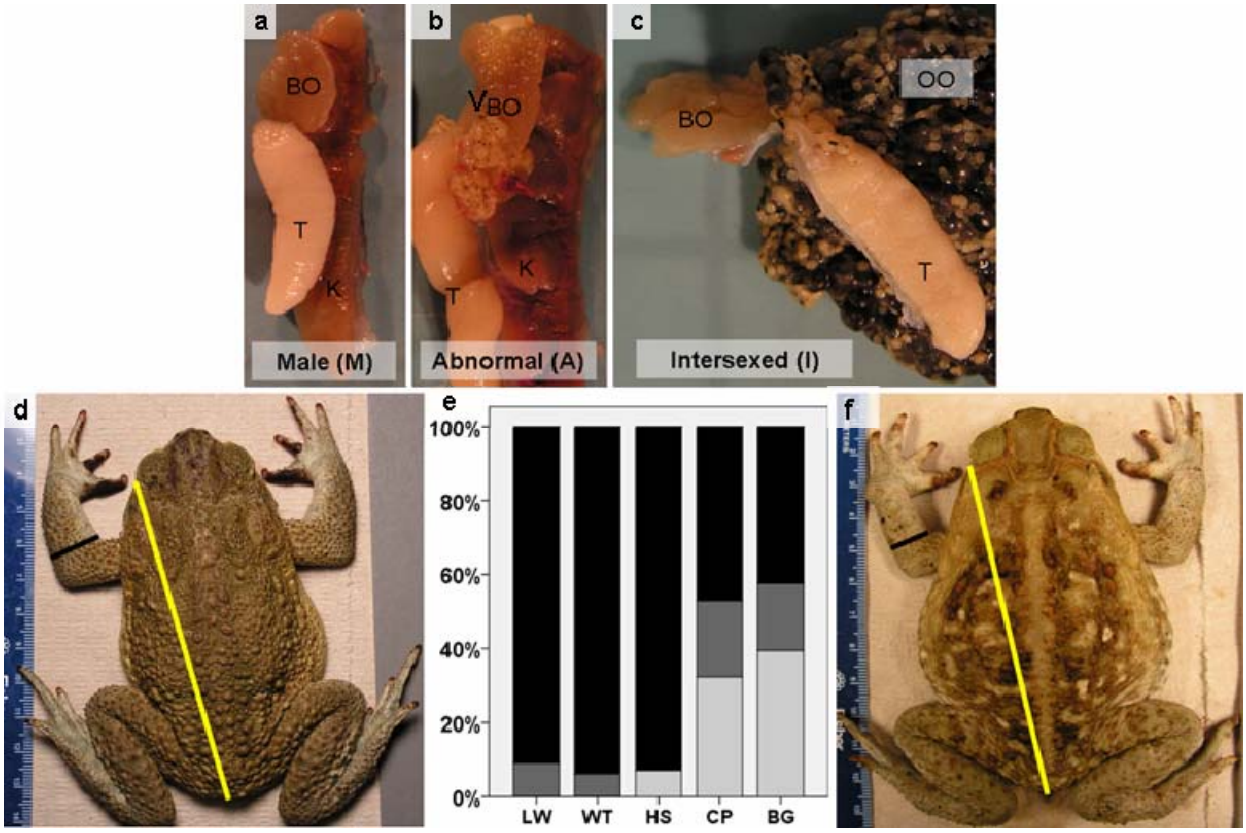

Figure 3-1 Examples and summary data of primary and secondary morphological traits of *Bufo marinus* collected from habitats that vary in the intensity of agriculture. a. Normal male (M) gonads have testes (T), a non-vitellogenic Bidder's organ (BO), and no ovaries or oviducts. b. Abnormal male (A) gonads have testes (T) vitellogenic or oogenic Bidder's organs (VBO), but no ovaries or oviducts, c. Intersexed gonads (I) have both testes (T) and ovaries (OO) or oviducts. Bidder's organs and ovaries were distinguished by the density of the tissue. Bidder's organs are thicker and more dense and ovaries are thin, sac-like, and convoluted. Ovaries were also completely separate from the Bidder's organ. d. Photograph of a male from a non-agricultural site; Solid coloration, large forelimb width, and dark multiple nuptial pads are diagnositic. e. Percentage of individuals that were classified as male (black), abnormal (dark gray), and intersexed (light gray) at each site. Agricultural intensity increases along the X-axis. f. Photograph of an intersexed toad. Similarly sized intersexed toads had more mottling, smaller forearm widths and fewer nuptial pads.

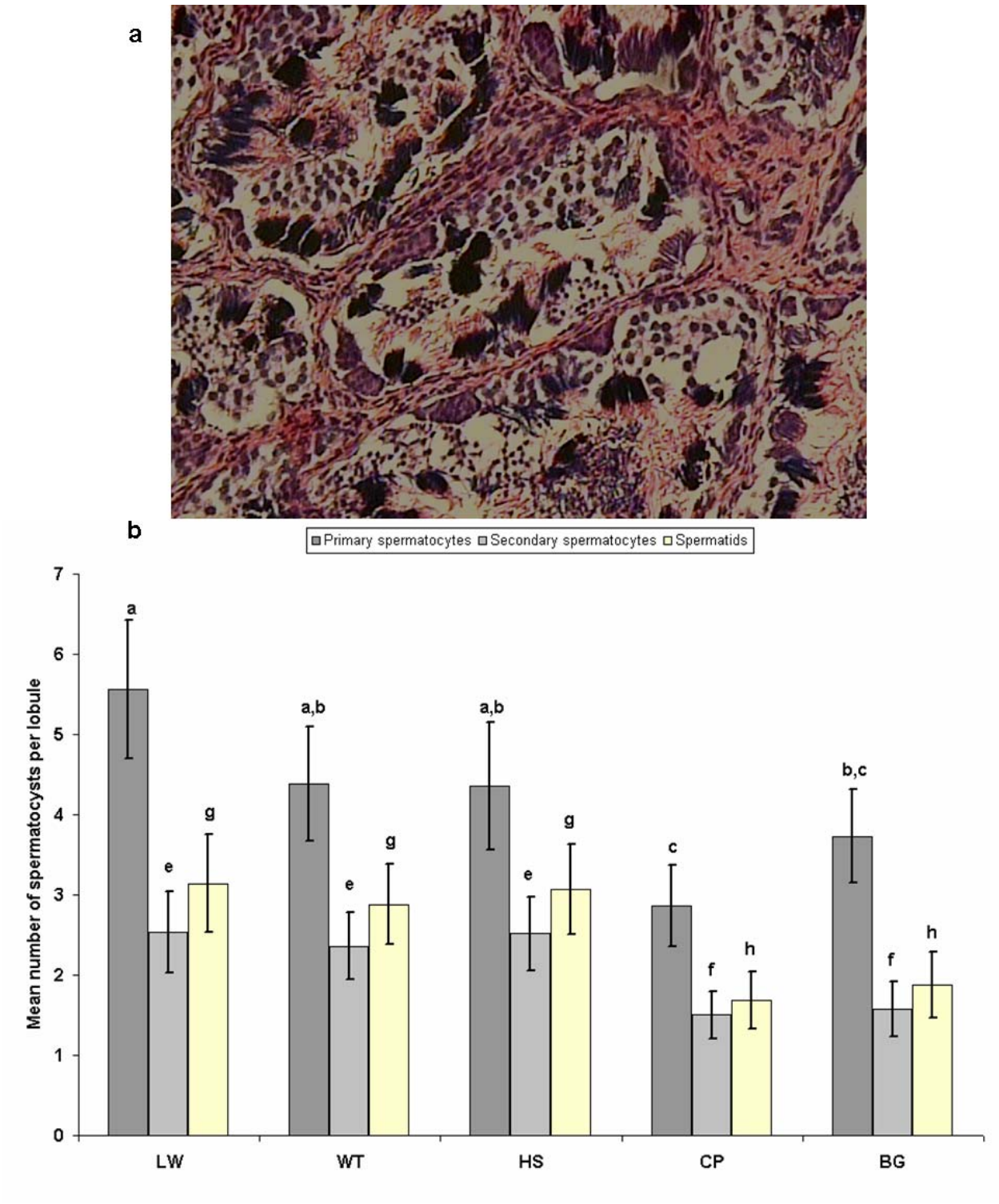

Figure 3-2 a. Cross section of a testis. The round lobule in the center of the photograph contains several groups of spermatogenic cells. Each group is called a spermatocyst. Spermatocysts contain only one cell type either: primary spermatocytes, secondary spermatocytes, sprematids (round or elongate), and sperm. Sperm can also be loose which is indicative of reproductive readiness. The largest round cells are primary

spermatocytes and include the more obvious groups of cells along the edge of the lobule (light purple cysts). The smaller round cells are secondary spermatocytes. No round spermatids are observed in these lobules but they are easily distinguished from secondary spermatocytes by their small size. Elongated spermatotids are light purple whereas sperm is dark purple to black. b. Mean number of sprematocysts of each cell type per lobule across sites of varying agricultural intensities. Agricultural intensity increases from left to right. Letters a, b, and c represent significant differences among sites for primary spermatocytes, e and f denote differences for secondary spermatocytes, and g and h denote differences among sites for spermatids. Bars are 95% confidence intervals.

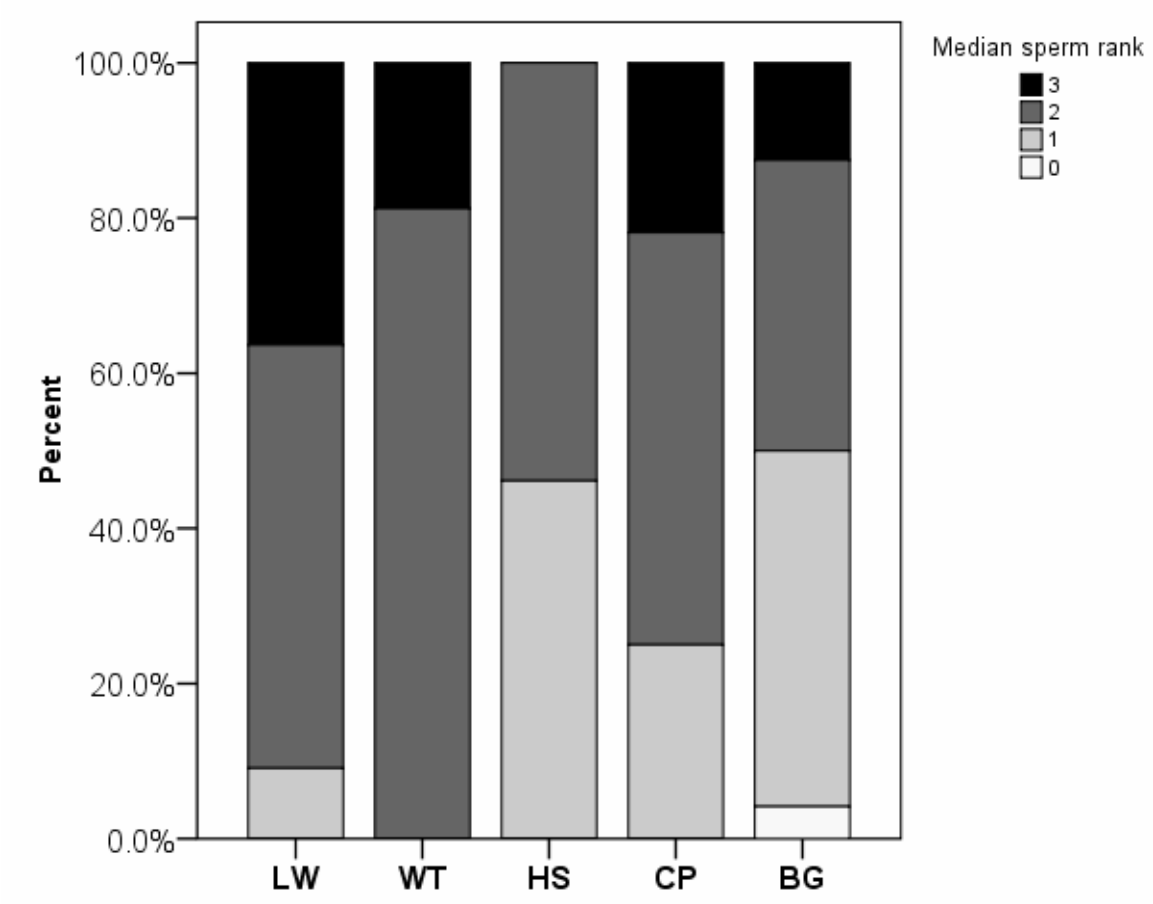

Figure 3-3 Percentage of males with a median sperm rank of 0-3 across sites. Agricultural intensity increases from left to right along the X-axis.

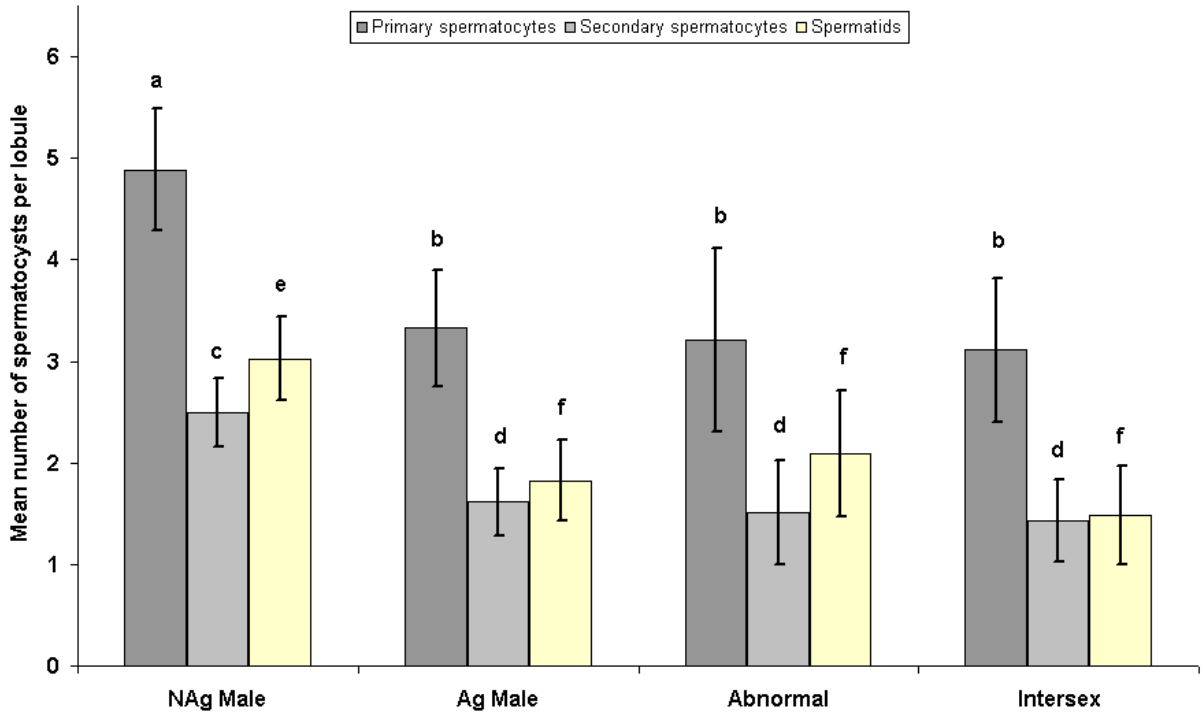

Figure 3-4 Mean number of sprematocysts of each cell type per lobule across sex groups including non-agricultural males (NAgM), agricultural males (AgM), agricultural abnormal toads (A), and agricultural intersexed toads (I). Letters a and b represent significant differences based on post hoc a Tukey's test among sex groups for primary spermatocytes, c and d denote differences for secondary spermatocytes, and e and f denote differences among sites for spermatids. Bars are 95% confidence intervals.

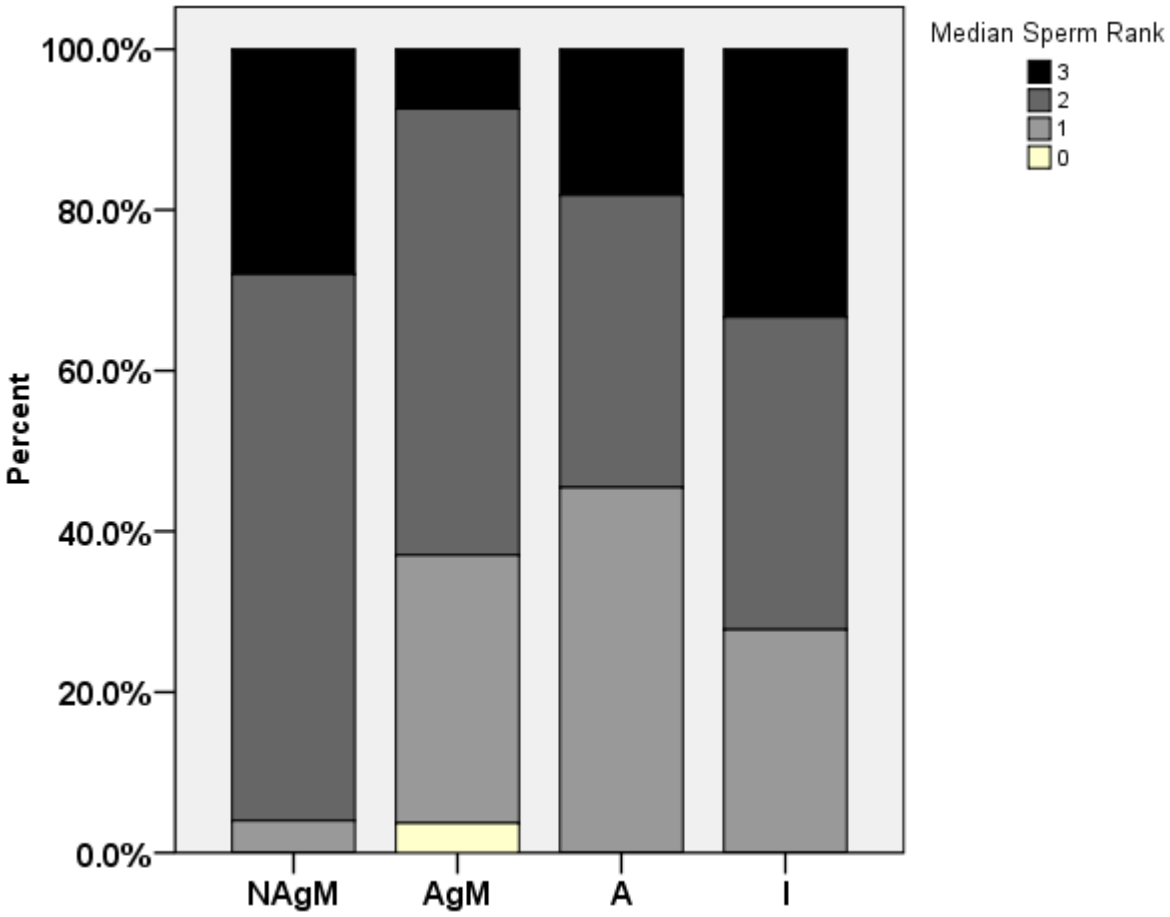

Figure 3-5 Percentage of toads with a median sperm rank of 0-3 by sex group including non-agricultural males (NAgM), agricultural males (AgM), agricultural abnormal toads (A), and agricultural intersexed toads (I).

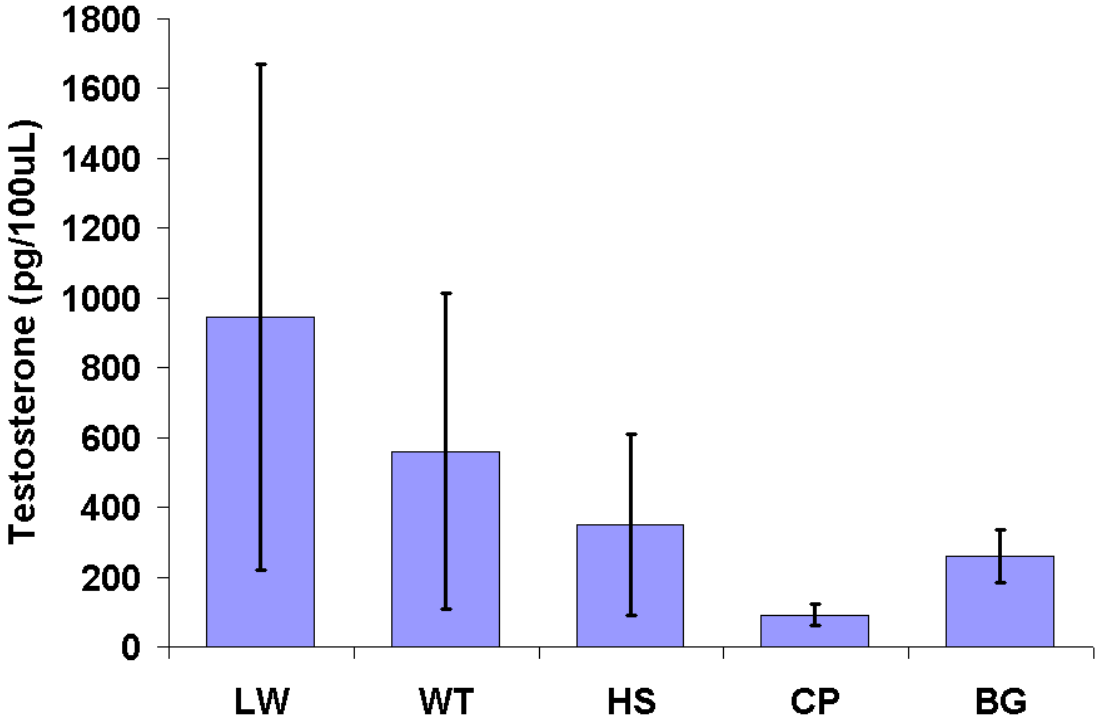

Figure 3-6 Testosterone concentration by site. The most agricultural site is on the right. Bars are 95% confidence intervals.

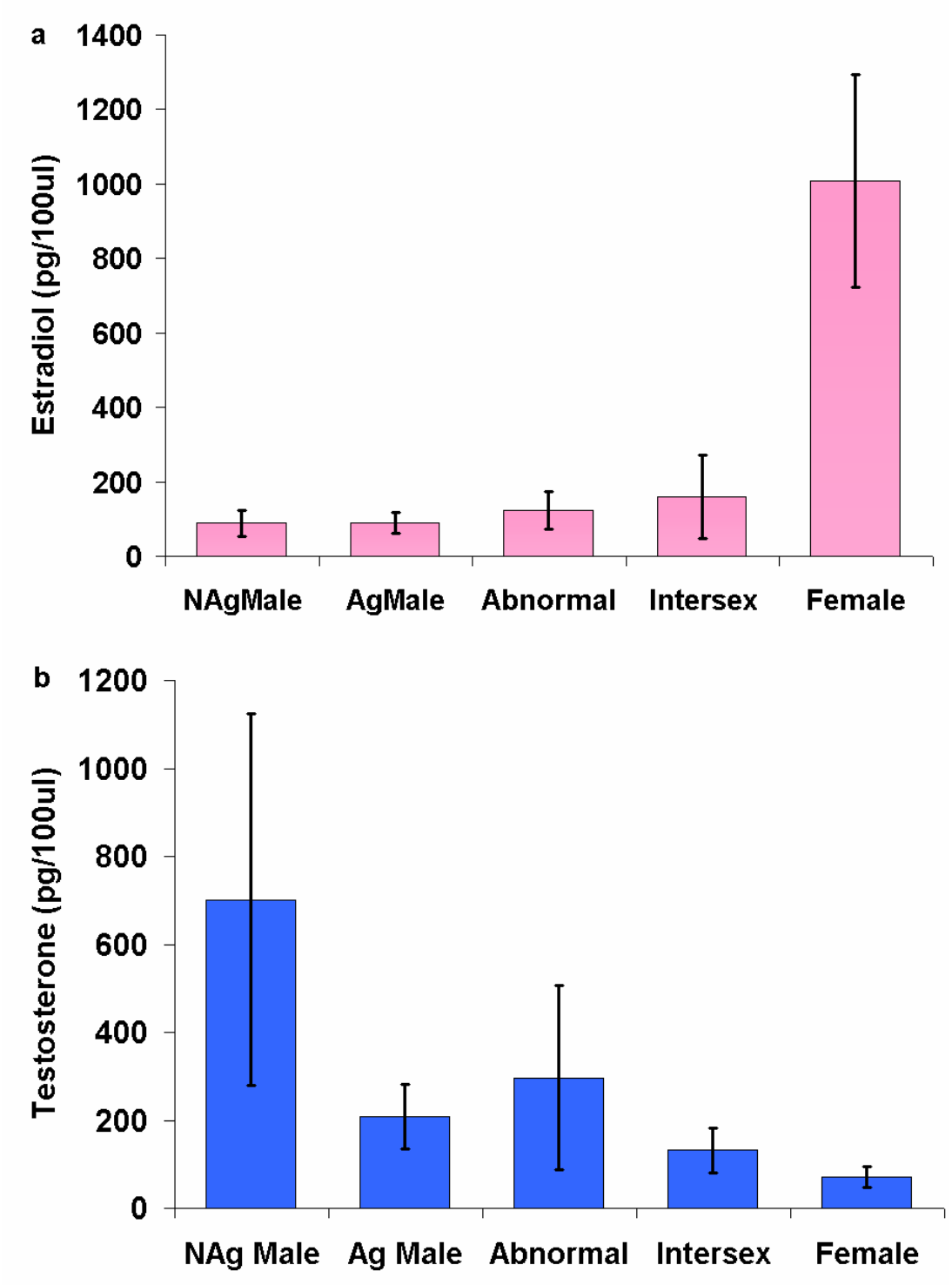

Figure 3-7 Hormone concentrations. a Estradiol 17β and b Testosterone concentration across sex groups. Non-agricultural males (NAgM), agricultural males (AgM), agricultural abnormal toads (A), and agricultural intersexed toads (I). Bars are 95% confidence intervals around the mean of the untransformed data.

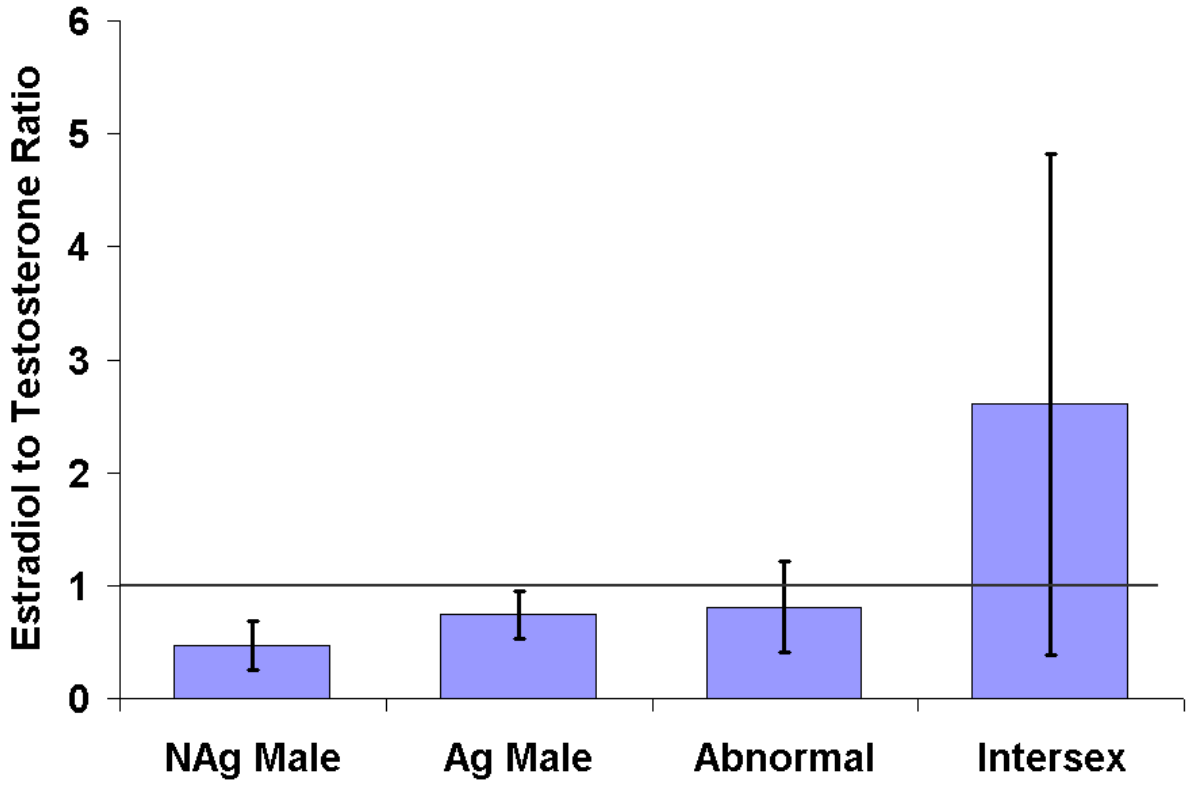

Figure 3-8 Estradiol 17β to testosterone concentrations among sex groups including non-agricultural males (NAgM), agricultural males (AgM), agricultural abnormal toads (A), and agricultural intersexed toads (I). Letters represent significant differences among sex groups. Bars are 95% confidence intervals. Solid line indicates a ratio of one.

CHAPTER 4
GENE EXPRESSION PROFILE DIFFERENCES AMONG OVARIAN, TESTICULAR, AND INTERSEX GONADAL TISSUE OF WILD CAUGHT ADULT MARINE TOADS

Introduction

Over the last century, human-induced environmental changes have occurred on a global scale. Extensive urbanization, industrial farming practices, and the use of chemicals, such as pesticides and pharmaceuticals, have resulted in increased concentrations of environmental contaminants (Swarup & Patra, 2005). Many of these pollutants alter the endocrine systems of wildlife and humans and induce reproductive abnormalities (Colborn & Clement, 1992). All classes of vertebrates and many invertebrates experience developmental and functional abnormalities of the reproductive system after exposure to endocrine disrupting chemicals (EDCs: (Edwards, Moore & Guillette, 2006; Edwards & Myers, 2007; Milnes *et al.*, 2006; Porte *et al.*, 2006; Weltje & Schulte-Oehlmann, 2007). In many cases, different chemicals induce similar reproductive abnormalities (phenotypes) across diverse vertebrate taxa (Milnes *et al.*, 2006).

Similarity in response is likely due to conserved mechanisms of genetic and endocrine control of gonadal development, growth, and function among vertebrates. In other words, developmental and functional abnormalities observed in EDC-exposed animals could stem from disruption of conserved genetic networks. Identification of gene networks disrupted by contaminant exposure can improve characterization of the physiological and genetic mechanisms involved in toxin-induced pathologies. Monitoring gene expression profiles of exposed animals could permit the identification of chemical signatures that can be used as biomarkers, and can be linked to specific mechanisms of toxicity as well as whole organism effects. In addition, this approach promotes a system wide understanding of the effects of pollutants and can be more

sensitive than morphological or developmental endpoints (Denslow, Garcia-Reyero & Barber, 2007; Helbing *et al.*, 2007).

DNA arrays are commonly used to rapidly identify alteration in 100s to 1000s of mRNA transcripts and allow for the identification of genes and gene networks that underlay phenotypic traits associated with contaminant exposure. Several laboratory studies have discovered genes whose expression levels are significantly altered by exposure to specific chemicals, leading to identification of novel mechanisms of toxicity and potential biomarkers of exposure (Denslow *et al.*, 2007). Field studies have compared gene expression of organisms collected from polluted and reference environments demonstrating differential expression of specific genes associated with polluted environments (Denslow *et al.*, 2007). Field studies are important because gene expression is highly dependent on environmental context, and exposure to contaminants under controlled conditions likely induces very different genetic responses relative to responses generated in a variable environment. Therefore, field studies provide information about the mechanisms through which natural populations are affected by toxins. Field studies are challenging, however, because many variables are not controlled (e.g., length of time of contaminant exposure), and the additional variation can decrease statistical power and mask the effects of contaminants. Relating gene expression to specific phenotypes or pathologies ("phenotypic anchoring") can help explain some of this inherent variation making comparisons more powerful, and help link alteration in gene expression changes to traditional toxicological endpoints (Denslow *et al.*, 2007; Schimidt, 2003). Indeed, Denslow et al. (2007) recently argued that establishing the relationship between altered gene expression and traditional markers of toxicology (i.e., phenotypic traits) is a critical step required to advance the study of aquatic toxicology. The emerging field of ecotoxicogenomics seeks to do this (Schimidt, 2003).

In previous studies, I showed that the incidence and severity of gonadal abnormalities (indicative of endocrine disruption) in *Bufo marinus* are associated with changes in land use patterns (chapter 1 and 2 of this volume). The frequency of gonadal pathologies in these toads was higher in areas characterized by intensive agricultural practices (primarily sugar cane farming) than in suburban areas. Toads in agricultural areas had a greater incidence of intersex gonads and abnormal Bidder's organs, feminized skin coloration and demasculinized forelimb morphology. Furthermore, spermatogenesis and testosterone levels were reduced in male and intersex toads living in agricultural habitats.

In this study, I characterized gene expression profiles of gonadal tissues from normal female (only ovary tissue), normal male (only testis tissue), and intersex (testis and ovary tissue) *B. marinus* using an anuran DNA array (Crump *et al.*, 2002b). First, I characterize gene expression and identify genes that have correlated expression patterns among the different gonadal tissues. Second, I identify potential biomarkers of sex by determining specific genes that are most closely associated with each gonadal type. Third, I test for differences in the levels of gene expression of a subset of genes to identify those that are differentially expressed in each reproductive organ. This study is the first to investigate genome wide changes in gene expression associated with normal and intersex gonadal tissue in amphibians.

Methods

Gonadal tissues used in this study were collected during the summer of 2005. Toads from 2 sites with low or intermediate agriculture (Wellington and Homestead) and 2 agricultural locations (Canal Point and Belle Glade) were collected and euthanized with an overdose of MS222 (0.3% tricaine methanesulfonate, pH 7). One gonad from each individual was placed in RNALater (Ambion, Inc) for gene expression analyses and the other gonad was preserved for histological description (reported in chapter 3). Gonadal tissues from 5 individuals of each

sexual condition (normal male, normal female and intersex) were hybridized to separate arrays (N = 15). Ovaries and testes were from toads from Homestead and Wellington, whereas intersex gonads were collected from Belle Glade, and Canal Point, FL, USA.

Gene expression is dynamic and can respond to environmental variation by changing over a period of minutes to hours, handling and capture stress were minimized and similar among sites and sexes. Toads were captured, handled, and held (15min-2 hours) under similar conditions. In addition, gene expression data were carefully screened for high variation and genes that varied significantly within a sex condition (e.g. perhaps due to differences in handling) were excluded from further analyses (see statistics section in methods). This means that several potentially important genes associated with variation in gonadal function within a sex group were not analyzed in this study. For example, each of the five females used in this study varied in stage of vitellogenisis from non- vitellogenic (and regressed) to moderately vitellogenic. Therefore, a portion of the genes associated with vitellogenisis could have been left out of this study due to high, among female, variation in their expression levels. Although, these genes are not studied here, this data is available for future work, and should be investigated further.

Isolation of RNA

For intersex gonads, I separated and weighed the ovarian and testicular portions and isolated RNA separately so that RNA of each tissue type could be quantified in future studies. For this paper, I was interterested in mRNA expressed in the entire gonad, so after RNA isolation (see below), I constituted the total RNA (prior to cDNA preparation) by mixing the appropriate ratio (determined by mass) of testicular and ovarian RNA extract. None of the ovaries of intersex individuals were vitellogenic. For the non-intersex gonads, I weighed each tissue and extracted RNA as indicated below.

Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Invitrogen Canada Inc., Burlington, Ontario, Canada). Gonadal tissue was homogenized in 1 mL TRIzol reagent for each 100 mg of tissue with a 3 mm diameter tungsten-carbide bead in safe-lock Eppendorf 1.5 ml microcentrifuge tubes. For homogenization, a Retsch MM301 Mixer Mill (Fisher Scientific Ltd, Ottawa, ON) was used at 20 Hz for 6min intervals until all tissue was completely homogenized. Mixing chambers were rotated 180 degrees at each 6-min interval. After phase separation with chloroform and RNA precipitation with isopropanol, the isolated RNA was re-suspended in up to 40µl diethyl pyrocarbonate (DEPC)-treated RNase-free water and stored at -70°C.

cDNA preparation and labeling

cDNA was prepared according to the manufacturer's protocol (Invitrogen) except single stranded cDNA was purified using QIAquick PCR purification columns (Qiagen) prior to the double strand synthesis step. SMART cDNA was radiolabled with [α-^{32}P] dATP using a HexaLabel DNA labeling kit (Fermentas). The labeled cDNA was purified using QIAquick PCR purification columns (Qiagen). Radioactivity was quantified for each sample so that standard quantities of radioactive cDNA could be added to each array. Immediately prior to hybridization, the radiolabeled cDNA was heat denatured for 5 min at 95°C and quickly cooled on ice for 5 min.

Gene Expression Profiling

The MAGEX cDNA array, which is a heterologous frog multi-gene cDNA array containing gene probes originating from conserved protein-encoding sequence regions, was purchased from Viagen X Biotech Inc. (Victoria, BC, Canada), and is described in detail elsewhere (Crump, Lean & Trudeau, 2002a; Veldhoen *et al.*, 2006). Briefly, a cDNA sequence for each gene target was spotted in duplicate at adjacent grid positions on a nylon membrane.

Approximately 90 percent of the 420 gene sequences on the array originated from *Xenopus laevis*, and the others were isolated from *Rana catesbeiana*. Each membrane contained three intron controls to monitor for genomic DNA contamination. Prehybridization, hybridization, and posthybridization washes were performed at 48°C.

Five replicate hybridizations were performed for each gonad type, and each gonad was collected from separate individual. Testes were collected from males from Wellington (3) and Homestead (2). Ovaries were collected from Wellington (2), Homestead (2), and Belle Glade (1). Intersex tissue was collected from intersex toads from Belle Glade (2) and Canal Point (3). A single ovary was collect from Belle Glade an agricultural site because it was most similar to the other ovaries in its stage of oogenesis. No reproductive abnormalities have been identified in female *Bufo marinus* from Belle Glade. Hybridizations were carried out in 20 ml of hybridization solution containing 4x SSC, 10% (w/v) dextran sulfate, 1.0% (w/v) SDS, and 0.5% (w/v) Blotto. Pre-warmed hybridization solution was added to each hybridization tube (35 mm internal diameter x 150 mm length; Amersham) containing an array membrane and allowed to pre-hybridize for 2 h. Radiolabeled cDNA samples were then added to a final concentration of 8 x 10^7 cpm/ml and allowed to hybridize overnight. After hybridization, the membranes were rinsed briefly with 50 ml 2x SSC, washed twice with 50 ml 2x SSC/0.1% SDS for 15 min, once with 50 ml 0.1x SSC/1.0% SDS for 25 min, and rinsed with 50 ml 0.1x SSC.

The arrays were placed on 3M filter paper (Rose Scientific Ltd, Edmonton, AB, Canada) soaked with ddH_20 and wrapped with plastic wrap. Each processed membrane was exposed to a phosphorimager screen (Molecular Dynamics Inc., Sunnyvale, CA, USA) for five days. Hybridization signals were collected using a Storm 820 Gel and Blot Imaging System (Amersham) at 50 μm resolution. The resulting image data were converted to a standard 8-bit

TIFF file using Photoshop V5.0 (Adobe Systems Inc, San Jose, CA, USA), and auto-leveled which darkens the blots increasing the signal intensity. Both non auto and auto-level images were prepared for analysis to account for signal saturation.

Relative expression for each gene target was collected from the image data using ImaGene Version 5.6.1 (BioDiscovery Inc, El Segundo, CA, USA). Some genes were blotted more than twice so within array variation could be assessed. Signal intensities for each gene and blank positions were determined from the median spot pixel intensities and corrected by subtracting the local median background pixel intensities. Signal intensities that were derived from areas of non-specific hybridization on the arrays were not included in the final analysis. A non-signal background was determined from the median intensity value plus one standard deviation of blank positions across the auto-level data set. Signal intensities for gene positions exhibiting values below median background were adjusted to this value so that background variation was illuminated and would not be mistaken for treatment variation. Saturated gene positions identified in auto-level data were replaced across all data sets by the corresponding values obtained in the non auto-level analysis.

Both non auto- and auto-leveled data for each of the 15 arrays (5 per sex group) were normalized using a geometric mean derived from the median signal intensities from the following genes: ribosomal protein L8, GAPDH, cytoplasmic beta actin, NM23/nucleoside diphosphate kinase, and ubiquitin. The choice of gene transcripts used for normalization was dictated by spot quality, consistency, and passing a robust estimated standard deviation cut-off (see Statistics below).

Quality Control and Statistical Analyses

Non-specific binding and radiation contamination can occur on an array making it impossible to accurately detect the expression of genes in that area. Contamination is easily detected because it forms a splotch that does not match the grid within which the genes are spotted. A gene was also excluded from analyses if it was represented by fewer than 5 data points (at least one spot on each array) or 3 arrays per gonad type (each having at least two spot intensities for that gene). Gene expression data obtained for each gonadal type were analyzed for consistency using a intra-class correlation with a 2-way random effects model (reliability analysis SPSS Version 12.0, Chicago, IL, USA). The correlation coefficient for each set of arrays was never less than 0.85 (Ovary -0.918, Testis -0.848, Intersex -0.922). As an additional quality measure, and to reduce the chance of making a type I error, I excluded genes that showed high variance among arrays within a gonadal type. The variance cut off was based upon a robust estimated standard deviation for each gene among replicate arrays [(maximum value-minimum value)/2]. If the robust estimated standard deviation was greater than 2 (i.e., if the maximum expression for a gene was >4-times the minimum expression within a gonad type) the gene was excluded from the final list of genes considered in further analyses.

For the remaining genes, I calculated the relative expression (i.e., fold-change) of ovary (or testis) to intersex (i.e., ovary/intersex or testis /intersex) or ovary relative to testis (ovary/testis). Only genes exhibiting positive or negative fold change values greater than or equal to 1.5 for at least one comparison were used in any analysis. Traditionally, array studies use a 2 fold threshold, but since the goal of this work was to identify as many differentially expressed genes as possible I relaxed this (to 1.5) to increase the total number of genes available for consideration. Others were considered to be equivalent across all sex groups and thus unimportant for this study. Pearson correlations between gene expression values in ovaries

versus intersex tissue, testis versus intersex tissue, and ovaries versus testis tissue were conducted in SPSS 15.0.

Identifying correlated gene expression patterns-cluster analysis

Fold change values were imported into Cluster 3 (Eisen *et al.*, 1998), $\log_2$ transformed, and average linkage clustering was used to produce a hierarchical cluster tree which was visualized in Treeview (Eisen *et al.*, 1998). Genes that showed similar relative expression patterns (fold change) across the sex groups were identified. For example, genes that were similarly expressed between ovary and intersex tissue but were expressed at relatively lower levels in testis tissue clustered together.

Biomarker identification-correspondence analysis

Correspondence analysis was used to identify gene transcripts that had strong expression levels that were associated with a particular gonadal type. Since the data sets were a combination of data generated from non auto-leveled and auto-leveled data (which were on different scales), I could not directly perform CA on the signal intensity values. Instead, the fold changes of one sex relative to another were used (ovary/intersex, testis/intersex, and ovary/testis). To make increases and decreases symmetrical around zero, the fold changes were natural log transformed. Thus, an increase (e.g., a doubling: ln (2) = 0.69) would be seen as equivalent in magnitude to a comparable decrease (e.g., a halving: ln (0.5) = -0.69).

Test for differential gene expression-robust analyses

All tests for differential gene expression were performed in the R statistical programming environment (R development core 2007). To look for significant differences in gene expression across sex groups, each of the 89 differentially expressed genes were analyzed in separate robust analyses of deviance tests. This analysis is part of a family of statistical techniques (Robust Statistics) for estimating the parameters of parametric models while dealing with violations of

idealized assumptions (Huber 1981, Hampel et al 1986, Venables and Ripley 1999). For example, many types of data contain extreme values or outliers resulting in departures from normality or other parametric assumptions. Robust statistics emulate classical methods, but are not prone to estimation errors when data include outliers or exhibit other small departures from model assumptions. Multiple comparisons inflate the probability of type I errors. However, because the goal of this analysis was to generate hypotheses about mechanisms and to identify candidate genes for further study, inferences based on a Bonferoni corrected alpha level of 0.0005 would rule out many potentially important genes. Thus, I present non-corrected p-values, but also note the genes whose expression patterns are significantly different after Bonferoni correction.

Results

General Characterization of Gene Profiles

Differential gene expression across the different gonadal types is shown for a representative region of the MAGEX array (Figure 1). Of the 420 genes on the array, 259 gene transcripts were detectable above background expression levels. Of the original 259 genes expressed, 89 met all data quality requirements and displayed a fold-change in relative gene expression of greater than 1.5 or less than 0.67 in at least one sex comparison (Table 1). These genes varied in their function and included: 12 genes involved in cell growth control, 4 involved with chromatin structure, 11 in signal transduction, 29 with transcription, 5 with hormonal regulation, 5 with metabolism, 6 with transport/ and binding, 6 with apoptosis/protein processing, 7 involved with cell structure, 1 of unknown function, and 3 involved with protein translation.

A high percentage of genes were down regulated in ovaries relative to intersex (50%) or testicular (46%) tissues (Figure 2). Twenty percent of the genes in ovaries were up regulated

relative to testes (O/T), and 11% were up regulated relative to intersex gonadal tissue (O/I). Thirty eight percent of the genes show similar expression levels between ovaries and intersex tissues (O/I) but were expressed at higher or lower levels in testis tissue, whereas 32% of genes had similar levels of expression in testes and intersex gonads and were higher of lower in ovaries. In general, males seem to have roughly equivalent percentages of genes that are up regulated, equivalent, or down regulated relative to intersex gonads (Figure 2). Although gene expression among these tissues is strongly correlated, overall gene expression between ovaries and intersex tissue were more highly correlated (R= 0.92) than between testes and intersex tissue (R= 0.83) or ovaries and testes (R= 0.74, Figure 3).

Correlated Gene Expression Patterns

Nine gene clusters were identified, each with correlation coefficients (CC) > 0.90 (Figure 4). Gene cluster one (CC=0.94; Figure 4), includes many genes that are important in cell cycle regulation and are involved in both oogenesis and spermatogenesis (cyclin H and B2, nerve growth factor, inportan alpha 1a, suvivin). Gene cluster one (CC=0.94; Figure 4), includes many genes that are important in cell cycle regulation and are involved in both oogenesis and spermatogenesis (cyclin H and B2, nerve growth factor, inportan alpha 1a, suvivin). This cluster in characterized by similar gene expression in ovaries and intersex gonads, whereas testes showed lower expression levels. Gene cluster two (CC=0.93; Figure 4) includes genes that are typically thought to regulate development (retinoic acid converting enzyme, sonic hedgehog, and Pax-2) and function as transcription factors in adults. Altered regulation of these genes has been associated with development of cancers (Shang, 2006), and according to the cluster analysis both ovaries and testes had lower expression relative to intersex gonads, whereas ovaries and testes had comparable expression levels. Gene cluster three (CC=0.90; Figure 4) predominantly includes genes that are involved in cell growth and transcription; ovaries and testes had lower

expression relative to intersex gonads, but ovaries also tended to have lower expression than testes (compared to cluster #2). Cluster four (CC=0.95; Figure 4) contains a variety of genes involved in transcription, and translation. Ovaries had lower levels of gene expression than either intersex gonads or testes, whereas testes and intersex gonads had similar expression levels. Cluster five (CC=0.98; Figure 4) also included genes associated with transcription and in this case ovaries had lower gene expression than intersex gonads and testes, whereas testes had higher levels of gene expression than intersex gonads. Gene cluster six (CC=0.95) also included several genes associated with transcription and was characterized by ovaries with variable expression levels (both higher and lower) relative to intersexes, but testes had higher expression levels relative to intersexes and ovaries. Cluster seven (CC=0.96) included two genes expressed in ovaries at higher levels than intersexes or testes, whereas testes and intersex tissue were similar. Cluster eight (CC=0.98) included genes involved in cell growth and signal transduction that were expressed similarly between ovaries and testes and were higher relative to intersex tissue. Cluster nine (CC=0.99) included two genes one of which was relatively similar across all groups and the other tended to be expressed at higher levels in ovaries and testes relative to intersex tissue and was lower in ovary compared with testis.

Biomarker Identification

The correspondence analysis identified 18 genes with expression patterns that were strongly associated with specific sexes and might serve as biomarkers of sex type (Figure 5, Table 2). This association could be due either to a specific gene being highly expressed or relatively absent in a specific gonad type. For example, if a specific gene was expressed in males and females but was very low in intersex gonads the correspondence analysis would identify its absence as being associated with intersex condition. The expression patterns of four genes were associated with ovary tissue. These included one gene involved in cell growth, two

controlling chromatin structure, and one involved in gene transcription (Table 2). Five different genes showed a strong association with testicular tissue. These included three signal transduction genes, one associated with transcription, and another involved in ion transport. Three genes were identified to be closely associated with intersex tissue. One of these genes is associated with cell growth, another with signal transduction, and the third with transcription.

Three other groups were identified that were associated with two sex groups, but not the third including: one metabolic gene that was similar in testis and intersex tissue, two signal transduction genes and one transcription gene were associated with ovary and testicular tissues, and two cell growth genes associated with ovary and intersex tissue (Table 2).

Expression Level Comparisons

Twenty five of the 89 genes differed significantly in their levels of gene expression (alpha = 0.05), and eight differed significantly after Bonferronni correction (alpha=0.0005, Table 3). Of the eight genes that differed after Bonferronni correction five (adenosine A1 receptor a1R gene, adenosine A1 receptor a1R gene, Na K transporting ATPase beta subunit, E2 ubiquitin conjugating enzyme Ubc9, and collagenase 4 precursor) were significantly different between testicular and intersex tissue (alpha=0.0005), and not between ovarian and intersex (lowest alpha=0.131). Two (mitochondrial cytochrome c oxidase subunit 1 and elongation factor 1 alpha chain) were similar between testicular and intersex tissue, and differed in the ovary (one higher one lower). One gene (cyclin H) was significantly different from intersex tissue and ovarian tissue which expressed higher levels and sifferent from testicular tissue expressed which expressed lower quantities. None of these genes were identified as biomarkers of gonadal type.

Two genes, osteogenic protein-1 homolog precursor (cell growth), and retinoic acid converting enzyme (hormonal regulation), were significantly (alpha=0.05) up regulated in both

males and females relative to intersexes (Table 3), whereas sonic hedgehog (transcription factor) were down regulated in both males and females relative to intersexes.

Twelve genes differed significantly in their relative levels of expression in females relative to intersexes but not in males relative to intersexes. However, only two of the 12 were significantly up regulated in females relative to intersex gonads: elongation factor 1 alpha chain (protein translation) and RAG-2 (chromatin structure). The remaining 9 genes were all down regulated including 4 genes involved with signal transduction (colorectal cancer tumor suppressor, casein kinase 1 epsilon, activated protein kinase C receptor, calcineurin A), two transcription factors (Pitx1, and liver helicase), three genes involved in metabolism (ornithine decarboxylase, aldolase B, mitochondrial cytochrome c oxidase subunit 1), and one structural gene (alpha 1 collagen type II).

Nine genes differed significantly in males relative to intersexes but did not differ between females and intersexes. Two of these, E2 ubiquitin conjugating enzyme (involved in apoptosis and protein processing) and wee 1A kinase (involved in cell growth control), were significantly down regulated in males (Table 3). The remaining 7 genes were all up regulated in males relative to females and intersexes, including two genes involved with signal transduction (phospholipase C gamma 1a, adenosine A1 receptor), two genes involved with transport (sodium phosphate cotransporter, Na K transporting ATPase beta subunit), one involved in hormonal regulation (thyrotropin releasing hormone precursor), one involved with cell growth (cyclin B2), and one in apoptosis and protein processing (collagens 4 precursor).

Discussion

Correlated Gene Expression

The cluster analysis identified several clusters that contained genes associated with transcription that could be especially important since they control expression of other genes.

This group of genes was the most highly represented on the array (91 transcription related genes). The fact that these genes did not cluster together suggests that expression of different transcription related genes are more (or less) important for each gonadal. For example, both clusters three and four contained genes associated with transcription, but only in cluster three did intersex gonads have higher expression levels than ovaries and testes. Investigating these genes, and the genes they control, could help identify mechanisms involved in the morphology and function of this intersex pathology.

Comparing different clusters also helps to identify the mechanisms involved with the gonadal pathologies and normal gonadal function. Clusters one and six were similar in that ovary and intersex tissues had similar expression levels, and testicular tissue had either lower (#1) or higher (#6) expression. These genes could be important for understanding the mechanisms through which intersex tissues are feminized ("act" like ovary tissue), and for identifying genes and the genetic mechanisms that distinguish ovaries from testes. In clusters two and eight, ovarian and testicular tissue were relatively similar whereas intersex tissue had either higher (#2) or lower (#8) expression levels (relative to both ovaries and testes). Therefore, these genes distinguish intersex individuals from females and males, and identify possible genetic mechanisms involved in the structure and function of the intersex condition. Cluster four is characterized by genes that are similar in testes and intersex tissues, but are different in ovaries; thus they represent a suite of genes that could be important for ovary function.

Biomarkers of Sex

Three of the four genes associated with ovaries in the correspondence analysis are known to be expressed in vertebrate ovaries (Wee 1-A kinase, Cytosine 5- methyltransferase, and deoxyribonuclease gamma). In *Xenopus leavis*, Wee 1-A kinase increases phosphorylation of cdc2 (a cyclin-dependent kinase), and inhibits cells from undergoing mitosis (Mueller, Coleman

& Dunphy, 1995). In mammals, Wee 1 A kinase, cdc 2 and other cell cycle control proteins are involved in controlling the meiotic arrest of oocytes (El Touny & Banerjee, 2006; Park *et al.*, 2004).

Cytosine 5- methyltransferase (down) regulates gene expression and embryogenesis and is found in *Xenopus leavis* stage III oocytes. Concentrations increase as the oocytes mature and in late stage oocytes, DNA methyltransferase is translocated into the nucleus, where it down regulates gene expression and facilitates the formation of chromatin and rapid DNA replication. This gene expression down regulation occurs on a genomic scale and is maintained through fertilization and the early stages of embryogenesis. Reductions in DNA methyltransferase leads to premature gene transcription and body plan defects and thus has important implications for embryonic development (Kimura, Suetake & Tajima, 1999; Stancheva & Meehan, 2000).

Less is known about the function of deoxyribonuclease gamma in gonadal tissues, but it has been localized in both ovary and tetes and is involved in degrading DNA during apoptosis (Shiokawa *et al.*, 2006). Early response -1 (ER-1) has not previously been localized in the ovary but similarly to Cytosine 5- methyltransferase it represses gene expression (Ding, Gillespie & Paterno, 2003). Indeed, ovaries tended to have a higher percentage of genes that were down regulated relative to intersex and testicular tissues. DNA methyltransferase and ER-1 are likely involved in this down regulation (Ding *et al.*, 2003).

All of the genes associated with ovary tissue in the correspondence analysis clustered together in cluster #1, and intersex gonads displayed similar gene expression levels to females for each of these genes. Therefore not only do intersex gonads share a common pattern of gene expression with females for a "random" suite of genes, they similarly express the genes that are most closely associated with ovary tissue, or "define femaleness".

Less is known about the genes associated with testicular tissue in this study. There is much debate in the literature about the role of SERCA-1 in spermatogenesis and whether it is important for inducing the acrosome reaction required during fertilization (Harper *et al.*, 2005; Jimenez-Gonzalez *et al.*, 2006; Lawson *et al.*, 2007; Triphan *et al.*, 2007). SERCA-1 has been localized in sperm and spermatozoa (Jimenez-Gonzalez *et al.*, 2006; Lawson *et al.*, 2007), and is known to regulate calcium concentrations and calcium signaling which are essential for cell homeostasis (Chami *et al.*, 2001). Neuromedin is expressed at high levels in *Xenopus laevis* ovaries (Wechselberger, Kreil & Richter, 1992); however, in this study it was highly expressed in *Bufo marinus* testes. Indeed, neuromedin is more highly expressed in rat testes relative to ovaries, but there is no detailed information on its function in either of these reproductive tissues (Kilgore *et al.*, 1993; Rucinski *et al.*, 2007). Despite the lack of information on the role of these genes (their protein products) on gonadal function, the pattern derived from the array data are interesting as they identify genes that are expressed in a sexually dimorphic pattern in the gonads and could be important for testicular function. Expression levels of four out of the five genes that were associated with maleness, or testicular tissue, were more similar between ovaries and intersex gonads than between testes and intersex tissue suggesting that intersex tissue is demasculinized.

All three of the genes associated with the intersex condition modulate cell proliferation. Cyclin A2 has been localized in ovarian granulosa cells, is required for the S phase and G2 to M phase transitions in the cell cycle, and plays an important role in regulating both meiosis and mitosis of female germ cells (Persson *et al.*, 2005). However, cyclin A2 has also been localized in spermatogonia and regulates mitosis in male germ cells (Diederichs *et al.*, 2005; Kajiura-Kobayashi, Kobayashi & Nagahama, 2005; Ravnik & Wolgemuth, 1996). Cyclin A2 is up

regulated in intersex gonads relative to both ovaries and testes, and likely increases cell proliferation of male and female germ cells. Pax 2 was also up regulated in intersex gonads relative to ovaries and testes, and its expression is also linked to increased cell proliferation. Pax 2 is a transcription factor that is typically up regulated during development and then is switched off in adult life via increased methylation (Shang, 2006). If methylation of PAX 2 is disturbed, it becomes upregulated and leads to hyperplasia and malignancies, and thus is considered an oncogene (Shang, 2006). The up regulation of PAX 2 suggests that intersex individuals could have altered DNA methylation patterns. The third gene that is associated with intersex gonads, casein kinase 1-epsilon, phosphorylates many different substrates and modulates a variety of processes such as cell differentiation, proliferation, chromosome segregation and circadian rhythms. Alterations in casein kinase expression has been linked to neurodegenerative diseases and cancer (Knippschild *et al.*, 2005).

The ovaries of the intersex toads used in this study were non-vitellogenic whereas two of the female ovaries were undergoing early stages of vitellogenisis. Despite these differences, ovary and intersex gonadal tissue gene expression was similar. The strong correlation between ovarian and intersex tissue gene expression (Figure x) , and the fact that intersex toads express genes associated with ovaries similarly to females and do not express genes associated with testes similarly to males, suggests that intersex gonads are more ovarian than testicular. Intersex ovaries were relatively small, and the average ovary to testis mass ratio in intersex gonads was 0.33. Although the same amount of radiolabeled cDNA was added to each array, the intersex cDNA was a mixture of ovary and testicular cDNA whereas the female ovary was not. Thus, the similarity in gene expression between female and intersex gonads occurs in spite of the fact that the portion of intersex ovary was smaller than the female ovary added to the array. Therefore,

the ovary portion of the intersex gonad could be expressing higher levels of genes relative to the female ovary, or that the testicular portion is also expressing these genes. Studying the genes that are expressed differentially in the ovarian and testicular portions of intersex tissues can help identify underlying functional differences in these tissues relative to normal ovarian or testicular tissue. However, no study has analyzed each portion of intersex tissue separately.

Endocrine signaling pathways and the mechanisms underlying reproductive development are relatively conserved among vertebrates suggesting that specific suites of genes might be associated with normal gonadal function and fertility. Recent studies have identified genes or groups of genes that are important for fertility in mice and humans. Rockett et al (2004) found that 40 genes were differentially expressed between testicular tissue of fertile and infertile mouse lines. These mouse lines included animals that do not develop spermatogonia, or experience arrested spermatogenesis at the spermatocyte, or spermatid stage. In the same study, 19 genes were expressed differently between samples collected from humans with normal and abnormal spermatogenesis (Rockett *et al.*, 2004). Comparisons of the mouse and human gene expression profiles lead to the hypothesis that differential expression of genes associated with cell cycle control, heat shock proteins (hsp), and proteolytic proteins are mechanistically linked and are associated with altered spermatogenesis and infertility in mammals (Rockett *et al.*, 2004). Indeed, similar types of genes were differentially expressed in this study and could be associated with differences in fertility between normal and abnormal toads. Altered expression patterns of these or similar networks of genes could underlie the mechanisms of toxicity of EDCs and explain similarities in observed pathologies across taxonomic groups.

The use of cDNA arrays has advanced the field of ecotoxicology by providing a way to assess the effects of toxicants on multiple gene transcripts and biochemical pathways in a single

study (Denslow *et al.*, 2007). Arrays contribute to the development of a system wide understanding of toxicity by providing information on known and unknown pathways of toxicity and by linking those pathways to phenotypic changes. However, arrays have limitations because gene expression varies extensively with several inherent biological processes including age, sex, and genetic polymorphisms, as well as with external phenomena such as temperature, day length, and season (Denslow *et al.*, 2007). Arrays describe gene expression profiles for organisms collected at a single point in time under a specific suite of environmental conditions. Biological pathways could vary in sensitivity to contaminants across seasons, sexes, or genetically distinct populations. In addition, cDNA arrays do not provide a truly quantitative measure of gene expression, but describe relative changes (Denslow *et al.*, 2007).

To understand the effects of endocrine disrupting chemicals, multiple endpoints must be measured. Previous work in this volume has shown that the occurrence and severity of intersex gonads is higher in toads collected from agricultural sites relative to those found in suburban sites. However, the mechanisms through which these abnormalities occur have not been studied and much work is needed linking gene expression, morphology and physiological performance. This study has documented expression profiles associated with phenotypically normal ovarian and testicular tissue and compared those profiles to those of pathological intersex tissue. This approach has identified many different genes and clusters of genes with correlated expression patterns expressed across these gonad types. I have identified specific genes associated with each gonadal type that should be studied further to determine whether they are dependable biomarkers for each sex group. Future studies will continue to investigate these biomarkers of sex over a larger sample size of individuals using QPCR so that individual variation can be

investigated and quantified. This work shows that intersex *Bufo marinus* living in agricultural areas in South Florida express genes in a pattern that is more similar to females than to males.

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Table 4-1 Fold change differences. Ovary and intersexed tissue (Ovary/Intersex), testis and intersexed tissue (Ttestis/Intersex), and ovary and testis (Ovary/Testis). Cluster numbers refer to clusters from the hierarchical cluster analysis identified in Figure 4-4. Functional codes: 1) cell growth control, 2) chromatin structure, 3) signal transduction, 4) transcription, 5) hormonal regulation, 6) metabolism, 7) transport/ and binding, 8) apoptosis/protein processing, 9) structural, 10) unknown function, 11) protein translation.

Gene	GenBank	Cluster	Functional	Female Intersex	Male Intersex`	Female Male
alpha skeletal actin	X03470	1	9	0.02	-0.55	0.57
c-Jun	5650725	1	4	-0.11	-0.77	0.67
cyclin B2	AB005253	1	1	-0.57	-1.53	0.97
cyclin H	U20505	1	1	-0.42	-1.34	0.91
cytosine-5-methyltransferase	D78638	1	2	0.03	-0.91	0.94
deoxyribonuclease gamma	AF059612	1	2	0.08	-0.74	0.82
E2 ubiquitin conjugating enzyme (Ubc9)	U88561	1	8	-0.08	-0.68	0.60
ER1	AF015454	1	4	0.27	-0.95	1.23
importin alpha 1a	L36339	1	7	-0.07	-0.74	0.67
nerve growth factor	X55716	1	1	-0.15	-0.72	0.57
survivin	AW198821	1	8	-0.30	-0.84	0.54
wee 1A kinase	U13962	1	1	0.10	-0.63	0.73
adult beta-globin	J00978	2	7	-0.83	-0.94	0.11
aldolase B	AB030822	2	6	-0.72	-0.88	0.16
cdc2	AB005254	2	1	-0.45	-0.92	0.47
cyclin E	L23857	2	1	-0.44	-0.43	-0.01
GSK-3 binding protein	AF062738	2	3	-0.59	-0.65	0.07
katanin p60	AF177942	2	9	-0.32	-0.55	0.23
myelin proteolipid protein	Z19522	2	9	-0.47	-0.47	0.00
Pax-2	AF179300	2	4	-1.17	-1.44	0.27
PCNA	M34080	2	2	-0.28	-0.52	0.23
retinoic acid converting enzyme	AF057566	2	5	-0.53	-0.78	0.25
sonic hedgehog	L39213	2	4	-0.60	-1.15	0.55

Table 4-1 (continued)

Gene	GenBank	Cluster	Functional	Female Intersex	Male Intersex`	Female Male
AP-2	M59455	3	4	-0.73	-0.15	-0.57
B-50/GAP 43	X87582	3	1	-0.57	-0.36	-0.21
casein kinase 1-epsilon	AF183394	3	3	-0.99	-0.67	-0.33
Cu/Zn SOD	X16585	3	6	-0.60	-0.34	-0.26
cyclin A2	X85746	3	1	-1.10	-0.97	-0.13
ets-2a	M81683	3	4	-0.67	-0.46	-0.21
fos-related antigen-2	U37374	3	4	-0.79	-0.18	-0.60
iodothyronine 5'-deiodinase III	L28111	3	5	-0.51	-0.16	-0.35
MAP kinase phosphatase	X83742	3	3	-0.80	-0.64	-0.17
MCM2	U44047	3	1	-0.53	-0.18	-0.35
MCM5/CDC46	U44048	3	1	-0.58	-0.40	-0.17
Myf-5	X56738	3	4	-0.91	-0.65	-0.26
NEDD 8	Not Submitted	3	8	-0.77	-0.43	-0.34
ornithine decarboxylase	X56316	3	6	-0.70	-0.56	-0.14
p54 mRNA binding protein	M80257	3	4	-0.72	-0.33	-0.39
TBP-binding repressor Dr1	AB003582	3	4	-0.61	-0.37	-0.24
TFIID subunit	D50054	3	4	-0.77	-0.47	-0.29
Xefiltin	U63711	3	9	-0.86	-0.59	-0.27
activated protein kinase C receptor	AF105259	4	3	-0.31	0.15	-0.46
alpha-1 collagen type II	M63595	4	9	-1.05	-0.07	-0.98
ATP-dependent RNA helicase	X57328	5	4	-0.20	0.24	-0.44
beta tubulin	L06232	5	9	-0.22	0.22	-0.44
brain factor 1	AF101387	4	4	-0.46	0.28	-0.74
calbindin D28K	U76636	4	7	-0.71	-0.08	-0.63
cellular retinoic acid binding protein	S74933	5	5	-0.38	0.44	-0.82
c-myc I	X14806	5	4	-0.17	0.38	-0.55
colorectal cancer tumor suppressor	U10986	4	3	-0.91	0.57	-1.47
DLL3 Distal-less 3	L09729	4	4	-0.41	0.02	-0.43
DLL4 Distal-less 4	L09728	4	4	-0.59	0.30	-0.89
elongation factor-1 alpha chain	M25504	4	11	-0.91	0.09	-1.00

Table 4-1 (continued)

Gene	GenBank	Cluster	Functional	Female Intersex	Male Intersex`	Female Male
hsp30B	X02512	5	7	-0.47	0.59	-1.06
liver helicase	AF302423	4	4	-1.65	-0.05	-1.59
MAM domain protein	U37376	5	10	-0.21	0.20	-0.42
mitochondrial cytochrome c oxidase subunit 1	M10217	4	6	-3.30	0.08	-3.39
myelin basic protein	AB000736	4	9	-0.71	-0.03	-0.68
nervous system-specific RNA binding protein	M34894	5	4	-0.64	0.87	-1.51
neuromedin	L01530	5	3	-0.32	0.92	-1.24
nuclear factor I-C1	L43149	5	4	-0.35	0.53	-0.87
Oct-1	X17190	4	4	-0.46	0.12	-0.58
Pitx-1	AF155206	4	4	-0.71	0.27	-0.98
protein S10	S57432	4	11	-1.00	0.07	-1.06
ribosomal protein L32	X55030	4	11	-0.38	0.08	-0.46
thyroid-stimulating hormone beta	L07618	5	5	-0.36	0.46	-0.82
vitellogenin	65210	4	6	-0.44	0.26	-0.70
adenosine A1 receptor (a1R gene)	AJ249842	6	3	0.00	0.64	-0.64
CCCH zinc finger protein C3H-3	AF061982	6	4	-0.05	0.63	-0.68
circadian CLOCK	AF203107	6	4	0.22	0.84	-0.62
collagenase-4 precursor	L76275	6	8	0.00	0.56	-0.56
Fez	AF195021	6	4	0.16	0.84	-0.68
metalloproteinase (XMMP)	U82541	6	8	0.23	1.03	-0.80
Na+/K+ transporting ATPase beta subunit	M37788	6	7	0.00	0.74	-0.74
PKC-related kinase 2	AF027183	6	3	0.22	0.62	-0.40
SERCa1 fast skeletal muscle	X63009	6	3	0.68	1.74	-1.05
sodium phosphate cotransporter	L78835	6	7	-0.23	1.49	-1.72
thyrotropin-releasing hormone precursor	X64056	6	5	0.37	1.29	-0.92
Xsal-1	L46583	6	4	0.00	0.40	-0.40

Table 4-1 (continued)

Gene	GenBank	Cluster	Functional	Female Intersex	Male Intersex`	Female Male
recombination activation (RAG-2)	L19325	7	2	0.95	0.00	0.95
Tcf-3 co-repressor CtBP	AF152006	7	4	0.92	0.28	0.64
BMP1b	Y09660	8	8	0.74	0.61	0.13
calcineurin A	AF019569	8	3	1.77	1.22	0.55
DNA binding protein E12	X66959	8	4	0.78	0.56	0.22
L-myc	L11363	9	4	0.31	0.45	-0.14
Mad1	L77888	8	1	0.45	0.44	0.01
Max2/Max4	L09738	8	4	1.62	1.12	0.50
osteogenic protein-1 homolog precursor	U40034	8	1	0.75	0.69	0.06
phospholipase C-gamma-1a	AF090111	9	3	1.28	1.68	-0.39

Table 4-2 Genes identified in correspondence analysis as being associated (or disassociated) with a specific reproductive organ type. Function codes: 1) cell growth control, 2) chromatin structure, 3) signal transduction, 4) transcription, 5) hormonal regulation, 6) metabolism, 7) transport/ and binding, 8) apoptosis/protein processing, 9) structural, 10) unknown function, 11) protein translation.

Gene Code		Functional code
	Associated with ovary tissue	
FJ7	wee 1-A kinase	1
FM55	DNAse gamma	2
FM62	cytosine 5-methyltransferase	2
FN44	ER-1	4
	Associated with teticular tissue	
FM24	SERCa1 fast skeletal muscle	3
FI9	neuromedin	3
FB2	colorectal cancer tumor suppressor	3
FH20	nervous system-specific RNA binding protein	4
FH15	sodium phosphate cotransporter	7
	Associated with intersex tissu:	
FJ30	cyclin A2	1
FJ20	casein kinase 1-epsilon	3
FD9	Pax-2	4
	Low in ovary but equal in testicular and intersexed tissue	
FM40	mitochondrial cytochrome c oxidase subunit 1	6
	Low in intersex, but equal in testicular and ovary tissue	
FM34	phospholipase C-gamma-1a	3
FN17	calcineurin A	3
FJ8	Max2/Max4	4
	Low in testis, but equal in ovary and intersexed tissue	
FJ36	cyclin H	1
FJ38	cyclin B2	1

Table 4-3 Gene expression level comparisons. Estimates of levels of expression, standard errors and pvalues.

Gene	Female	SE	F vs. I Pr> t	Intersex	SE	M vs. I Pr>t	Male	SE
Female and different from intersex								
osteogenic protein 1 homolog precursor	5.2E+03	1.2E+03	0.033 +	2.7E+03	2.0E+02	0.042 +	6.0E+03	1.6E+03
*cyclin H	3.3E+03	8.9E+02	0.015 -	4.6E+03	4.2E+02	<0.001 -	1.2E+03	8.4E+02
sonic hedgehog	1.0E+04	1.2E+04	0.044 -	2.4E+04	5.7E+03	0.013 -	6.3E+03	1.2E+04
retinoic acid converting enzyme	6.8E+03	3.6E+03	0.017 +	1.2E+04	1.7E+03	0.002 +	4.9E+03	3.5E+03
Female and intersex significantly different								
recombination activation RAG 2	2.8E+03	NA	<0.001 +	1.1E+03	NA	0.295	1.1E+03	1.3E+00
colorectal cancer tumor suppressor	1.2E+03	1.5E+03	0.045 -	2.9E+03	7.4E+02	0.454	4.5E+03	2.8E+03
casein kinase 1 epsilon	1.3E+04	9.8E+03	0.028 -	2.7E+04	4.0E+03	0.065	1.6E+04	9.4E+03
activated protein kinase C receptor	3.1E+04	1.4E+04	0.033 -	5.1E+04	5.7E+03	0.674	5.6E+04	1.7E+04
calcineurin A	6.6E+03	1.4E+03	0.001 +	1.4E+03	2.3E+02	0.726	2.9E+03	4.4E+03
Pitx 1	1.3E+04	1.1E+04	0.043 -	2.6E+04	5.6E+03	0.59	3.2E+04	1.6E+04
liver helicase	1.7E+03	3.0E+03	0.007 -	6.5E+03	1.5E+03	0.933	6.4E+03	3.2E+03
ornithine decarboxylase	1.5E+04	7.9E+03	0.01 -	2.7E+04	3.8E+03	0.441	2.0E+04	1.2E+04
aldolase B	1.1E+04	5.7E+03	0.001 -	2.3E+04	2.9E+03	0.142	1.3E+04	9.6E+03

Table 4-3 (continued)

Gene	Female	SE	F vs. I Pr> t	Intersex	SE	M vs. I Pr>t	Male	SE
*mitochondrial cytochrome c oxidase subunit 1	1.6E+03	6.6E+03	<0.001 -	4.2E+04	3.3E+03	0.391	4.5E+04	7.1E+03
alpha 1 collagen type II	2.0E+03	1.9E+03	0.03 -	4.5E+03	8.8E+02	0.83	5.0E+03	2.8E+03
*elongation factor 1 alpha chain	7.4E+02	3.0E+02	<0.001 +	1.6E+03	1.4E+02	0.509	1.7E+03	4.2E+02
Male and intersex significantly different								
wee 1A kinase	9.0E+03	4.2E+03	0.954	9.1E+03	1.8E+03	0.022 -	3.9E+03	3.8E+03
cyclin B2	1.8E+04	1.2E+04	0.098	3.0E+04	6.0E+03	0.005 +	7.5E+03	1.2E+04
*adenosine A1 receptor a1R gene	1.1E+03	7.3E+01	0.823	1.1E+03	NA	<0.001 +	2.6E+03	<0.001
phospholipase C gamma 1a	3.9E+03	1.5E+03	0.076	1.7E+03	3.2E+02	0.012 +	5.9E+03	1.7E+03
thyrotropin releasing hormone precursor	4.0E+03	2.0E+03	0.408	2.9E+03	7.5E+02	0.036 +	8.3E+03	3.0E+03
*sodium phosphate cotransporter	1.2E+03	8.0E+02	0.287	1.7E+03	4.0E+02	<0.001 +	3.9E+03	8.2E+02
Na K transporting ATPase beta subunit	1.1E+03	2.0E+02	0.789	1.1E+03	1.0E+02	<0.001 +	3.0E+03	2.0E+02
*E2 ubiquitin conjugating enzyme Ubc9	1.2E+03	2.2E+02	0.131	1.5E+03	8.8E+01	<0.001 -	7.1E+02	2.0E+02
*collagenase 4 precursor	1.1E+03	NA	0.349	1.1E+03	NA	<0.001 +	4.7E+03	NA
Not significantly different								
nerve growth factor	7.0E+03	7.0E+03	0.306	1.1E+04	3.3E+03	0.092	4.6E+03	6.8E+03
Mad1	2.4E+03	1.6E+03	0.896	2.5E+03	7.2E+02	0.45	3.4E+03	1.9E+03
cyclin A2	1.1E+03	3.4E+02	0.271	1.3E+03	1.7E+02	0.473	1.2E+03	3.5E+02

Table 4-3 (continued)

Gene (not significantly different)	Female	SE	F vs. I Pr> t	Intersex	SE	M vs. I Pr>t	Male	SE
cyclin E	1.1E+03	1.2E+03	0.387	1.7E+03	6.2E+02	0.537	1.2E+03	1.3E+03
cdc2	1.3E+04	1.5E+04	0.832	1.5E+04	4.5E+03	0.102	7.0E+03	9.0E+03
MCM5 CDC46	1.0E+04	1.1E+04	0.433	1.4E+04	5.3E+03	0.526	1.1E+04	1.1E+04
MCM2	4.5E+03	1.7E+03	0.156	6.2E+03	5.8E+02	0.449	5.6E+03	1.4E+03
B 50 GAP 43	1.8E+04	1.3E+04	0.083	3.0E+04	6.1E+03	0.34	2.2E+04	1.4E+04
PCNA	2.2E+04	1.3E+04	0.739	2.5E+04	5.4E+03	0.183	1.7E+04	1.1E+04
deoxyribonuclease gamma	3.7E+03	3.0E+03	0.835	4.0E+03	1.3E+03	0.136	1.8E+03	2.7E+03
cytosine 5 methyltransferase	1.2E+04	6.4E+03	0.856	1.1E+04	2.4E+03	0.057	5.8E+03	5.1E+03
neuromedin	1.8E+03	7.5E+02	0.634	2.0E+03	3.1E+02	0.317	3.8E+03	2.1E+03
MAP kinase phosphatase	1.6E+04	1.0E+04	0.056	2.8E+04	4.5E+03	0.143	1.7E+04	1.1E+04
PKC related kinase 2	1.5E+03	8.2E+02	0.653	1.3E+03	4.4E+02	0.109	2.4E+03	1.1E+03
GSK 3 binding protein	1.5E+04	1.3E+04	0.104	2.7E+04	6.1E+03	0.152	1.6E+04	1.3E+04
SERCa1 fast skeletal muscle	3.6E+03	1.6E+03	0.188	1.9E+03	4.0E+02	0.525	5.4E+03	5.7E+03
ets 2a	2.6E+03	3.6E+03	0.337	4.5E+03	1.8E+03	0.478	3.0E+03	3.8E+03
c myc I	1.3E+04	8.4E+03	0.455	1.7E+04	4.0E+03	0.998	1.7E+04	1.1E+04
L myc	5.2E+03	2.9E+03	0.995	5.2E+03	1.3E+03	0.854	5.6E+03	3.7E+03
circadian CLOCK	2.2E+03	9.7E+02	0.79	2.0E+03	4.0E+02	0.905	2.3E+03	2.5E+03
c Jun	3.1E+04	2.4E+04	0.814	3.4E+04	1.1E+04	0.131	1.7E+04	2.1E+04
nuclear factor I C1	2.0E+04	2.1E+04	0.214	3.4E+04	1.0E+04	0.537	4.2E+04	2.3E+04
X1 Oct	1.1E+04	9.8E+03	0.271	1.7E+04	4.9E+03	0.944	1.8E+04	1.3E+04
AP 2	1.3E+03	1.5E+03	0.157	2.4E+03	7.7E+02	0.755	2.2E+03	1.6E+03
TBP binding repressor Dr1	1.7E+03	1.6E+03	0.536	2.2E+03	7.5E+02	0.364	1.5E+03	1.5E+03

Table 4-3 (continued)

Gene (not significantly different)	Female	SE	F vs. I Pr> t	Intersex	SE	M vs. I Pr>t	Male	SE
TFIID subunit	1.7E+04	1.7E+04	0.223	2.8E+04	8.1E+03	0.476	2.2E+04	1.7E+04
Tcf 3 co repressor CtBP	4.2E+03	2.0E+03	0.145	1.6E+03	3.8E+02	0.779	1.8E+03	8.5E+02
brain factor 1	2.1E+04	1.5E+04	0.157	3.3E+04	6.7E+03	0.858	3.5E+04	1.8E+04
CCCH zinc finger protein C3H 3	2.0E+04	1.1E+04	0.964	2.0E+04	3.9E+03	0.208	3.1E+04	1.3E+04
DLL4	1.5E+04	1.2E+04	0.146	2.5E+04	5.6E+03	0.876	2.6E+04	1.6E+04
DLL3	4.3E+02	2.7E+02	0.402	5.5E+02	1.2E+02	0.851	5.8E+02	2.5E+02
Fez	4.9E+03	2.5E+03	0.823	4.6E+03	1.2E+03	0.213	9.4E+03	4.8E+03
DNA binding protein E12	2.5E+03	7.8E+02	0.078	1.5E+03	2.8E+02	0.28	2.3E+03	9.9E+02
Xsal 1	1.2E+03	1.3E+02	0.798	1.2E+03	5.6E+01	0.344	1.4E+03	2.6E+02
p54 mRNA binding protein	3.7E+04	4.1E+04	0.115	7.3E+04	2.0E+04	0.451	5.4E+04	4.4E+04
Myf 5	1.1E+03	3.0E+03	0.24	3.0E+03	1.5E+03	0.284	1.3E+03	3.0E+03
ATP dependent RNA helicase	1.5E+03	8.7E+02	0.819	1.4E+03	2.4E+02	0.472	1.7E+03	7.2E+02
fos related antigen 2	1.5E+03	2.0E+03	0.179	2.9E+03	1.0E+03	0.952	2.8E+03	2.2E+03
ER1	3.1E+03	1.6E+03	0.551	2.4E+03	4.7E+02	0.113	1.4E+03	1.0E+03
cellular retinoic acid binding protein	1.5E+03	1.4E+03	0.596	1.9E+03	7.4E+02	0.618	2.5E+03	1.9E+03
thyroid stimulating hormone beta	1.2E+03	3.0E+02	0.178	1.4E+03	1.5E+02	0.78	1.8E+03	1.7E+03
iodothyronine 5 deiodinase III	1.9E+04	1.6E+04	0.243	3.0E+04	7.6E+03	0.216	1.8E+04	1.6E+04
Cu Zn SOD	1.8E+04	1.9E+04	0.231	3.1E+04	9.1E+03	0.247	1.9E+04	1.9E+04
vitellogenin	1.1E+04	1.1E+04	0.162	1.9E+04	5.3E+03	0.583	2.3E+04	1.2E+04
importin alpha 1a	6.6E+03	4.1E+03	0.91	6.9E+03	1.3E+03	0.072	3.7E+03	2.9E+03
calbindin D28K	1.6E+03	1.1E+03	0.275	2.3E+03	4.6E+02	0.8	2.1E+03	1.1E+03
adult beta globin	1.1E+04	4.2E+04	0.522	2.5E+04	2.1E+04	0.5	9.8E+03	4.2E+04

Table 4-3 (continued)								
hsp30B	2.1E+03	1.1E+03	0.507	2.5E+03	4.3E+02	0.352	4.1E+03	2.0E+03
NEDD 8	3.2E+04	2.6E+04	0.091	5.8E+04	1.2E+04	0.37	4.4E+04	2.7E+04
Gene (not significantly different)	Female	SE	F vs. I Pr> t	Intersex	SE	M vs. I Pr>t	Male	SE
metalloproteinase XMMP	4.9E+03	1.9E+03	0.219	3.1E+03	4.6E+02	0.073	9.2E+03	3.6E+03
survivin	6.9E+04	3.4E+04	0.145	1.0E+05	1.4E+04	0.054	4.9E+04	3.8E+04
BMP1b	2.1E+03	7.2E+02	0.126	1.3E+03	2.4E+02	0.212	1.9E+03	6.8E+02
katanin p60	6.4E+03	1.1E+04	0.271	1.3E+04	5.2E+03	0.196	5.7E+03	1.1E+04
myelin proteolipid protein	1.1E+03	9.6E+01	0.382	1.2E+03	4.7E+01	0.209	1.1E+03	9.5E+01
myelin basic protein	1.4E+03	2.1E+03	0.307	2.5E+03	1.0E+03	0.984	2.4E+03	2.5E+03
beta tubulin	3.9E+02	2.6E+02	0.839	4.2E+02	1.2E+02	0.343	5.5E+02	2.6E+02
Xefiltin	5.5E+03	1.1E+04	0.162	1.4E+04	5.4E+03	0.302	6.8E+03	1.2E+04
alpha skeletal actin	8.2E+02	1.8E+02	0.923	8.3E+02	5.3E+01	0.169	6.0E+02	2.1E+02
MAM domain protein	4.6E+02	6.1E+01	0.088	5.3E+02	2.1E+01	0.101	6.3E+02	7.6E+01
protein S10	1.9E+04	1.9E+04	0.139	3.5E+04	9.0E+03	0.529	4.2E+04	1.9E+04
ribosomal protein L32	2.8E+02	1.4E+02	0.253	3.7E+02	6.6E+01	0.436	4.9E+02	2.2E+02
Pax 2	1.6E+03	NA	0.833	1.1E+03	NA	0.276	1.1E+03	NA

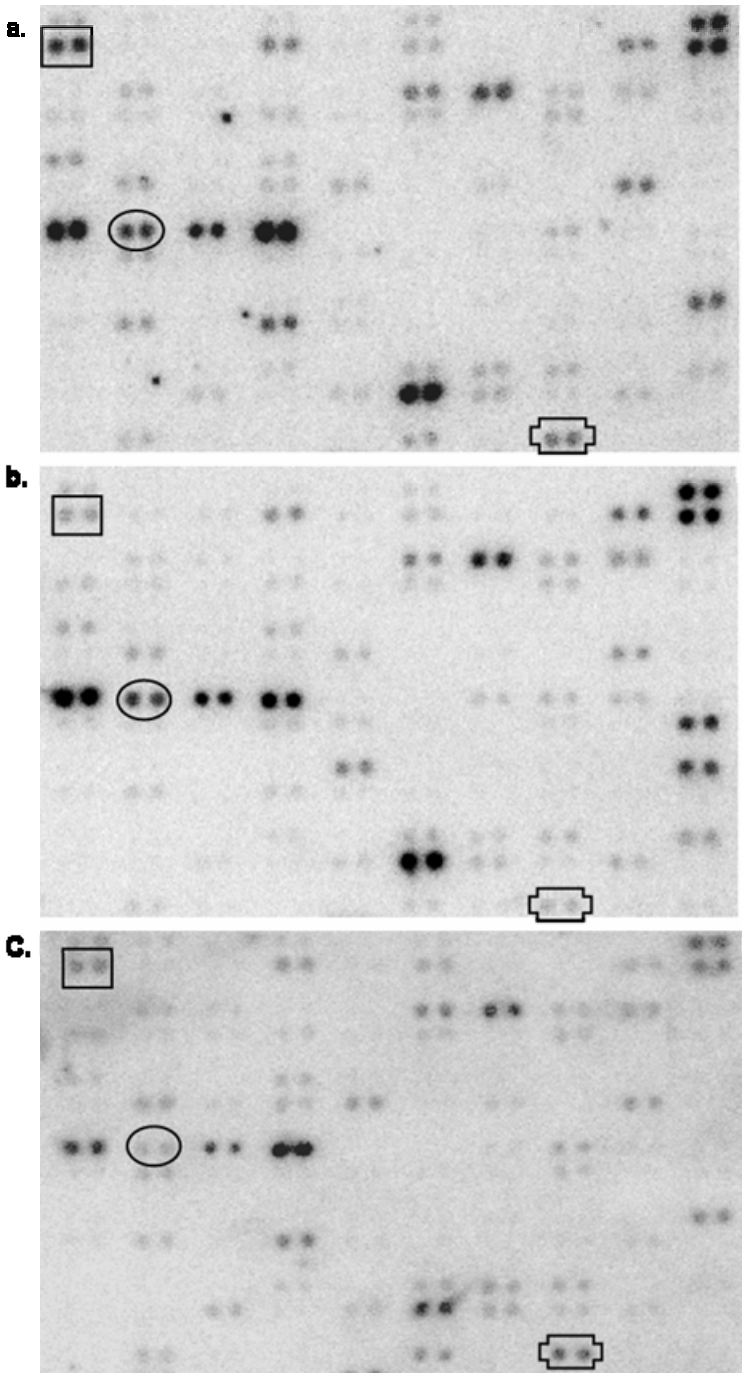

Figure 4-1 Representative region of three arrays depicting differences in gene expression among sex groups. a. Array of ovary tissue from a female toad. b. Array of testis tissue from a male toad. c. Array of an intersexed gonad. Genes are spotted in duplicate, and were differentially expressed across sex groups. Boxes indicate a single gene that was up regulated in ovaries relative to testes and intersexed tissues. Ovals indicate a gene that that expressed similarly in ovaries and testes, but down regulated in intersexed tissue. Crosses indicate a gene that was similar in ovaries and intersexed tissue, but down regulated in testes.

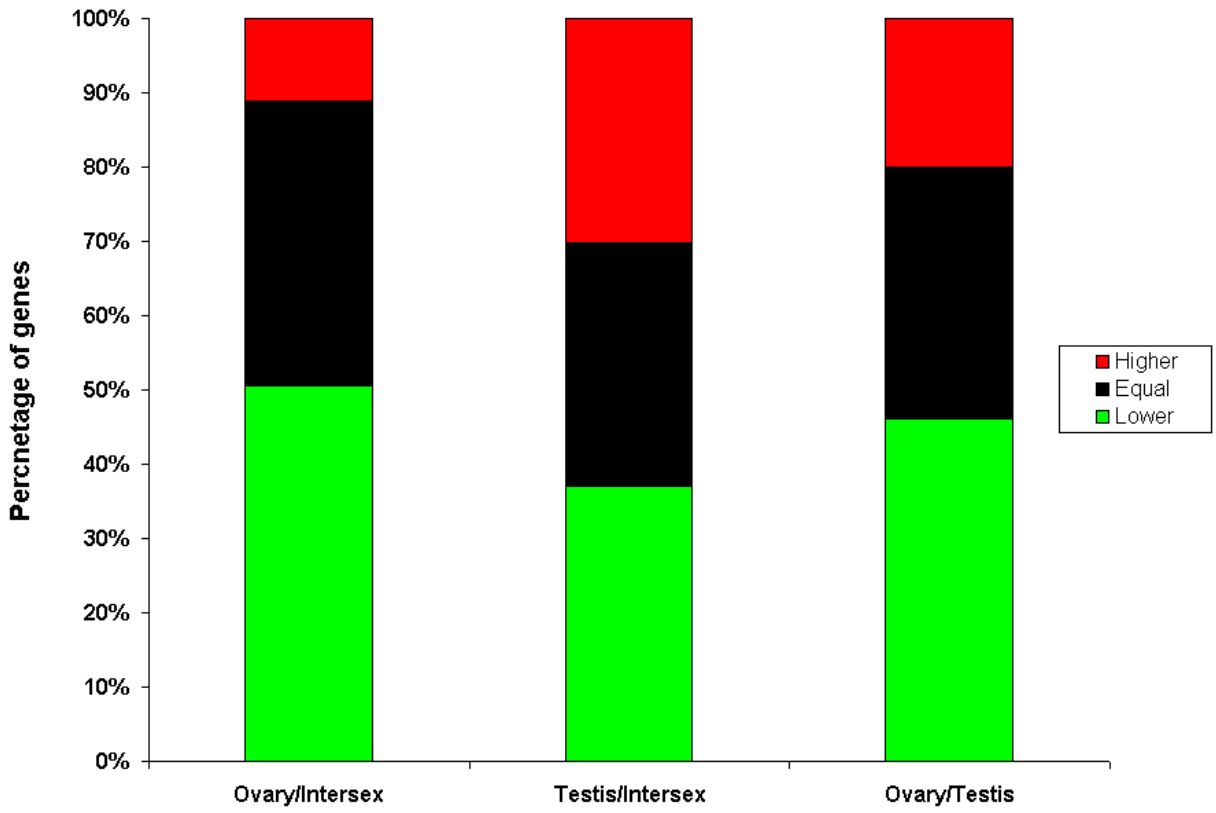

Figure 4-2 Percentage of genes that had a fold change that was higher, equal, or lower relative to another sex group. Fold change equals the median expression value of one sex organ (e.g. ovary) divided by the median expression level of another (e.g. intersex).

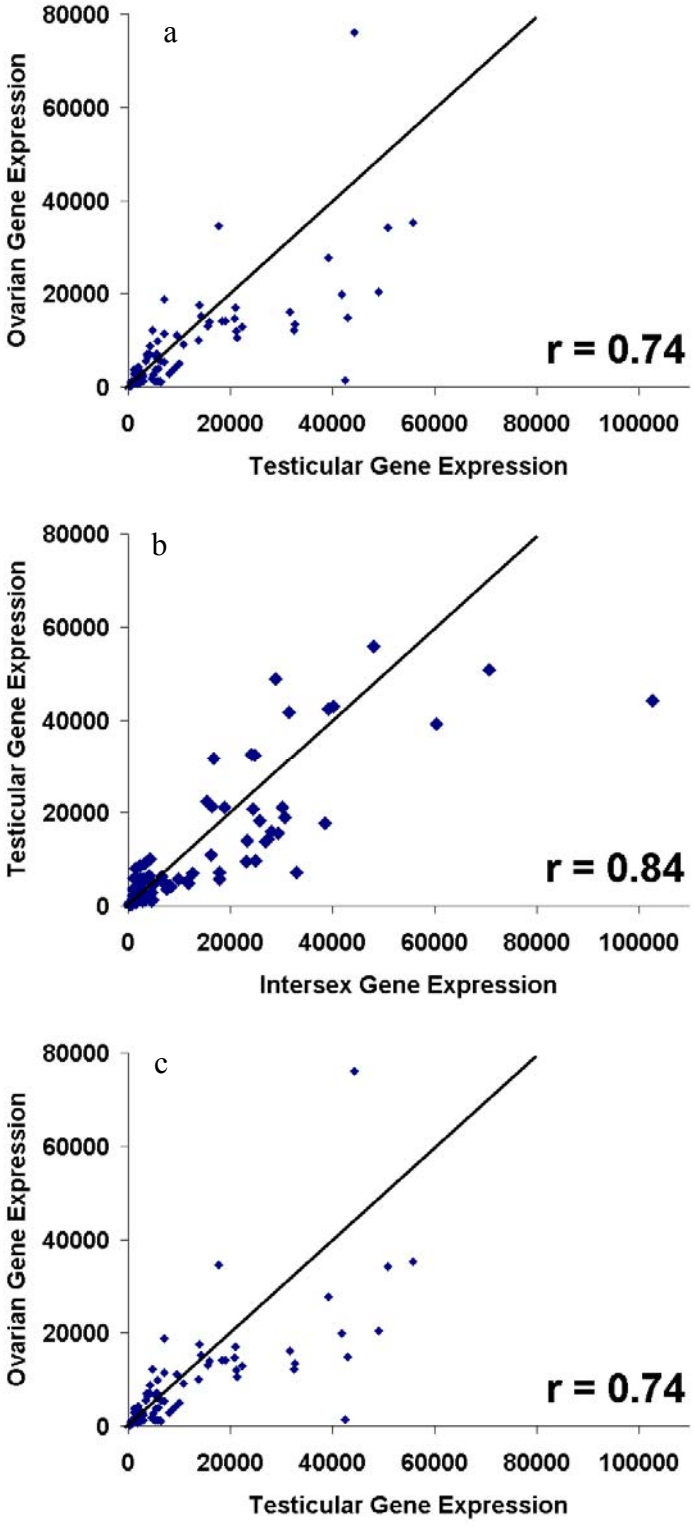

Figure 4-3 Gene expression correlations. a. ovary and intersexed tissue gene expression, b. testis and intersexed tissue gene expression, and c. ovary and testis tissues. Solid line represents the one to one line.

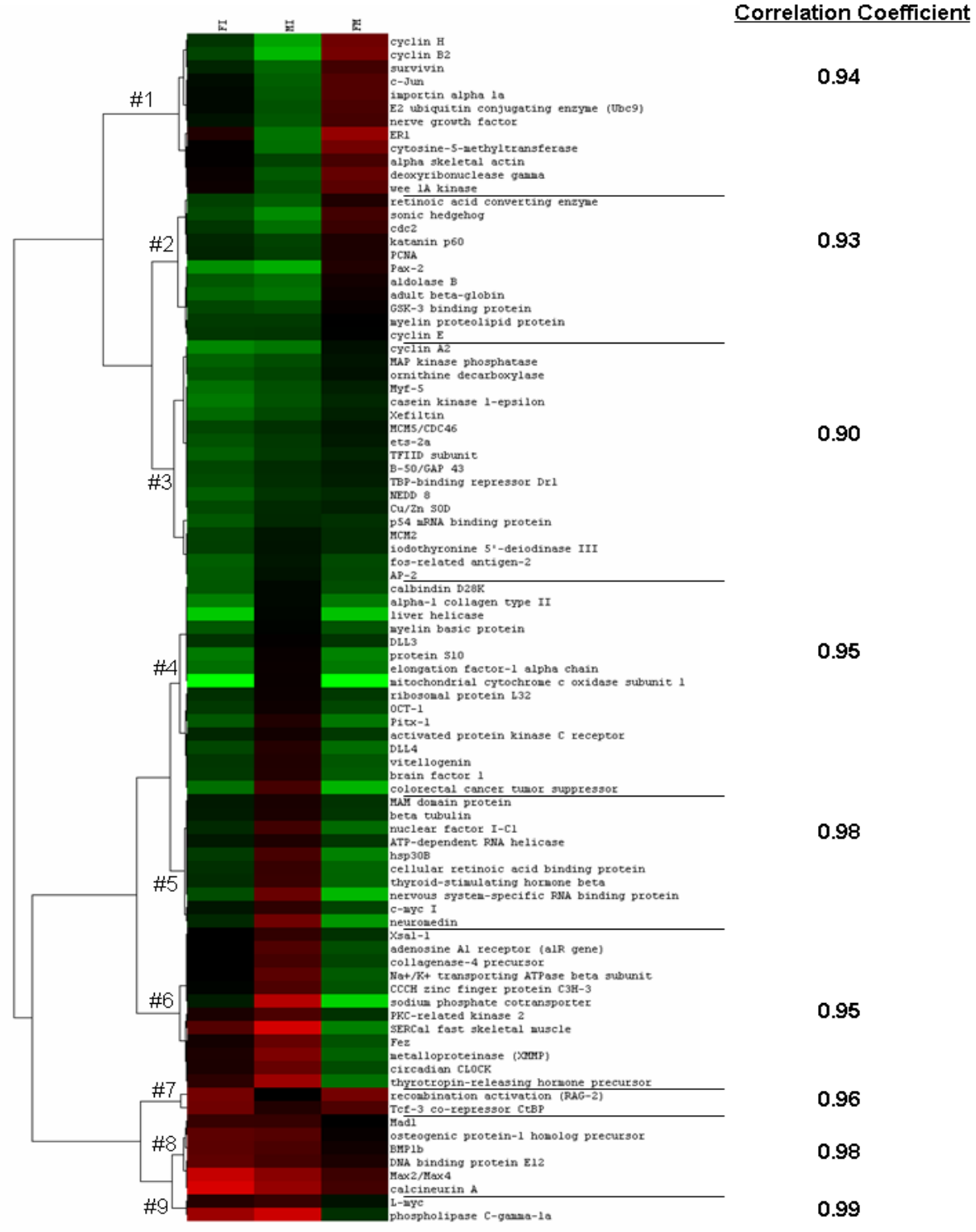

Figure 4-4 Cluster analysis of fold change expression levels of each gene that met the data quality requirements and showed a fold change higher than 1.5 increases or 0.67 decreases in at least one category. Numbers indicate clusters that had correlation coefficients of at least 0.90.

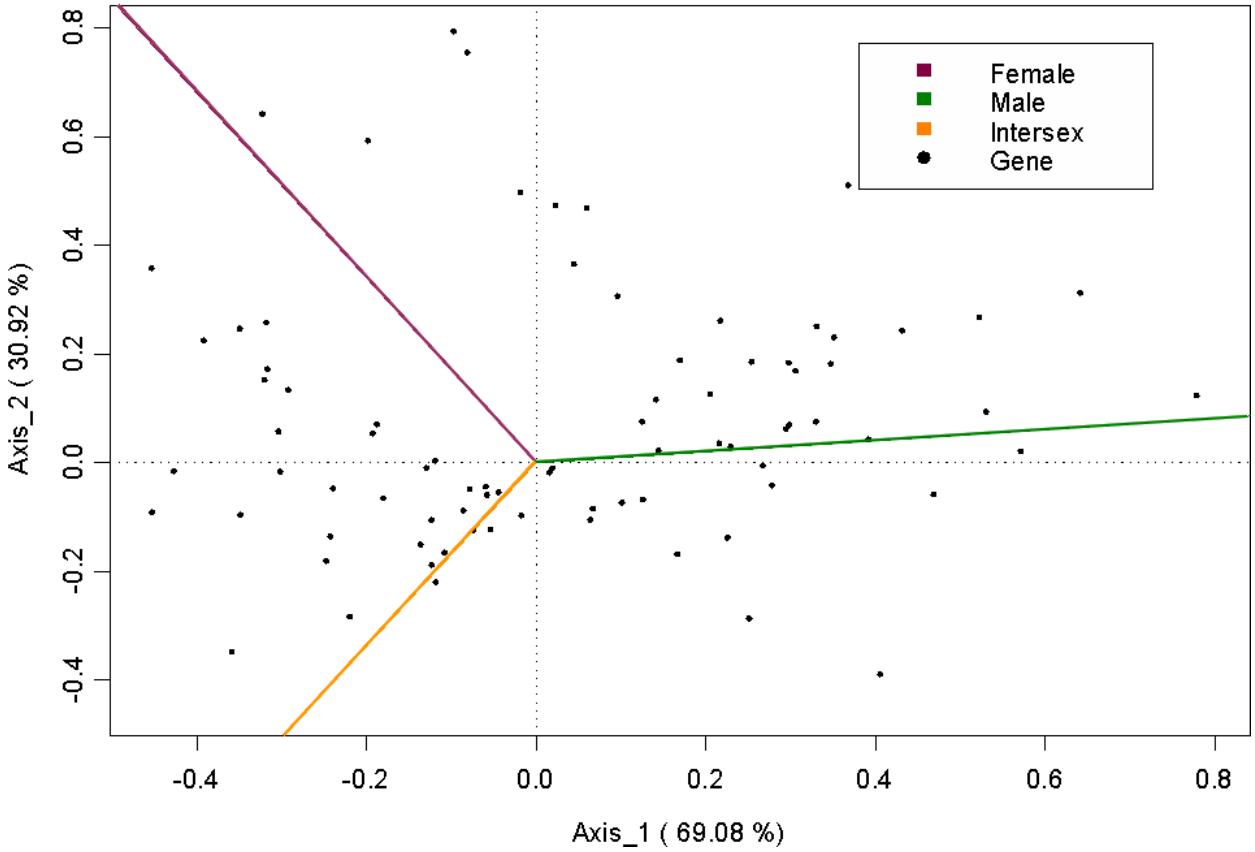

Figure 4-5 Association among gene expression and gonadal type. Black points represent a gene transcript. The further away a gene transcript is relative to the origin the more that gene deviates from an average response. If the point lies close to or along the line representing a gonad type (purple ovary, green testis, orange intersex), then it is more strongly related to that gonadal type. If gene are located in the same half of the plot, those gonadal tissue types are more related. This is particularly true for the halves represented by the *X*-axis which accounts for >65% of the data variation. Therefore, ovarian and intersex gene expression patterns are more similar to each other than either is to testicular gene expression. The names of the genes identified as closely associated with each gonadal type are listed in Table 2. This figure was generated by Dr. Mary Lesperance University of Victoria, British Columbia Canada.

CHAPTER 5
RENAL PATHOLOGIES IN MARINE TOADS ARE ASSOCIATED WITH LAND USE PATTERNS

Introduction

Many pollutants are broadly distributed as a result of wide scale use, run off, and fallout in precipitation (Davidson & Knapp, 2007; Lie *et al.*, 2003; Sparling, Fellers & McConnell, 2001; Thurman & Cromwell, 2000). Even areas once believed to be pristine are now known to be affected by environmental pollutants (e.g. (Lie *et al.*, 2003; Thurman *et al.*, 2000). As a result, environmental contaminants are a pan global health concern for humans and wildlife (Colborn & Clement, 1992; Colborn, vom Saal & Soto, 1993; Edwards, Moore & Guillette, 2006; Guillette *et al.*, 1995; Guillette & Gunderson, 2001; Norris & Carr, 2006; Vos *et al.*, 2000).

Kidneys are the principle excretory organs for all vertebrates, filtering toxins from the blood and excreting them, so excessive or chronic exposure to exogenous toxins can lead to renal pathologies. Many endogenous metabolic toxins and exogenous contaminants are removed from the body via active transport across the proximal tubule which makes it a target for toxin-induced pathologies (Sweet, 2005). For example, acute tubular necrosis (a disease of the kidney's proximal tubules) occurs in humans that have ingested medications or pesticides, or in wildlife that live in polluted habitats (Chan *et al.*, 1998; Ortiz, De Canales & Sarasquete, 2003; Rankin, 2004), and leads to loss of tubular brush boarders, epithelial cell thinning, vacuolization, and degeneration (Kern, 1999; Ortiz *et al.*, 2003).

Interstitial tissue around the proximal tubule is also an important target for toxin-induced pathologies such as interstitial nephritis, which involves infiltration of lymphocytes and leads to inflammation. Chronic interstitial nephritis typically occurs after long term exposure to pharmaceuticals or environmental toxins, and is further associated with interstitial fibrosis, an irreversible renal injury characterized by the accumulation of matrix proteins in the interstitium

(Eddy, 1996; Kern, 1999; Ortiz *et al.*, 2003). Interstitial fibrosis is the most important cause of chronic renal failure in humans (Sipes, McQueen & Gandolfi, 1997).

Renal pathologies associated with pollutant exposure have been documented in a diverse array of wildlife. For example, the kidneys of herring gulls (*Larus argentatus*) from the Great Lakes (USA) region contaminated with planar halogenated aromatic hydrocarbons (e.g., used in solvents and some pesticides) had diagnostic pathologies consistent with interstitial nephritis (Fox, Grasman & Campbell, 2007). In most instances of interstitial nephritis and tubular necrosis, glomeruli are unaffected. However, Baltic grey seals (Halichoerus grypus), polar bears (*Ursus maritimus*), and sledge dogs (Canis familiaris) exposed to DDT, polychlorinated biphenyls (PCBs), or organohalogens had glomerular pathologies including large hyaline bodies, diffuse thickening of the glomerular capillary walls, and sclerosis (Bergman, Bergstrand & Bignert, 2001; Sonne *et al.*, 2006; Sonne *et al.*, 2007b). Polar bears (*Ursus maritimus*) from eastern Greenland had several renal pathologies that were correlated with body burdens of specific contaminants. For example, the occurrence of interstitial fibrosis in Polar bears from eastern Greenland was correlated with polybrominated diphenyl ether (a flame retardant) concentrations in their blubber, whereas diffuse glomerular capillary wall thickening was found to be associated with chlordane (a pesticide) concentrations (Sonne *et al.*, 2006). As evidenced by these examples, studies of the effects of pollutants on renal physiology have focused predominantly on mammals. No studies have characterized toxin-induced renal pathologies in amphibians.

Although many studies have linked exposure to specific chemicals with specific renal diseases, none have compared renal pathologies occurring in animals living in different types of human-dominated landscapes. Urban and suburban areas are typically polluted with

polyaromatic hydrocarbons (PAHs) associated with road runoff (Crosbie & Chow-Fraser, 1999; Maltby *et al.*, 1995), whereas agricultural areas are more often polluted with several pesticides used to control weed, insect, and rodent populations (Crosbie *et al.*, 1999; Kreuger, 1998). These associations are so strong that PAH and pesticide (i.e. metolachlor) concentrations have been used to estimate the proportion of urbanized and agricultural land, respectively, among different watersheds (Crosbie *et al.*, 1999; Standley, Kaplan & Smith, 2000). Thus, both urbanized and agricultural habitats are polluted with mixtures of chemicals that likely induce renal pathologies. The goal of this study was to investigate renal pathologies diagnostic of toxin exposure in *Bufo marinus* living in suburban and agricultural habitats.

Methods

Characterizatizing Land Use Type

I collected *Bufo marinus* from five sites in South Florida with different suburban and agricultural land use. Land use type for each site was determined by importing Google Earth digital satellite images (downloaded August 20, 2007) and a distance key of each site into Image Pro-Plus (Media Cybernetics Inc) image analysis program. I centered a 5.6 km^2 grid (with 9 ~622 m^2 cells) over each collection site. The home range of *B. marinus* is ~2km^2 (Zug, Lindgren & Pippet, 1975), so this grid includes the area likely to be experienced by a toad from each local site.

Land use type for the five sites was defined by the percentage of agricultural land, proximity to nearest agriculture (for those sites lacking agriculture), and amount of suburban development (i.e. roadways) within the 5.6 km^2 area. Agricultural landuse in this study is prodominantly sugarcane (northern sites), or mixed vegetable and silviculture (southern site), and not livestock ranches. I estimated the percentage of agricultural area (km^2) per cell and averaged the percentages across the grid to determine the mean percentage of agriculture for

each site. The percent agricultural coverage among the sites varied from 0 to 97% (Belle Glade - 97% Canal Point - 51.2%, Homestead - 34%, Wellington - 0%, and Lake Worth-0%). Belle Glade and Canal Point were almost entirely agricultural land with few roads, whereas Homestead included a mixture of agricultural fields, buildings, and roadways. Wellington and Lake Worth, had zero agriculture and were distinguished from each other by the distance to the closest agricultural area, amount of buildings and roadway cover. Wellington was 5.18 km from agriculture and had less suburban development, whereas Lake Worth was 22 km from agriculture and had more suburban development.

Toads collected from suburban areas often were found in shopping center parking lots where they are likely exposed to oil residues (polyaromatic hydrocarbons PAHs), antifreeze (e.g. ethylene glycol), and car exhaust, but they were also collected from lawns next to roads and parking areas. Suburban lawns are often treated with a variety of chemicals some of which are similar to those used at agricultural sites. Toads collected from agricultural areas were collected from within agricultural fields, grassy areas on the margins of agricultural fields and from the parking lots of the field stations. Thus, in addition to exposure to high levels of agricultural contaminants it is likely that these toads were also exposed to pollutants from cars, albeit to a lesser degree (the parking lots a these sites were only used by station employees and were not as busy as the shopping mall parking lots in the suburban sites).

Sample Collection, and Histology

After toads were captured, they were euthanized with an overdose of the anesthetic MS222 (0.3% Tricaine Methanesulfonate, pH 7), the kidney-gonad complex was removed, and one kidney and gonad from each individual was fixed in neutral buffered formalin for histological analysis.

Kidney tissue from 82 toads was evaluated. Some of the toads had gondal abnormalties or intersex gonads. However, there was no association between gonadal abnormalities (e.g. intersex gonad or mishaped tistis) and kidney abnormalities within each site. For example, the presence of testicular abnormalities in toads from Lake Worth and Belle Glade were not assocaited with the total number of kidney abnormalities (LW=X^2 = 2.36, df=4, p=0.67; BG=X^2 = 8.61, df=4, p=0.20). Therefore, all individuals within sites were pooled for site comparisons of renal pathologies, regardless of the presence of gonadal abnormalities. Kidney tissue from 68 male and 14 intersex toads was dehydrated, embedded in paraffin, and sectioned at 7-8 □m. Approximately 10 slides were made for each individual; every other slide was stained with hematoxylin and eosin. To ensure that the same cells were not counted more than once, one section per stained slide (total of 5) was evaluated per individual, and sections were at least 90 μm apart. At least 11 toads from each site were analyzed (LW=11 male, 0 intersex; WT=16 male, 0 intersex; HS=11 male, 3 intersex, CP=16 male, 4 intersex; BG=14 male, 7 intersex).

Response Variables and Statistics

I recorded the presence or absence of vaculization, degraded tubule cells, dilated tubules, interstitial lymphocyte infiltration, and interstitial scarring for each toad (assessed across the five sections). Each of these responses was examined using a chi square analysis to determine if the incidence of each pathology varied among sites more than expected by chance.

I counted the number of fungal elements, and granulomas in each section and averaged these counts across sections for each toad. These data were analyzed using Kruskal Wallis one-way analyses of variance to determine if the abundances of these abnormalities (number per section per toad) differed among sites.

I also combined some of the response variables to examine pathologies indicative of toxin-induced tubular necrosis and chronic interstitial nephritis (Kern, 1999). I determined the number

of pathologies present in a toad that were indicative of toxin-induced tubular necrosis and scored each toad 0-3 depending on the presence of vacuolization, degrading tubule cells, and dilated tubules. I also determined the number of pathologies indicative of interstitial nephritis and toads received a score of 0-3 based on the presence of interstitial lymphocyte infiltration, presence of granulomas, or interstitial scarring. These responses were analyzed with Kruskal Wallis one-way analyses of variance to determine whether the pathologies associated with each renal disease were common at specific sites.

Results

Several renal pathologies diagnostic of exposure to toxicants were identified in this study (Figure 1 A-E). The presence of vacuolization of tubule cells varied among sites more than expected by chance ($X^2 = 15.868$, df=4, p=0.003). Approximately 55% of the toads at Lake Worth and Canal Point had vaculized tubule cells whereas only 20-25% of the Homestead and Belle Glade toads and 0% of the Wellington toads had this pathology.

Approximately 25% of the toads at Lake Worth, the most suburban site, and Belle Glade, the most agricultural site, had dilated tubules, whereas very few if any toads at the other three sites had this pathology ($X^2 = 15.044$, df=4, p=0.005; Figure 1 C and D;and Figure 3A).

Degenerating (severely vacuolarized) tubule cells (Figure 1 B, Figure 3B) and interstitial fibrosis (epithelial scarring and hyperplasia Figure 1E; Figure 3C) varied in frequency among sites ($X^2 = 23.773$, df=4, p<0.001 and $X^2 = 17.359$, df=4, p=0.002 respectively). Degenerating tubule cells were most common at Belle Glade and Canal Point, and epithelial scarring was most common at Belle Glade. Lymphocyte infiltration was not significantly different among sites ($X^2 = 7.961$, df=4, p=0.093 Figure 3D).

Pathogenic fungal infections were found only in six toads across all sites. Lake Worth (LW) had no individuals with fungal infections whereas West Palm (WP), Homestead (HS), and

Canal Point (CP) each had one, and Belle Glade (BG) had three toads with fungal bodies. Therefore, statistical analysis was not conducted for this pathology. The number of granulomas was significantly different across sites (KW = 10.218, df=4, p=0.037; Figure 2). Toads obtained from the most agricultural site, Belle Glade had more granulomas (mode = 5; median 7.5) than toads from any other site (Figure 2). However, one toad from Wellington had 51 granulomas which is higher than any animal from BG (max 47).

The number of abnormalites consistent with toxin-induced tubular necrosis were significantly different among sites (KW = 23.7, df=4, p<0.001, Figure 3E). Lake Worth (most suburban) and Canal Point and Belle Glade (agricultural) had the highest percentage of individuals with at least one pathology associated with tubular necrosis (73%, 80%, and 71% respecitvely), whereas 12.5% and 29% of the individuals at Wellington and Homestead repectively had at least one.

The number of pathologies consistent with interstitial nephritis were significantly different among sites (KW = 15.3, df=12, p=0.004, Figure 3F) and occurred more commonly than those indicative of tubular necrosis. Toads with all three interstitial pathologies investigated where found at every site except for Wellington (Figure 3F). Fifty percent of the individuals at Wellington had at least one pathology consistent with interstitial nephritis. However, 70%-75% of the toads at Lake worth, Homestead, and Canal Point, and 95% of the toads collected at Belle Glade had at least one pathology associated with interstitial nephritis. The number of pathologies associated with interstitial nephritis were lowest at Wellington and highest at Belle Glade (Figure 8B).

Discussion

A wide variety of drugs and environmental pollutants induce nephrotoxicity. For example, chronic renal failure in humans is associated with long term use or abuse of analgesics (Amdur *et*

al., 1996). The kidney is unusually susceptible to environmental pollutants for several reasons. It receives more blood, relative to organs of similar volume and thus, the kidney is exposed to greater quantities of toxins. The processes involved with filtering metabolic waste products and forming nitrogenous waste for excretion also concentrate toxins in the tubular fluid. As water is reabsorbed, toxins in the tubule fluid become highly concentrated, which can lead to their diffusion into the tubular cells (Amdur *et al.*, 1996; Sipes *et al.*, 1997). This concentrates toxins within the tubule cell. In addition, many pollutants are metabolized in the kidney, which contributes to its susceptibility, as chemicals can be bioactivated and made more toxic during their metabolism (Amdur *et al.*, 1996; Sipes *et al.*, 1997).

The specific pathologies identified in this study have been associated with toxicant exposure in other organisms, including humans, polar bear, dogs, birds, and fish (Chan *et al.*, 1998; Eddy, 1996; Fox *et al.*, 2007; Ortiz *et al.*, 2003; Rankin, 2004; Sonne *et al.*, 2006; Sonne *et al.*, 2007a). In addition, the dominant types of pollutants in a specific area depend on local land use (Crosbie *et al.*, 1999). For example, Crosbie and Chow-Fraser (1999) demonstrated that polycyclic aromatic hydrocarbons (PAHs) were most concentrated in urban sites, whereas metolachlor was most concentrated in agricultural sites. These associations were so strong that the authors argued that the pollutants could be used to determine land use practices or as surrogates to land use measures (Crosbie *et al.*, 1999). However, many of the pollutants present at each of the five study sites in this study are unknown (but see appendix 1 for some known pollutants). Each site has a milieu of pollutants, and the mechanisms of toxicity leading to renal pathologies is complex. For example, the agricultural sites have high pesticide (e.g., atrazine and glyphosate), and fertilizer (leading to high levels of nitrates, a potential renal toxin) application. Urban settings likely have high PAHs (due to higher traffic and road density), as well as

residential application of fertilizers and pesticides. In addition, the mixtures of chemicals at the two most agricultural sites (CP and BG; predominantly sugarcane) likely differ from the agricultural contaminants used at the farms around Homestead which grow other types of plants (e.g., trees and vegetables).

Although it is known that certain renal pathologies are associated with pollution exposure and that pollutants vary with land use, this is the first study that examines the occurrence of particular renal pathologies in toads from habitats that vary in land use. This study demonstrates that renal pathologies consistent with toxin exposure were present in *Bufo marinus* collected from all sites. Granulomas, degrading (necrotic) tubule cells, and interstitial fibrosis occurred more frequently at the most agricultural sites (Belle Glade and Canal Point), whereas dilated tubules occurred only occurred at the most suburban *and* the most agricultural sites. The more frequent occurrence of some toxin-induced pathologies at the most agricultural and suburban sites suggests that toads in these areas may experience greater exposure to toxins or to a greater variety of toxins.

Toxin-induced tubular necrosis is characterized by proximal tubular epithelium necrosis where tubule cells become vacuolized, and necrotic. As the tubule cells are destroyed, only the basement membrane around the tubule remains (Figure 1). This study demonstrates that pathologies associated with this tubule disease were generally more common in agriculture. A higher percentage of the animals at the agricultural sites had two of the three (recorded) pathologies diagnostic of this disease. The only animals that had all three pathologies consistent with tubule necrosis were collected from the most agricultural site. However, a high percentage of toads at the most suburban site (70%) had at least one pathology associated with this debilitating disease which was much higher than the sites with intermediate agricultural and

suburban exposures. The presence of tubular pathologies across sites concomitant with specific pathologies occurring most often at agricultural sites (degenerating tubule cells) suggests that there are chemicals that have the same mechanisms of toxicity across sites, but that agricultural areas have additional chemicals that work through other mechanisms to induce additional pathologies. Alternatively, the kidney could respond to toxic chemicals of various forms in a similar manner. That is, specific renal pathologies are not due to specific chemicals, but represent a generalized response to perturbation by toxic chemicals.

Interstitial nephritis is characterized by the presence of inflamation in the renal interstitium (Kern, 1999). Inflammation is a natural biological response to cellular or tissue level damage and involves the infiltration of plasma and leukocytes from the blood into the injured tissues. Interstitial nephritis can be acute or chronic, and the diagnostic feature of chronic interstitial nephritis is the presence of interstitial fibrosis (Kern, 1999). Fibrogenesis is characterized by excessive formation of extracellular matrix proteins such as collagen in response to tissue damage or pathogens. In humans, progressive interstitial fibrosis is the strongest determinant of chronic renal failure, and the most common cause is the use of medications, and thus is "toxin"-induced. For example, in a recent study, 92% of the interstitial nephritis cases investigated were drug induced (Clarkson *et al.*, 2004). However, interstitial nephritis can also be induced by bacterial or viral infections, or rarely, by hereditary diseases. Differential diagnosis among drug/toxin or bacterial/viral induced interstitial nephritis depends on the dominant types of leukocytes present in the interstitium (Kern, 1999). A predominance of neutrophils, which have diagnostic saddle shaped nuclei, in the inflammatory infiltrate indicates the presence of bacterial infection (Kern, 1999). However, lymphocytes and eosinophils predominate in drug induced interstitial nephritis. In addition, granuloma formation (dense clusters of lymphocytes and

multinucleated giant cells) occurs in approximately 30% of drug-induced interstitial nephritis cases, and adverse reactions to drugs is thought to be the most common cause of these formations (Kern, 1999). However, the presence of fungal infection can also induce granuloma formation.

The majority of leukocytes observed infiltrating the renal interstitium in this study were lymphocytes, which have large nuclei and small amounts of cytoplasm relative to other leukocytes, and are associated with toxin induced interstitial nephritis. When examined specifically, lymphocyte infiltration (although not significantly), and the number of granulomas were highest at the most agricultural site. Some of the observed granulomas were due to fungal infection, as they encapsulated fungal bodies. However, fungal infections were rare (~ 7%) and granulomas occurred when fungal bodies were not present.

Tubular injury and necrosis can induce a cascade of physiological events that lead to interstitial inflammation and fibrogenesis. As interstitial fibrosis worsens it can lead to further necrosis and reduced filtration rate. These diseases either alone or in conjunction are associated with renal failure, which occurs when glomerular filtration rate decreases to a level at which kidneys can no longer function properly. As kidneys fail many other systems are affected. Blood pressure can become increased due to excess fluids. Urea and other chemicals such as potassium accumulate in the blood, which can lead to edema and electrolyte imbalances. In addition, the kidney stops producing proper amounts of bicarbonate, which can lead to decreased blood pH, and death. The pathologies I document here are expected to have negative effects on the health of *Bufo marinus*, and are likely occurring in and affecting other wildlife species in these or similar habitats. The similarity between the renal pathologies found across diverse taxa

exposed to toxicants, and those found in humans taking medications such as antibiotics is striking.

The specific milieu of chemicals present at each site is expected to induce different pathologies depending on the tissue type. In previous studies I have demonstrated that morphological and functional gonadal abnormalities occur more often in toads living in agricultural areas where endocrine disrupting chemicals known to alter the reproductive system are used. Agricultural toads also exhibit renal pathologies that are diagnostic of pollution exposure. However, there is no association between gonadal and renal pathologies. Although gonadal abnormalities occur more often in toads living in agricultural areas toxin-induced renal pathologies are not limited to agricultural sites. Therefore, toads across all five sites are exposed to pollution, but the specific pollutants used in the most agricultural sites (appendix 1) induce endocrine disruption altering gonadal development whereas pollutants at the other sites do not. This suggests that the chemiacal milues at agricultural sites work though different mechanism of toxicity relative to those at the suburban sites.

Often, scientists think of animals as models for understanding human disease. In this study, a detailed understanding of human renal diseases was used to understand those of wildlife. Future research should focus on understanding the similarities and differences in the mechanisms through which environmental contaminants influence renal physiology and pathogenesis of kidney diseases in wildlife and humans. A more detailed understanding of the mechanisms of toxin-induced renal pathologies, as well as the chemicals involved, could help devise strategies to avoid or mitigate the impacts of toxins on humans and animals living in human-modified environments.

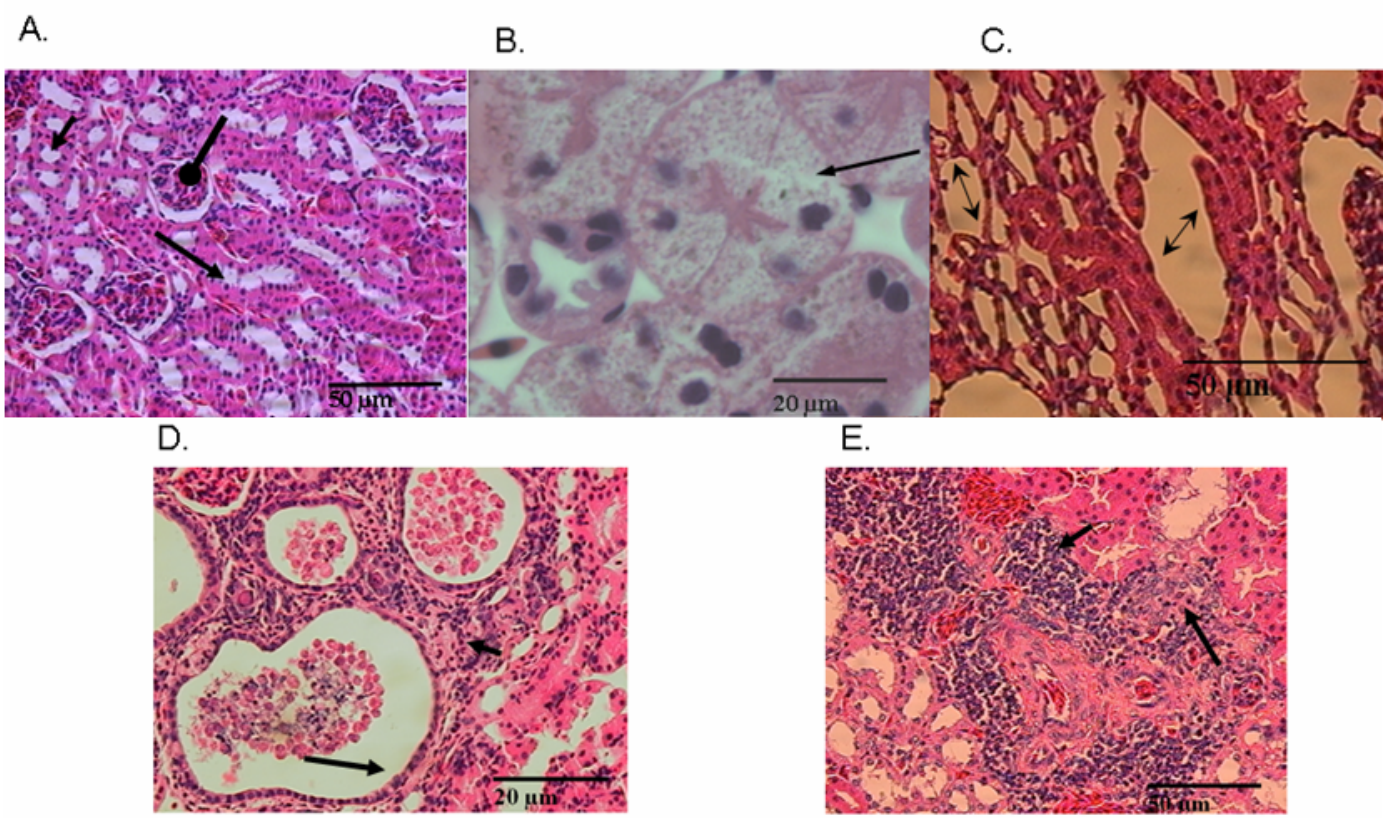

Figure 5-1 Selected renal pathologies. A. A normal kidney section. The distal tubules (short arrow), proximal tubule with brush borders (long arrow), and glomeruli (round arrow head) are distinguishable (40X bar is 50 µm). B. Tubule cell vacuolization indicated by (small arrow) has become so extensive that the cells are degrading. C. Dilated tubules and thinning tubule cells. Proximal tubules have no brush borders (double headed arrow). D. Tubular necrosis, dead and dying tubule cell casts are located within the tubules. Lymphocyte infiltration is extensive (short arrow), and tissue in this area is becoming fibrous and scarred. As the tubule cells are destroyed, only the basement membrane around the tubule remains (long arrow). The accumulation of lymphocytes in the renal tissue indicates an immune response. High infiltration of lymphocytes can cause the degradation of the adjacent tissue structures. E. Chronic interstitial nephritis is associated with interstitial fibrosis an accumulation of matrix proteins in the interstitium (long arrow), hyperplasia of interstitial epithelial cells, and lymphocyte infiltration (short arrow).

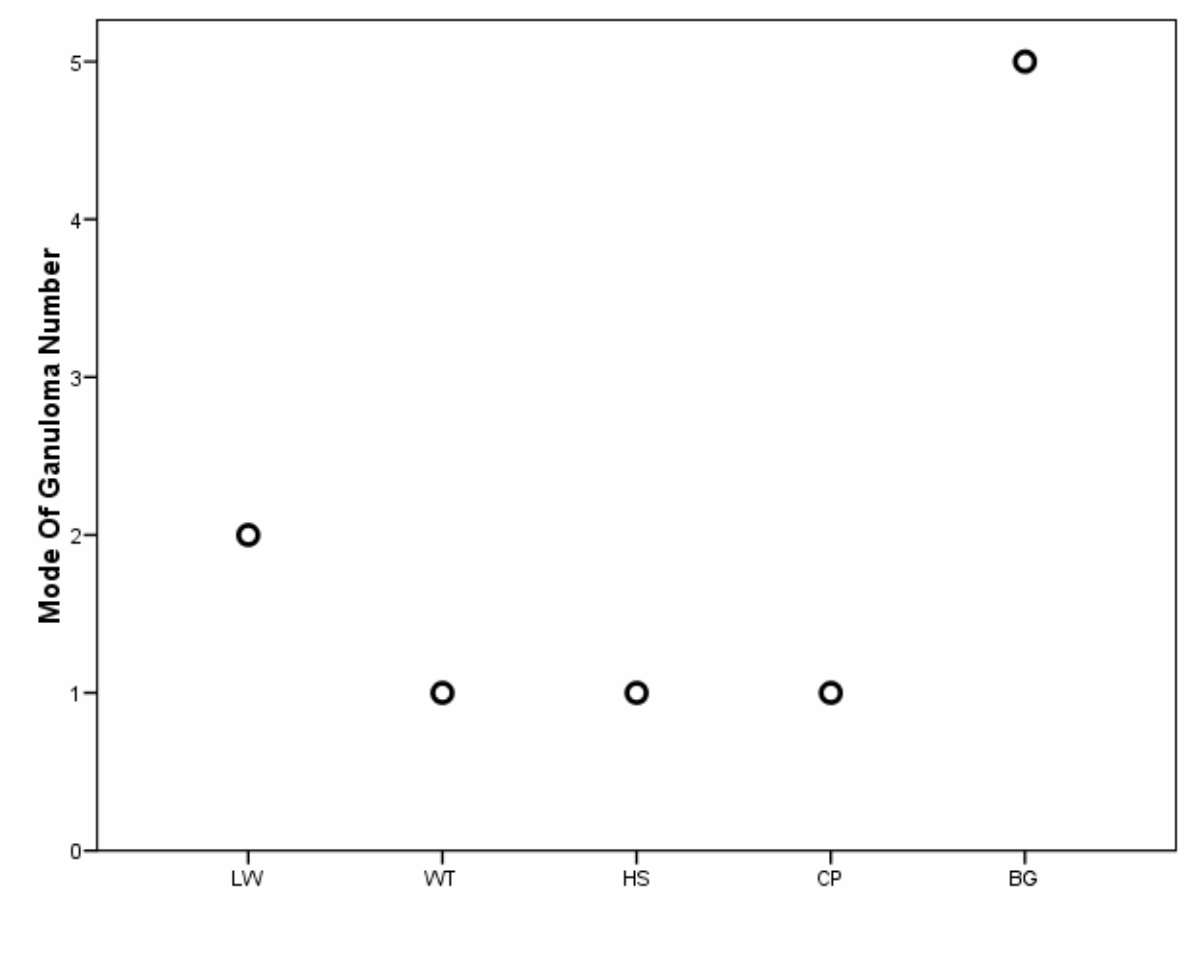

Figure 5-2 Mode of granuloma number across sites. The most suburban site is on the left and agriculture increases along the x-axis.

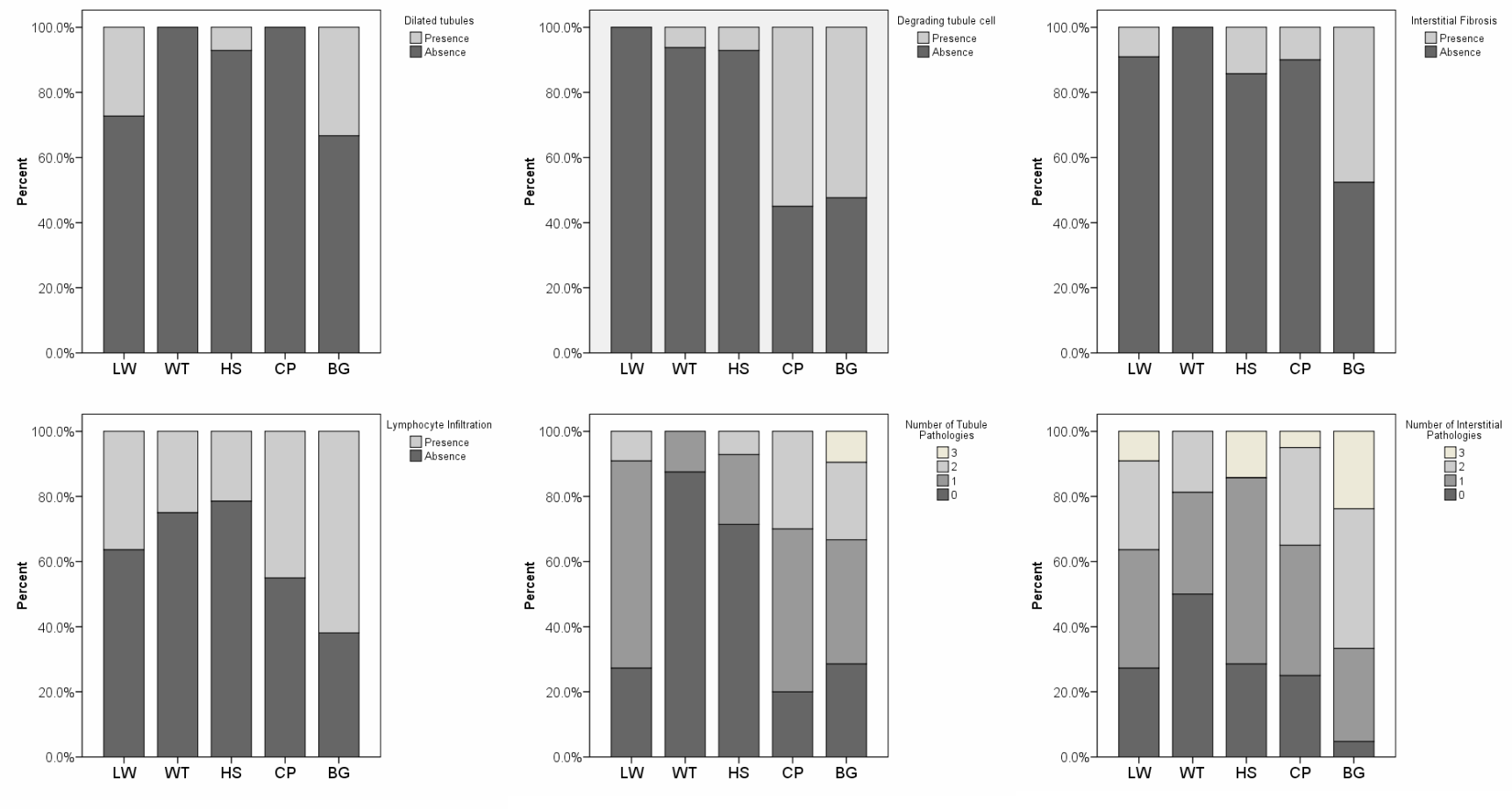

Figure 5-3 **A.** The percentage of male toads with the presence vs. absence of dilated tubules across five study sites. **B.** Presence and absence of degrading (necrotic) tubule cells across sites. **C.** Presence and absence of interstitial fibrosis across sites. **D.** Presence and absence of lymphocyte infiltration across sites. **E.** Percentage of toads found at each site with zero - three pathologies diagnostic of toxin-induced tubular necrosis (maximum value is 3). **F.** Percentage of toads found at each site with zero – three pathologies diagnostic of toxin induced interstitial nephritis (maximum value is 3). The most suburban site is on the left and the most suburban site is on the right. (LW=11 male, 0 intersex; WT=16 male, 0 intersex; HS=11 male, 3 intersex, CP=16 male, 4 intersex; BG=14 male, 7 intersex)

APPENDIX
CHEMICAL USAGE LIST

Table A-1 A list of chemicals used at each agricultural site were provided by the farm managers at each facility. Information on what the chemical is used for and whether or not it is a known endocrine disruptor was derived from the Pesticide Action Network North America (http://www.pesticideinfo.org/Index.html). Literature citations under "sources" include primary literature sources that report endocrine disruptive effects of each known endocrine disruptor.

Chemical trade name	Active ingredient(s)--chemical class	Use	Endocrine disruptor	Site applied	Source(s)
Atrazine	Atrazine--Triazine	Herbicide	Known Reproductive	BG and CP	Danzo, 1997; Fan et al., 2007; Hayes et al., 2006b; Sanderson et al., 2002
Ametryn	Ametryn--Triazine	Herbicide	Unknown	BG	
Asulox	Asulam sodium salt--Carbamate	Herbicide	Unknown	BG and CP	
Envoke	Trifloxysulfuron-sodium--Sulfonylurea	Herbicide	Unknown	BG and CP	
Mocap	Ethroprop--Organophosphate		Unknown	BG	
(active ingredients are ethroprop and PNCB)	PNCB--Substituted Benzene	Nematicide-insecticide	Suspected Thyroid		Hurley et al., 1998
PROWL 3.3 EC	Pendimethalin--2,6-Dinitroaniline	Herbicide	Suspected Thyroid		

- Table A-1 (Continued)

Roundup UltraMAX	Glyphosate, isopropylamine salt--phosphonoglycine	Herbicide	Known Reproductive	BG and CP	Howe et al., 2004; Hurley et al., 1998; Oliveira et al., 2007; Walsh et al., 2000
Sencor DF	Metribuzin--Triazine	Herbicide	Suspected Thyroid	CP	Porter et al., 1993
Thimet 20-G	Phorate--Organophosphate		Unknown	BG and CP	

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BIOGRAPHICAL SKETCH

Krista McCoy has spent much of her time as a student being a teacher and a mentor. As an undergraduate, she volunteered for two years at the Virginia Living Museum in Newport News Va. where she interpreted exhibits for the public and conducted live animal programs. After she graduated from Old Dominion University she worked for two years at Three Lakes Nature Center in Richmond Va. where she conducted nature hikes, hands-on animal programs, and nature programs that followed Virginia's "standards of learning" for students from grades K-12. After receiving her master's degree with Reid Harris at James Madison University, she co-instructed a course for the Smithsonian National Museum of Natural History's Monitoring and Assessment of Biodiversity Program (SI/MAB). This program was designed to teach wildlife managers from diverse countries and cultures (e.g. Nepal, India) wildlife monitoring techniques to enhance their ability to manage and conserve biodiversity in their own countries.

As a PhD student at the University of Florida, Krista was selected to be an NSF GK-12 fellow in the "Science Partners in Inquiry Based Collaborative Education" (SPICE) program. While in this program she taught middle school students the value of ecosystem health and biodiversity while instilling an appreciation of learning through inquiry based projects. Through SPICE she also had the opportunity to mentor students from diverse backgrounds typically underrepresented in science.

During summer 2004, she invited two of her middle school students to come to the University of Florida and help conduct her dissertation research. She also mentored a high school student through an experiment that was presented at the Science Fair. The student won first prize at the regional show and was invited to the State where she won third prize, and to the International Competition where she earned honorable mention. Krista has also mentored two other high school students through the University of Florida's Student Scientist Training

Program (SSTP). These students spent 35 hrs per week in the laboratory with Krista, and she taught them how to care for experimental animals, and collect and analyze data to be presented in both poster and lecture formats.

Krista has also mentored many undergraduate students by providing them with undergraduate research opportunities related to her research, or by helping them develop their own independent projects. Four of her students have completed independent honors projects and graduated with highest honors. She has also co-authored two papers with undergraduates, and she is currently working on two additional papers with undergraduates that will be submitted for publication before the end of the year. In addition, Krista has participated in the Strategies for Ecology, Education, Development, and Sustainability program (SEEDS: http://www.esa.org/seeds/) at the 89th annual meeting of the Ecological Society of America. This program is designed to help students from underrepresented groups navigate a large scientific meeting and to network with other scientists without becoming overwhelmed and intimidated.

Krista will continue to mentor, teach, and learn as she conducts postdoctoral research, through Boston University, at the Smithsonian Tropical Research Institute in Gamboa, Panama. She looks forward to continuing her dissertation research and beginning a variety of new projects.