

Effects of the Environmental Estrogen 17 α -Ethinylestradiol on Early Development of Green
Frogs (*Rana clamitans*) and Mink Frogs (*R. septentrionalis*) at the Experimental Lakes Area
(Ontario, Canada)

By

Bradley J. Park

A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Zoology

University of Manitoba

Winnipeg, Manitoba

© June, 2003

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FACULTY OF GRADUATE STUDIES

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Abbreviations

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
cpm	Counts per minute
dd	Double distilled
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
dph	Days post-hatch
E2	17 β -estradiol
EE2	17 α -ethynylestradiol
ELA	Experimental Lakes Area
h	Hours
NSB	None-specific binding
NSB/I-	None-specific binding/iodide
NSD	No significant difference
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyl
RIA	Radioimmunoassay
rT ₃	Reverse triiodothyronine
SD	Standard deviation
T ₃	3,5,3'-triiodothyronine
T ₄	Thyroxine, tetraiodothyronine
TCR	Total counts recoverable
TH	Thyroid hormone
TR	Thyroid hormone receptor
WC	Water control

Acknowledgements

Thanks to my advisor, Dr. Karen Kidd, for her guidance and support throughout my thesis research. Thanks also to Dr. J. Geoff Eales and Dr. Gordon Goldsborough for their valuable input as members of my thesis advisory committee.

There are many others I would like to thank for their contributions. I received a great deal of help from summer research assistants Paul Mutch, Tristan Hodge, and Natalie Asselin. Thanks also to the ELA Fish Crew: Dr. Ken Mills, Sandy Chalanchuk, and "Uncle Doug" Allen, for their assistance in the field. I am particularly indebted to Robert E. Evans for taking the time to teach me everything I know about histology. Further, I greatly value the advice and friendship I received from the staff and students in the endocrinology lab – James Plohman, Glenn Fines, and Suzanna Wiens. I would also like to thank the following people from the Freshwater Institute and the ELA for providing valuable advice, time, and resources: Kerry Wautier, Vince Palace, Lenore Vandenbyllardt, Paul Blanchfield, Cheryl Podemski, Mark Lyng, Morris Holoka, Len Hendzel, Marnie Potter, Ian Delorme, Neil Fisher, and Stephen Page. I am grateful to Beverly Horne and Dr. Ken Stewart for kindling an early interest in herpetology, and to Dr. Gordon Robinson for his guidance in the undergrad years.

Finally, to the most almighty powerful people in the world – my circle of family and friends – thank you all for helping me along the way.

Abstract

Environmental contaminants can have deleterious effects on wildlife through modulation of normal endocrine system function. Some of these contaminants, termed environmental estrogens, are commonly detected in aquatic systems and can mimic the effects of endogenous estrogens, thereby inducing physiological responses in exposed organisms. Given the recent declines in wild amphibian populations, there is dire need to elucidate the effects of environmental estrogens on aquatic organisms using ecologically relevant exposures.

A multidisciplinary study is being conducted at the Experimental Lakes Area (ELA, Ontario, Canada) to determine the impacts of an environmental estrogen on aquatic biota in a previously undisturbed boreal lake. In May to October 2001, Lake 260 was dosed with 17 α -ethynylestradiol (EE2), a potent synthetic estrogen commonly detected in sewage treatment effluents. The mean concentration of EE2 in the water column (6.1 ng/L \pm 1.5) was comparable to that detected in polluted waterways.

Endocrine-disrupting chemicals, including environmental estrogens, have adverse effects on amphibians, and may ultimately contribute to population declines. However, field-based data are lacking. As part of the ELA research effort, embryos and larvae (i.e. tadpoles) of two indigenous Ranid species (mink frog - *Rana septentrionalis*, and green frog – *R. clamitans*) were examined. Data were collected from Lake 260 and from undisturbed ELA reference lakes in 2000 and 2001, i.e. prior to and during the EE2 additions. Three studies were conducted to assess potential effects of an ecologically relevant estrogen exposure on aspects of early development in these species.

Contaminants can disrupt embryonic and larval development of amphibians. In the first study, hatching success and larval growth and development rates were examined. Hatching success in green frog eggs exposed to EE2 *in situ* was significantly lower than in unexposed eggs, whereas

there was no effect on mink frog eggs. There was no consistent effect of EE2 on growth or development rates of tadpoles of either species exposed *in situ* from hatching to the mid-larval phase. Wild mink frog tadpoles exposed to EE2 were significantly heavier at later developmental stages than those from reference lakes in 2001, whereas there was no difference between lakes in 2000. Green frog tadpoles exposed to high concentrations of EE2 (80, 1000 ng/L) during the early larval phase in a laboratory study were significantly developmentally advanced relative to controls, whereas there were no differences at low concentrations (5, 20 ng/L).

Environmental contaminants, including estrogens, can disrupt normal development in larval anurans by altering thyroid system function. In the second study, thyroid hormone (TH) content was assessed in tissues of metamorphic green frog tadpoles reared *in situ* on Lake 260 and on reference lakes. Mean tissue TH concentrations were not significantly different among EE2-exposed and reference tadpoles.

Finally, contaminants can induce reproductive defects in wild amphibians. In the third study, a histological analysis of gonad development was conducted in wild and cage-reared tadpoles. In wild mink frog tadpoles there was no apparent effect of EE2 on sex ratios, and low incidence of intersex gonads occurred in EE2-exposed tadpoles but not in the reference tadpoles. In the mink and green frog tadpoles reared *in situ* from the egg to the mid-larval stage there was no apparent effect of EE2 on sex ratios. There were no intersex gonads in green frog tadpoles; however, low incidence of intersex gonads occurred in EE2-exposed mink frogs and not in reference samples.

Results from this study indicate that ecologically relevant EE2 exposure disrupted aspects of development, but had no discernable effect on thyroid system function at the time of metamorphosis. Reproductive impairment may impact population sustainability.

Chapter 1: Introduction

Endocrine Disrupting Chemicals

Vertebrate endocrine system function is dependant upon chemical signaling. Chemicals (i.e. hormones) are produced by numerous glands and tissues, and are transported to target cells where they induce specific regulatory effects, ultimately guiding the processes of growth, development, reproduction and behaviour (Chester-Jones et al. 1987).

There is increasing evidence that anthropogenic substances in the environment can interfere with aspects of normal endocrine function in wildlife, i.e. the synthesis, secretion, transport, metabolism, action and excretion of chemical signals (Ankley et al. 1998). These substances are broadly referred to as endocrine disrupting chemicals (Kavlock 1996). Structurally diverse chemicals from industrial and domestic sources have been implicated as endocrine disruptors (Sumpter et al. 1997), and their effects have been reported in wildlife populations. For example, fish exposed to sewage effluents in United Kingdom waterways exhibit striking reproductive defects, including high incidence of intersex gonads, i.e. the presence of both male and female reproductive tissue in an individual (Lye et al. 1997; Jobling et al. 1998). Alligators (*Alligator mississippiensis*) from Lake Apopka, Florida (USA) exhibit reduced testicular development and depressed plasma testosterone concentrations resulting from an organochlorine pesticide spill (Crain et al. 1998; Guillette 2000). Bird populations in the Great Lakes (Ontario, Canada) have shown skewed sex ratios, deformities, and a range of reproductive abnormalities that have been linked to the presence of DDT (dichlorodiphenyltrichloroethane) and PCB (polychlorinated biphenyls) (Fox 2001). High accumulations of PAHs (polycyclic aromatic hydrocarbons) in tissues of beluga whales (*Delphinapterus leucas*) have been associated with abnormalities in reproductive, immune, and adrenal function (Fox 2001). In humans, increases in male reproductive abnormalities may be the result of increased levels of environmental chemical exposure (Carlsen et al. 1995). Thus, it appears that environmental endocrine disruptors are

diverse and widespread, that they affect a broad range of taxa, and that their effects are potentially detrimental for wildlife populations.

Environmental chemicals interact with the endocrine system through various mechanisms. Exogenous chemicals can affect the synthesis, secretion, transport, and elimination of endogenous hormones, thus altering the amount available to target tissues (McNabb et al. 1999). Circulating hormones are typically bound (reversibly) to proteins that facilitate their transport in the bloodstream. Contaminants can bind these proteins, reducing the amount of endogenous hormone available to target tissues (Yamauchi et al. 2000). Hormones are metabolized and converted to new forms by endogenous enzymes, a process critical for determining their biological activity (Eales and Brown 1993; Petrini and Zaccanti 1998). Xenobiotics can inhibit enzyme function in wildlife, therefore affecting hormone activity (Gillesby and Zacharewski 1998). Contaminants can also interact with hormone receptors. An exogenous substance can bind to a hormone receptor and either induce an effect similar to that of the natural ligand (i.e. act as an agonist or hormone mimic) or block the natural ligand from binding its receptor (an antagonist) (Gillesby and Zacharewski 1998; McNabb et al. 1999). Further, contaminants may interfere with signal transduction that occurs subsequent to receptor binding (Fairbrother et al. 1999). Endogenous hormones can induce physiological responses at extremely low plasma concentrations (McNabb et al. 1999), therefore small doses of contaminants may be sufficient to modulate endocrine function. Clearly, there are many potential sites and modes of disruption. Given that organisms are exposed to multiple contaminants at polluted sites, there is great opportunity for disruption.

Environmental Estrogens

Environmental chemicals that simulate the effects of endogenous estrogens, termed environmental estrogens (or xenoestrogens), have been identified in aquatic environments, and are the most well studied class of endocrine disruptors (Rudel et al. 1998; Snyder et al. 1999; Ternes et al. 1999). These include organochlorine pesticides, PCBs in paints and industrial

chemicals, PAHs from petroleum-based sources, and various pharmaceuticals and personal care products. In addition, natural hormones (estrone, 17 β -estradiol, 16 α -hydroxyestrone) excreted from humans or livestock, as well as phyto- and myco-estrogens (plant and fungi derived, respectively) are also present in aquatic environments (Sumpter et al. 1997). Chemical structures of environmental estrogens may or may not resemble those of endogenous estrogens, thus predicting the estrogenic potential of contaminants is difficult (Gillesby and Zacharewski 1998).

Estrogenic chemicals have been identified in sewage treatment effluents and receiving waters (Ternes 1999), in groundwater (Rudel et al. 1998), and in estuarine and offshore marine environments (Lye et al. 1997; Allen et al. 1999). Natural and synthetic estrogens are frequently detected in sewage treatment effluents due to their incomplete removal in the treatment process (Ternes et al. 1999). Synthetic hormones are used in pharmaceuticals such as birth control pills, and are designed to be highly potent, highly specific to humans or animals, and highly stable (Sumpter et al. 1997; Larsson et al. 1999; Snyder et al. 1999). 17 α -ethynylestradiol (EE2) is a synthetic estrogen used in various pharmaceuticals, such as oral contraceptives, anti-cancer drugs, and hormone replacement therapy (Sumpter et al. 1997). EE2 has garnered some attention because it is a potent estrogen and has been detected in aquatic environments in which feminized fish populations occur (Ternes et al. 1999). Environmental occurrence of EE2 is summarized in Table 1.1 (p.5). Estrogens are typically excreted in conjugated forms that become incorporated into domestic sewage. They may be released unchanged or in the deconjugated (and therefore more potent) free form (Snyder et al. 1999; Ternes 1999; Ternes et al. 1999).

Effects of xenoestrogens are typically weaker than those of endogenous estrogen. For example, Lutz and Kloas (1999) demonstrated that E2 had a higher affinity for the hepatic estrogen receptor of *Xenopus laevis* than did several environmental estrogens, including bisphenol A, nonylphenol, octylphenol, and DDT. However, many xenoestrogens are highly lipophilic and can thus biomagnify in exposed organisms (Sumpter et al. 1997). Accumulation of estrogenic substances at concentrations approximately 100-fold higher than ambient levels has been

documented in fish downstream of sewage treatment plants (Larsson et al. 1999). Transgenerational effects of xenoestrogens are possible, because lipids and lipid-bound contaminants are mobilized and incorporated into growing ovarian follicles (Palmer and Palmer 1995). Additive effects of multiple estrogens are probable at polluted sites (Kortenkamp and Altenburger 1999; Larsson et al. 1999). Finally, it has been proposed that xenoestrogens in mixtures may have synergistic effects, although this remains controversial (Arnold et al. 1996; McLachlan et al. 1997; Ramamoorthy et al. 1997; Bergeron et al. 2002).

Xenoestrogens typically bind the cytosolic estrogen receptor in target cells and induce a cascade of events similar to those of endogenous estrogens, i.e. binding of the ligand-receptor complex and promoting estrogen-specific gene expression (Kloas et al. 1999). Gillesby and Zacharewski (1998) proposed several other potential mechanisms of exoestrogen action in vertebrates. Endocrine disrupting substances may alter the bioavailability of endogenous steroids through interactions with plasma steroid binding proteins. Displacement by xenobiotics could make endogenous hormones more bioavailable, thereby disrupting regular endocrine function. The authors state that intracellular exoestrogen action occurs via three pathways: (i) direct binding and activation of the estrogen receptor (ER), (ii) binding of other nuclear receptors that interact with the estrogen responsive element (ERE) and (iii) other signal transduction pathways that alter estrogen signaling. Endocrine disrupters may bind the estrogen or androgen receptors, at which point the receptor-mediated signal transduction may be activated or inhibited (Gillesby and Zacharewski 1998). Various members of the nuclear receptor superfamily aside from the ER can bind the ERE. This has been demonstrated for the retinoid X receptor (RXR), although resulting gene expression has not been demonstrated (Gillesby and Zacharewski 1998). Exogenous chemicals that interact with these receptors may be able to modulate the endocrine system (Gillesby and Zacharewski 1998).

Table 1.1 EE2 concentrations in various waterways and effluents. ST= sewage treatment, ND= not detected.

Source	EE2 Concentration (ng/L)	Reference
Domestic ST effluent (Britain)	ND to 7.0	Desbrow et al. 1998
Effluents and surface waters (U.S. rivers)	ND to 0.76	Snyder et al. 1999
ST effluent (Sweden)	4.5	Larsson et al. 1999
ST effluent (Germany)	ND to 15	Ternes et al. 1999
ST effluent (Canada)	ND to 42	Ternes et al. 1999
Streams, rivers (Germany)	ND	Ternes et al. 1999

Global Amphibian Decline

Recent reports indicate that declines, extinctions, and range reductions of amphibian populations are occurring at an alarming rate, a phenomenon broadly referred to as “amphibian decline” (Blaustein and Wake 1990). On a global scale, amphibian populations have been declining since the late 1950s and continue to do so (Houlahan et al. 2000).

Although widespread reports of declining amphibian populations date back several decades, the possibility of a global amphibian crisis first became apparent in 1989 at the first World Congress of Herpetology (Barinaga 1990; Phillips 1990; Wyman 1990). Participants from around the world presented scientific reports and anecdotal accounts of decreases in study populations. Some of these declines were explained by direct human impacts, while others lacked obvious explanations (Barinaga 1990; Wyman 1990). Though the bulk of the evidence up to this time was anecdotal, many scientists found the number and timing of these reports quite disturbing. Concerns raised at

this meeting led to the organization of a conference by the U.S. National Research Council Board on Biology in 1990 to address the nature of the noted declines and potential causes. Several conclusions were reached at this meeting: (1) some amphibians are “disappearing at an alarming rate”, even in relatively undisturbed habitats (2) declines are widespread and have been occurring since the late 1970s (3) amphibians may be the best suited vertebrates for use as biological indicators of environmental degradation (4) data required for monitoring populations and determining causes of declines are lacking (Wake and Morowitz 1990).

Declines in amphibian populations have been reported in various habitat types over a broad geographic range (Corn 2000). The majority of reports are from North, Central and South America and Australia, with some from Europe, and far fewer from Asia and Africa. This largely reflects the amount of research that has been conducted in each region, rather than the actual geographic extent of declines (Corn 2000). In North America, amphibian declines appear to be most pronounced in the western mountainous region. In the 1970s and '80s declines in northern leopard frog (*Rana pipiens*) populations were documented in Colorado (Corn and Fogleman 1984). Overall distribution declines have been documented for all native amphibian species in California's Central Valley (Fisher and Shaffer 1996). Populations of the mountain yellow-legged frog (*Rana muscosa*) and the Yosemite toad (*Bufo canorus*) have declined in the Sierra Nevada mountains (Bradford et al. 1994). In addition, declines have been reported for the boreal toad (*B. boreas*) in the Rocky mountains of Colorado and Wyoming, and the Cascade frog (*R. cascadae*), western spotted frog (*R. pretiosa*), and the red-legged frog (*R. aurora*) in Oregon (Blaustein and Wake 1990; Bradford et al. 1994). In the eastern U.S., 139 out 205 populations of *Plethodon* salamanders showed no significant declines in the 1950s through 1980s, but over 70% of these populations showed declines in the 1990s (Highton 2000).

It is broadly recognized that many vertebrate and invertebrate taxa are suffering declines and extinctions that are the result of anthropogenic factors. It is unclear whether amphibian decline is a distinct biological phenomenon or simply a part of the overall “biodiversity crisis”, i.e. are

amphibians being impacted more than other organisms? Many researchers propose that amphibians are particularly sensitive to harmful environmental effects relative to other taxa because of their physiology and life history, and may serve as effective bioindicators of environmental degradation (Greenhouse 1976; Cooke 1981; Vitt et al. 1990; Wyman 1990; Blaustein 1994; Pollett and Bendell-Young 2000). The permeable skin, biphasic life history, and unprotected embryonic stage in amphibians likely increase the susceptibility to deleterious effects in this class of organisms (Vitt et al. 1990). Amphibians meet all the requirements of effective biomonitors of organic contaminants (hence their increasing use in toxicology studies), and can be excellent early indicators of environmental health problems that are likely to affect other organisms, including humans (Heyer et al. 1994; Sparling et al. 2000).

In early discussions of amphibian declines it remained unclear whether a single factor was responsible for the apparent global amphibian crisis. Although widespread, it was noted that declines are not seen in all locations. Declining and non-declining species co-occur in some areas, and in some cases are members of the same genus (Blaustein and Wake 1990). Furthermore, declines have been documented in seemingly pristine areas while evidence of significant declines is lacking in some industrialized areas with obvious alteration of habitat (Blaustein and Wake 1990). Many authors have cited various causes for specific declines, and it is now evident that a myriad of factors contributes to amphibian declines worldwide.

There is little doubt that habitat alterations from human settlement have adverse effects on amphibian populations in many areas. Wetland drainage, forest harvesting, agriculture, and roadways can interfere with mating, foraging, hibernation, and dispersal (Reaser 2000; Seburn and Seburn 2000). Habitat fragmentation is of particular concern because it can block recolonization of a habitat after a local extinction event (Pechmann and Wilbur 1994). Hecnar and M'Closkey (1996) cite the loss of forest cover as a likely cause of decreased amphibian species richness in southwest Ontario, Canada, because woodland species rely on forest habitat for hibernation and foraging. Busby and Parmelee (1996) report that the herpetofaunal assemblage

found within a protected military reserve in Kansas has changed very little in the past 70-100 years. This is likely attributable to the maintenance of vast tracts of uncultivated land with native vegetation. They noted that in areas with heavy vehicle traffic, effects on vegetation and soil characteristics negatively impacted the herpetofaunal community.

Global climate changes have significant potential to impact amphibian populations. Anomalous climatic events such as drought, mid-winter thaws, and increased mean annual temperature can decrease habitat quality and incur physiological stress in amphibians (Reaser 2000; Seburn and Seburn 2000). Pounds and Crump (1994) propose that climate disturbances associated with the 1986-1987 El Niño/Southern Oscillation may be responsible for the extinction of the golden toad (*Bufo periglenes*) at Monteverde, Costa Rica. They hypothesize that warm, dry conditions may have increased physiological stress, created a pulse of contaminants, or enhanced pathogen outbreaks. A field study by Lizana and Pedraza (1998) demonstrates that increased UV-B (resulting from ozone depletion) reduces embryonic survival and causes developmental abnormalities in two *Bufo* species. They state that those species with low levels of DNA repair enzymes are particularly sensitive to these effects.

Pathogens such as Iridovirus, Chytridiomycosis, and various water molds have caused mass die-offs in some amphibian populations (Crawshaw 1997; Corn 2000; Reaser 2000), and mass epidemic disease has been proposed as the cause of extensive declines in Australia (Laurance et al. 1996). Introduced predators such as fish, bullfrogs (*Rana catesbiana*), and crayfish are also contributing to declines. Significant inverse relationships between the presence of exotic species and the presence of native anurans have been established in some North American populations (Fisher and Shaffer 1996). Non-native bullfrogs (*R. catesbiana*) and carp (*Cyprinus carpio*) are thought to be partly responsible for the decline of Blanchard's cricket frogs (*Acris crepitans blanchardi*) in Ontario, Canada (Lannoo et al. 1994).

Anthropogenic chemicals are also contributing to the loss of amphibians. Acidic deposition in amphibian habitats has been shown to reduce embryonic and larval survival in numerous species (Wissinger and Whiteman 1992; Freda and McDonald 1993; Bradford et al. 1994). Insecticides used in agriculture and forestry have been linked to increased mortality at metamorphosis, increased disease susceptibility, and decreased abundance (Marian et al. 1983; McAlpine et al. 1998; Taylor et al. 1999). Some chemicals can modulate the endocrine system, creating inappropriate responses in development and reproduction that can impact survival (Corn 2000).

Endocrine Disruption in Amphibians

Endocrine disruption has been reported in amphibians, although they are less well-studied than other vertebrates. A summary of amphibian endocrine disruption studies is given in Table 1.2 (p.16). Amphibians undergo sex steroid- and thyroid hormone-induced development processes (the role of thyroid hormones in larval development is particularly well documented), and as such are effective models for studying endocrine disruption (Hayes 2000). There is currently a well established laboratory contaminant screening method using amphibians, the FETAX assay (Frog Embryo Teratogenicity Assay – *Xenopus*). See Table 1.3 (p.18) for commentary on amphibian models in endocrine disruptor research. In the field, amphibians serve as sensitive indicators of environmental degradation (See Sparling et al. 2000 for review). Despite recent concern that endocrine disruption may be contributing to current trends of declining populations (Carey and Bryant 1995), few field-based studies devoted to effects of endocrine-active contaminants have been conducted.

In amphibians, the testes and ovaries typically develop from an undifferentiated gonad, a process which appears to be controlled by endogenous sex steroids (Hayes 1998). Gonad development can be altered by exposure to environmental chemicals in the wild (Reeder et al. 1998) and by steroids (natural and synthetic) in laboratory manipulations (Hayes 1998; Kloas et al. 1999), resulting in intersex individuals and skewed sex ratios (see Chapter 4). Kloas et al. (1999) reported that exposure of *Xenopus laevis* tadpoles to 17 β -estradiol (E2), nonylphenol (NP),

octylphenol (OP), bisphenol A, and butylhydroxyanisol (HA) resulted in a higher percentage of female phenotypes relative to controls. Reeder et al. (1998) examined the prevalence of intersex and sex reversal in wild Cricket frogs (*Acris crepitans*) by comparing individuals from unpolluted sites to those from sites with point sources of polychlorinated biphenyl (PCB) and polychlorinated dibenzofuran (PCDF). A relationship between sex ratio reversal and PCB/PCDF contamination was detected. Such disruption may ultimately interfere with reproductive function in wild amphibians (McNabb et al. 1999; Hayes 2000).

Vitellogenin (VTG) is a yolk protein precursor that is produced when endogenous estrogen binds hepatic estrogen receptors, a process that occurs in mature females (Palmer and Palmer 1995; McNabb et al. 1998; Hayes 2000). Vitellogenesis has been demonstrated in male amphibians exposed to xenoestrogens (Palmer and Palmer 1995; Palmer et al. 1998; Kloas et al. 1999; Mosconi et al. 2002). Laboratory studies indicate that exogenous estrogens and some xenobiotics can induce vitellogenin synthesis in *Xenopus laevis* males. Diethylstilbestrol (DES), dichlorodiphenyltrichloroethane (o,p'DDT), and 17 β -estradiol (E2) administered by injection resulted in VTG induction *in vivo* (Palmer and Palmer 1995). Immersion in toxaphene and dieldrin also induced VTG (Palmer et al. 1998). Finally, immersion in E2, nonylphenol, and bisphenol A resulted in significant induction of vitellogenin mRNA in primary cultured hepatocytes *in vitro* (Kloas et al. 1999). The population-level significance of this form of endocrine disruption in amphibians is unclear. It is not known if inappropriate VTG induction affects reproductive ability in vertebrates. However, VTG synthesis is energetically costly, and may represent a significant depletion of resources and increased physiological stress (McNabb et al. 1999). The induction of abnormally high vitellogenin levels in males can be used as an indicator of estrogenic activity, and may be a valuable predictor of other deleterious receptor-mediated effects of environmental estrogens (Kloas et al. 1999).

Secondary sex characteristics are also affected by contaminants (McNabb et al. 1999).

Secondary sex characteristics are considered to be those features aside from the ovaries and

testes that differ between the sexes, such as colour, behaviour, nuptial pads, and gonads (Hayes 1999). They are required for mating, territoriality, and other essential activities, and are likely regulated by sex steroids (Hayes 2000). Steroidal effects of environmental contaminants are of concern because they may alter secondary sex differentiation, or induce development in the inappropriate sex, thereby disrupting recruitment and reducing reproductive output (Clark et al. 1998; Hayes 2000). Sex-based features such as markings and laryngeal development/vocalization may be disrupted by xenobiotics, which would have drastic impacts on recruitment and reproductive output (Hayes 2000). Studies by Norris et al. (1997) and Clark et al. (1998) demonstrate that exposure to exogenous steroids alters the development of the gonads in larval tiger salamanders (*Ambystoma tigrinum*). In these studies both estradiol and androgens stimulated mullerian duct development, while development of the wolffian ducts was stimulated by androgens and inhibited by estradiol. As a result of the differential response of the gonads to sex steroids, *A. tigrinum* larvae may serve as indicators of agonistic or antagonistic effects of environmental chemicals. Clark et al. (1998) exposed *Ambystoma tigrinum* larvae to environmentally relevant concentrations of DDT (dichlorodiphenyltrichloroethane) and its metabolite DDE, both alone and in combination with sex steroids. In this system DDT antagonized the effects of estradiol on mullerian ducts in females, and DDE alone was estrogenic. In other systems, DDT is estrogenic and DDE is antiandrogenic, implying that a given endocrine disruptor may have both steroidal and antisteroidal effects.

Oocyte maturation is the final phase of oogenesis in amphibians, and is required for successful fertilization of ova after their release. The process involves germinal vesicle breakdown (GVBD), which is stimulated by progesterone, and is sensitive to some xenobiotics (Baulieu et al. 1978; Lin and Schuetz 1983; Pickford and Morris 1999). Oocyte maturation was affected by E2 exposure in *Rana pipiens* and by 17 α -ethynylestradiol (EE2) exposure in *Xenopus laevis* (Baulieu et al. 1978; Lin and Schuetz 1983). Pickford and Morris (1999) examined the effects of exogenous estrogens and environmental contaminants on progesterone-induced GVBD. EE2, E2, and several estrogenic contaminants (o,p'-DDT, octylphenol, di-n-butylphthalate, bisphenol A) had no effect

on GVBD, whereas methoxychlor (a proestrogenic organochlorine pesticide) inhibited GVBD. Pickford and Morris (1999) examined several possible mechanisms of methoxychlor inhibition of progesterone induced GVBD. They proposed that the effect was non-estrogenic because exposure to known estrogens (natural and synthetic) and estrogenic contaminants did not produce an effect, nor did exposure to an estrogen receptor antagonist. Methoxychlor did not appear to be in direct competition for the oocyte membrane progesterone receptor (omPR), because methoxychlor activity was different from that of antiprogestins. Methoxychlor was not able to significantly displace radiolabelled progesterone from the membrane receptor. Therefore disruption may have occurred via non-competitive binding to the omPR receptor, alteration of adenylate cyclase or phosphodiesterase activity, or membrane disruption through changes in Ca^{2+} flux (Pickford and Morris 1999). Disruption of oocyte formation may be detrimental for wild populations, and the stages of oocytes in contaminant-exposed organisms may be used as indicators of disruption (Ankley et al. 1998).

The hypothalamo-pituitary-interrenal (H-P-I) axis in amphibians is the system involved in the stress response (Gendron et al. 1997). Acute stress acts as a stimulus that causes the release of corticotropin (ACTH) from pituitary corticotrope cells, which acts on the interrenal to synthesize and secrete corticosteroids into the bloodstream, resulting in an increase in blood glucose. A recent field study indicates that environmental contaminants can disrupt the stress response in amphibians (Gendron et al. 1997). Gendron et al. (1997) examined the surge of circulating corticosterone in the mudpuppy (*Necturus maculosus*) following standardized stress challenges, comparing groups from polluted and unpolluted sites. Individuals from polluted sites had significantly lower corticosterone surges in response to capture and confinement stress. Liver glycogen stores were significantly lower in individuals from contaminated sites, possibly due to reductions in corticosterone. Amphibians from the polluted sites also showed significantly lower corticosterone surges in response to ACTH challenges relative to those from reference sites. Gendron et al. (1997) proposed several mechanisms of H-P-I axis disruption. The reduced corticosterone response after ACTH challenge indicates that the effect is occurring at or

downstream of the pituitary corticotropes. Organochlorines may interfere with corticosterone synthesis and secretion, or disrupt binding of ACTH to the membrane receptors of the interrenals. They note that extensive exposure to environmental contaminants can cause prolonged activation of the HPI axis, resulting in a compensatory down regulation of ACTH receptors. Impaired stress response in the wild may ultimately impact predator avoidance and immune response.

Hayes (1997) examined the effects of corticosterone exposure on tadpole development. The exposure caused mandibular fenestration and other abnormalities in the mouth region that are very similar to the effects of DDT. He states that the mechanism of DDT action is unknown, but likely involves the stress axis. DDT may act as a corticosterone mimic through interactions with the corticosterone receptor, or it may cause an increase in corticosterone release. Conversely, increased corticosterone may mobilize DDT accumulations in fat stores. Ultimately, disruption of the H-P-I axis may result in alterations of the fight-or-flight response, carbohydrate profile, and regulation of the hypothalamo-pituitary-gonad axis (Gendron et al. 1997). Moore and Zoeller (1985) report that increased corticosterone due to stress may block the release of LH-RH from the hypothalamus, thus inhibiting reproduction in amphibians.

Binding of a ligand to the estrogen receptor (ER) is the prerequisite for initiating direct estrogenic or anti-estrogenic effects in an organism (Gillesby and Zacharewski 1998). Lutz and Kloas (1999) established a radioreceptor assay for [³H] 17β-estradiol binding to the liver ER in *Xenopus laevis*. The binding affinities of steroids (estrogens and other endogenous steroids) as well as environmental chemicals were tested. Competitive displacement experiments in this study demonstrated that the estrogen receptors showed high specificity for binding of estrogens, but not for other endogenous steroids. Displacement results also indicated that exogenous ligands such as alkylphenols and bisphenol A were able to compete completely with [³H]E2 for the ER. In addition, 3 out of 5 sewage effluent samples displaced more than 50% of [³H]E2 binding at their original concentration and composition.

A study by Nishimura et al. (1997) examined the effects of exoestrogens on embryonic and larval development in *Xenopus laevis*. E2 and DES induced embryo death and malformations (retarded development of the head and eyes, suppressed organogenesis of digestive organs and nervous system). In addition to developmental effects, treatment with E2 increased the expression of estrogen receptor (ER4) mRNA in unfertilized and fertilized eggs, embryos, and tadpoles. Therefore, the developmental effects of E2 on embryos and larvae may have resulted from direct interactions with the estrogen receptor, though this mechanism was not explicitly demonstrated. Environmental chemicals may have similar developmental effects.

In metamorphosing amphibians a surge of endogenous thyroid hormones (TH) induces transformation from larva to adult. The central hypothalamus-pituitary-thyroid (H-P-T) axis synthesizes TH and secretes it into the plasma, TH then circulate to specific tissues and induce the appropriate responses (Shi 2000). Various lines of evidence indicate that contaminants, including PCBs and pesticides, disrupt the anuran thyroid system (Burkhart et al. 1998; Gutleb et al. 2000). This could ultimately impact survival, fitness, and reproduction of wild populations (Denver 1997; see Chapter 3).

Xenobiotics can interfere with normal limb bud development resulting in gross deformities such as malformed, extra, or missing limbs (Burkhart et al. 1998; Gardiner and Hoppe 1999; Hayes 2000; Sower et al. 2000). Malformations such as missing or extra limbs are likely to reduce overall fitness and increase predation rates. Retinoic acid plays an important function in limb development in amphibians (Sessions et al. 1999). The presence of anthropogenic retinoids in the environment may induce supernumary limbs and other deformities in some amphibian populations, but this mechanism has not been clearly demonstrated (Sessions et al. 1999). Retinoids, pharmaceuticals used in treatment of cancer and skin disorders, exhibit receptor-binding activity which interferes with limb bud development and causes deformities (Daughton and Ternes 1999; Sessions et al. 1999). *X. laevis* reared in contaminated water from Minnesota

and Vermont ponds (sites with high incidence of deformities in native anurans) exhibited abnormal limb development, and inhibition of metamorphosis (Fort et al. 1999a; Fort et al. 1999b).

Thus, it is apparent that contaminants disrupt amphibians at multiple points. Though population-level significance of these effects is unknown, clearly reproductive success of wild amphibian populations may be compromised. Amphibian populations have been declining globally for several decades, and continue to do so (Houlahan et al. 2000). Endocrine disruptors are likely contributing to this phenomenon, though research efforts are generally lacking (Carey and Bryant 1995; Hayes 2000). Demonstrating mechanisms of disruption will benefit the development of effective bioassays for endocrine-active contaminants. Vitellogenin induction is a potentially valuable screening tool because of the specificity of response (which is well documented), and the ability to screen mixtures and elucidate synergistic effects. Sex differentiation and secondary sex characteristics require further understanding before they can be effectively used as biomarkers of endocrine disruption. Combined use of *in vitro* and *in vivo* assays is valuable in determining specific pathways of disruption while simultaneously demonstrating effects on the whole organism. Generally, a more representative range of study species is needed. Laboratory studies that closely approximate exposure to endocrine disruptors in the wild will provide valuable comparisons for field studies. Finally, it is essential to determine the significance of endocrine disruption at the population level in amphibians.

Species	Chemical	Dose	Effect	Reference
<i>Xenopus laevis</i> (stage 38/40)	E2 NP bisphenol A OP HA	10^{-7} , 10^{-8} M 10^{-7} M 10^{-7} M 10^{-7} M 10^{-7} M	significant feminization (higher % female phenotypes) at all doses	Kloas et al. 1999
<i>X. laevis</i> (adult males)	E2 DES o,p'-DDT	1 µg/g (injected) 1, 250 µg/g 1 µg/g	VTG induction " "	Palmer and Palmer 1995
<i>X. laevis</i> (immature and adult)	DES Chlordane Dieldrin Endosulfan Toxaphene Paired comb'ns (above 4)	1 ppm (immersion) " " " " "	VTG induction no effect VTG induction Toxic VTG induction no effect	Palmer et al. 1998
<i>X. laevis</i> (adult male 2-3 yrs. liver cells)	E2 NP bisphenol A	10^{-10} to 10^{-5} M " "	VTG mRNA @ 10^{-9} M VTG mRNA @ 10^{-8} M VTG mRNA @ 10^{-7} M	Kloas et al. 1999
<i>Ambystoma tigrinum</i> (Larvae)	E2 DHT DDT p,p'-DDE Paired comb'ns steroid and xenobiotic	12.5 µg (injected) " 0.01 ppm (imm) "	see text	Clark et al. 1998
<i>A tigrinum</i> (Larvae)	E2 DHT T E2+T E2+DHT	12.5 and 25 µg (inj) " 25 µg 12.5 µg + 12.5µg "	see text	Norris et al. 1997
<i>X. laevis</i> (defolliculated oocytes)	Progesterone E2 EE2 o,p'-DDT OP di-n-butyl phthalate bisphenol A Methoxychlor HPTE	2.5 to 400 nM 5 nM to 5 µM " 62.5 to 4000 nM " " " 1.95 to 1000nM ?	stim. oocyte mat'n no effect on GVBD " " " " " sig. inhib. of GVBD no effect on GVBD	Pickford and Morris 1999

Table 1.2 (Continued) Summary of methodologies and results from amphibian endocrine disruption studies				
Species	Chemical	Dose	Effect	Reference
<i>Kassina senegalensis</i> (Stage 39-40)	Corticosterone	4 µg/L (1.1 µM) immersion 5 d	sig. decreased growth, increase pathology of mouth region	Hayes et al. 1997
<i>X. laevis</i> (Liver cell estrogen receptors)	HA NP OP bisphenol A DEP DDT TCB	10 ⁻⁹ to 10 ⁻³ M " " " 7.5x10 ⁻¹⁰ to 2.5x10 ⁻⁴ 10 ⁻⁹ to 10 ⁻³ M 10 ⁻⁹ to 10 ⁻⁶ M	all (except DDT) completely competed with [3H]E2 for ER @ 10 ⁻³ M	Lutz and Kloas 1999
<i>X. laevis</i> (embryos and larvae)	E2 EE2 DES DHT Progesterone	10 ⁻¹⁰ to 10 ⁻⁵ M " " " "	deformities at 10 ⁻⁵ M no effect deformities at 10 ⁻⁵ M no effect "	Nishimura et al. 1997

Table 1.3 Commentary on the use of amphibian model systems in endocrine disruptor research

Model System	Comments	Reference
Vitellogenin Induction	<ul style="list-style-type: none"> • clear assessments of estrogenicity in amphibians can be accomplished by demonstrating the induction of the estrogenic biomarker vitellogenin • (RT-PCR of vitellogenin mRNA <i>in vitro</i>) can be performed faster than an ELISA detecting the protein vitellogenin • may serve as an excellent biomarker for xenobiotic estrogen exposure in reptiles and amphibians or other oviparous and ovoviviparous vertebrates • may be used as a rapid, sensitive, and economical initial screen, followed...by more costly screens to identify the specific contaminating compounds • have demonstrated the effectiveness of these assays at detecting exposure to environmental estrogens through immersion • (<i>in vivo</i> immersion protocol) effective with weakly estrogenic compounds • can be used to address questions about...synergy among compounds 	<p>Kloas et al. 1999</p> <p>Kloas et al. 1999</p> <p>Palmer and Palmer 1995</p> <p>Palmer and Palmer 1995</p> <p>Palmer et al. 1998</p> <p>Palmer et al. 1998</p> <p>Palmer et al. 1998</p>
Larval Sex Reversal	<ul style="list-style-type: none"> • clear assessments of estrogenicity in amphibians can be accomplished by demonstrating the induction of....feminization during larval development • could be added to a suite of screening methods • at present...not ready for adoption into a screening program because they lack sufficient validation with known EDCs, and the mechanism of sexual differentiation in amphibians is not completely understood 	<p>Kloas et al. 1999</p> <p>Ankley et al. 1998</p> <p>Ankley et al. 1998</p>
Estrogen Receptor Binding	<ul style="list-style-type: none"> • recommend that comparative binding data be collected... used to decide whether results from a single species could be extrapolated to the rest of the class and/ or whether binding affinities are similar enough to utilize data from other vertebrate classes 	<p>Lutz and Kloas 1999</p>

Table 1.3 (Continued) Commentary on the use of amphibian model systems in endocrine disruptor research		
Model System	Comments	Reference
Estrogen Receptor Binding	<ul style="list-style-type: none"> (radioreceptor assay for ER binding) advantage...is obvious for screening potential estrogenic activities of pure substances as well as complex mixtures of different compounds 	Lutz and Kloas 1999
Secondary Sex Characteristics	<ul style="list-style-type: none"> (use of <i>Hyperolius argus</i> in a bioassay) is promising because of the high sensitivity and specificity and the ability to simultaneously detect both androgenic and estrogenic effects (response of <i>Ambystoma tigrinum</i> gonaducts to steroids) provides an excellent model for testing steroid-mimicking or antagonizing effects...may be useful for studying mechanisms of interactions 	Hayes 2000 Clark et al. 1998
Germinal Vesicle Breakdown	<ul style="list-style-type: none"> antagonism of progesterone-induced GVBD using naked <i>Xenopus</i> oocytes offers little potential as an assay for functional estrogenicity of xenobiotics in amphibia gonadotropin-induced GVBD of follicle cell-enclosed oocytes might ...represent a sensitive end point for estrogenic activity in the adult amphibian ovary xenobiotics...can easily be assayed either <i>in vivo</i> or even <i>in vitro</i>...tests would be of short duration, and...would be interpretable in the context of a large body of literature in this model system 	Pickford and Morris 1999 Ankley et al. 1998
FETAX	<ul style="list-style-type: none"> could be adapted to include exposure scenarios and endpoints appropriate for detection of (anti-)estrogens/androgens models for assaying androgenic and thyroidogenic interactions and assessing developmental effects are greatly needed 	Ankley et al. 1998 Hayes 2000
<i>In vitro</i> assays	<ul style="list-style-type: none"> have the advantages of rapid detection and high throughput. They are particularly useful for large-scale screening of compounds 	Palmer BD et al. 1998

Table 1.3 (Continued) Commentary on the use of amphibian model systems in endocrine disruptor research		
Model System	Comments	Reference
<i>In vitro</i> assays	<ul style="list-style-type: none"> • can give false negative and false positive results when used to screen compounds for estrogenicity • (the use of a cell culture) is a very good tool to assay inducible effects, while excluding other endogenous influences present in an intact animal 	Kloas et al. 1999
<i>In vivo</i> assays	<ul style="list-style-type: none"> • assays for estrogenicity based on whole animal model systems are needed • <i>in vitro</i> studies are essential for resolving mechanisms of action within specific tissues, they must also be combined with <i>in vivo</i> studies that reflect actions within the entire organism 	Palmer et al. 1998 Clark et al. 1998
General	<ul style="list-style-type: none"> • development of amphibian laboratory models to identify measurable physiological endpoints that can be monitored in the field is a crucial first step in assessing the impact of endocrine disruptors on amphibians in the wild 	Hayes 2000

Endocrine Disruptor Research at the Experimental Lakes Area

Recently there has been a call for the collection of “environmentally/ecologically relevant” data in endocrine disruptor research, i.e. field-based studies and contaminant exposure methodologies which closely approximate those in the wild. The Experimental Lakes Area is a research facility in northwestern Ontario, Canada, specializing in whole-lake manipulation studies and long term environmental reference data for freshwater ecosystems (Appendix A). A multidisciplinary research project is being conducted at the ELA to address the effects of the potent environmental estrogen EE2 on aquatic biota using an ecologically relevant exposure. The general objectives of this project are to (1) determine the ecological relevance of current EDC screening tools, (2) determine impacts on fish populations and lower-trophic-level organisms, and (3) identify the most sensitive species and life history stages. In 1999 and 2000, background data were collected on the study lakes. In the summers of 2001, 2002, and 2003, Lake 260 was dosed with EE2 to an epilimnetic water column concentration of approximately 6 ng/L. The responses of various taxa to the EE2 additions were compared to information from ELA reference lakes and pre-addition data from Lake 260. Lake recovery will be studied in subsequent years. Reference lakes used in the study are undisturbed, and are part of the ELA Long Term Environmental Reference database. Baseline data are available for physical, chemical, and biological parameters on these lakes.

Ecology and Life History of Mink and Green Frogs

During preliminary data collection for the ELA endocrine disruptor project, tadpoles were observed in Lake 260 and were thus included in the study. Two Ranid species, the mink frog (*Rana septentrionalis* Baird) and the green frog (*R. clamitans melanota* Rafinesque) are indigenous to the region, and are locally common, based on collections from the current research and on historical records of tadpole catches from fish trapping efforts (Mills K and Chalanchuk S, unpublished data). However, based on visual searches (eggs, tadpoles and adults), tadpole trapping efforts, and listening for calling males, both species are scattered, i.e. large congregations were not detected.

Mink frogs are distributed throughout eastern Canada, south to the Great Lakes basin. Green frogs are distributed throughout eastern North America, overlapping much of the mink frog range and extending into the southeastern U.S. Both species typically inhabit margins of large, permanent waters with abundant aquatic vegetation (Conant and Collins 1991), and the adults are primarily aquatic, rarely being found away from water (Preston 1982).

Breeding takes place in nearshore areas, and occurred in late June to early July for both species at the ELA. Green frog egg clutches are deposited in a mass attached to vegetation at the surface, whereas mink frog egg clutches are attached to submerged vegetation approximately 30 cm below the surface, and typically slump to the bottom prior to hatching (Wright and Wright 1949; Corkran and Thoms 1996). Immediately after hatching, the larvae are relatively immobile, and remain close to the remnants of the egg clutch. After yolk nutrition is completely used up active feeding begins. Anuran larvae typically graze on periphyton (Kupferburg 1997). The early larval period (premetamorphosis) is characterized by active feeding, an increase in mass, and the development of rudimentary hind limbs, which constitutes the first metamorphic changes in gross morphology. In both species the tadpoles fail to transform in their first summer, thus they remain inactive over the winter months. Overwintering larvae, which also occurs in *R. catesbiana*, is an adaptation to short growing seasons in some northern populations (Carey and Bryant 1995). In the following spring, active feeding resumes, and tadpoles continue to grow and develop. Growth eventually decreases and development increases, particularly in differentiation of hind limb digits (prometamorphosis). Finally, at metamorphic climax, feeding stops as the head, jaw, and gastrointestinal tract are rapidly restructured to the adult form, the tail is resorbed, and the forelimbs emerge, completing the transformation to the adult form.

Research Summary

There is a dire need to study amphibians in undisturbed habitats and to understand the potential impacts of human activities on wild populations. Amphibians are a diverse and complex class of organisms, and play a critical ecological role as both predator and prey. Given the current trend of

declining populations, research is required to improve conservation efforts and prevent irreversible loss of biodiversity.

Currently, the effects of endocrine disruptors on amphibians are poorly studied. In amphibians, the bulk of endocrine disruptor research has been based on laboratory exposure studies. Studies focusing on endocrine responses of native anurans in the wild remain scarce, despite effects demonstrated in other wildlife (Gendron et al. 1997). Environmental chemicals may be contributing to the current global trend of declining amphibian populations (Carey and Bryant 1995). Therefore, field-based research examining endocrine responses of native species is beneficial.

The overall objective of this research was to assess the potential endocrine-disrupting effects of a common environmental estrogen on tadpoles of two native Ranid species at the ELA. These species may be highly prone to aquatic contaminant exposure because they have a prolonged larval period and because the adults are highly aquatic. The majority of the research presented here is based on specimens that had been exposed in the wild or *in situ* (i.e. reared in cages on study lakes) to concentrations of EE2 that are comparable to those detected in various aquatic systems, and is thus considered ecologically relevant. Effects were examined at several levels of biological organization, i.e. biochemical, tissue, and whole organism. Impacts on both the reproductive and thyroid axes were assessed. Finally, both the embryonic and larval stages were studied.

Chapter 2 addresses hatching success, growth and development in tadpoles exposed to EE2 in the laboratory, *in situ*, and in the wild. In Chapter 3, thyroid hormone levels in EE2-exposed and reference tadpoles are compared. Thyroid hormones are critical in directing metamorphosis, and may be impacted by environmental estrogens. In Chapter 4, the impact of EE2 exposure on larval gonad development is examined. Wild mink frog tadpoles and cage-reared mink and green frog

tadpoles were examined using histological methods to assess sex ratios and the occurrence of intersex gonads. The results and significance of the findings are discussed in Chapter 5.

EE2 exposure data from Lake 260 were contrasted with pre-exposure data (Lake 260) and reference lake data. Characteristics of lakes used in these studies are summarized in Appendix

B. The following criteria were used in selecting reference lakes:

- Support natural populations of the species of interest
- Unmanipulated, or previously manipulated and returned to baseline conditions
- Resemble Lake 260 in physical, chemical, and biological characteristics
- Close proximity to field station, to minimize traveling time (particularly for *in situ* studies which required frequent visits)

The presence of EE2 in reference lakes was not analyzed. EE2 is typically detected in receiving waters of sewage treatment operations from human settlements, and as such is not expected to occur in an unpopulated area. There is no industrial development in the region, and limited public recreational use. Therefore, study lakes are free of point sources of pollution, and there are no anthropogenic estrogen inputs in these systems.

Chapter 2: Effects of 17 α -ethynylestradiol on hatching and larval growth and development rates of green frogs (*Rana clamitans*) and mink frogs (*R. septentrionalis*)

INTRODUCTION

In the majority of amphibian species the embryos and larvae are fully aquatic (McDiarmid and Mitchell 2000) and are thus exposed to waterborne contaminants in polluted areas. Contaminants may be contributing to amphibian population declines by impacting growth and development in early life stages (Carey and Bryant 1995). The effects of environmental estrogens on the embryonic phase have received little attention; however, inhibitory effects of estrogens on the larval phase have been documented (Gray and Janssens 1990; Hayes et al. 1993). Because amphibians inhabit waterways that receive estrogenic inputs, the potential impacts of a xenoestrogen on Ranid embryos and larvae were examined using whole-organism endpoints to assess developmental effects.

Contaminant Effects on Embryos

There are several potential routes of contaminant exposure for embryonic amphibians. Contaminants taken up by mature females can be incorporated into developing oocytes and mobilized during yolk nutrition of the embryo (Henry 2000). During oviposition in aquatic-breeding amphibians, the egg is surrounded by oviducal secretions of mucopolysaccharides that swell on contact with water to form the egg coat. Embryos are sensitive to direct chemical exposure during this period of egg coat formation (Larsson and Thuren 1987). The egg coat is permeable, permitting gas exchange and passage of metabolites, thus contaminant uptake can also occur directly through the egg coat (Beebee 1996). Egg masses of some species slump to the bottom prior to hatching (Corkran and Thoms 1996), thereby exposing the eggs to sediment-bound contaminants in polluted sites.

Hormones do not likely play a role in normal embryonic development (Hayes 2000), and as a result, the effects of hormonally active contaminants on amphibian embryos have received little

attention. Few mechanisms of amphibian embryo toxicity have been clearly demonstrated. A “curling defect” has been described, in which the developing embryo curls as it elongates because the vitelline membrane surrounding the egg fails to expand (Dunson and Connell 1982). This may be the result of chemically induced enzyme inactivation and often inhibits hatching (Laposata and Dunson 2000). Hatchlings use muscle movements to escape the jelly layer (Deuchar 1966), thus contaminant-induced paralysis or deformities would decrease hatch success. Elucidating mechanisms of embryotoxicity would improve the ability to predict and screen potential contaminants.

Studies have demonstrated deleterious effects of various contaminants on hatching in anurans. In laboratory studies, a dose-dependant decrease in hatching success of *R. arvalis* occurred when eggs were exposed to ecologically relevant concentrations of sediment-borne di-2-ethylhexylphthalate (Larsson and Thuren 1987). Phthalate esters are estrogenic and highly lipophilic (Jobling et al. 1995). Exposure to ecologically relevant concentrations of ammonia caused reduced hatching success in *R. pipiens*, *R. clamitans*, and *Bufo americanus* (Jofre and Karasov 1999). In field studies, hatch success of three amphibian species was significantly lower in ponds irrigated with treated municipal wastewater effluent than in reference ponds, but the presence of hormonally active contaminants was not assessed (Laposata and Dunson 2000). Similarly, hatch success was reduced in *R. pipiens* eggs reared in stormwater retention ponds contaminated with a mixture of metals, PCBs and PAHs (Bishop et al. 2000). Specific mechanisms of toxicity were not addressed in these studies. Low levels of contaminants have been shown to enhance UV damage in embryos of some anurans, therefore synergisms between chemicals and other environmental factors are of concern in wild exposures (Hatch and Burton 1998).

In contrast, hatching success was not adversely affected in other studies. Embryos of *Rana sylvatica*, *R. pipiens*, *R. clamitans*, and *R. catesbiana* were exposed to fenitrothion, tebufenozide (insecticides), triclopyr, or hexazinone (herbicides), and there was no effect on hatch success

(Berrill et al. 1994; Pauli et al. 1999). The authors conclude that spraying of these chemicals in nature would have little effect on the embryonic stage. *R. sylvatica* exposed to endosulfan (an organochlorine insecticide) showed no decrease in hatch success (Berrill et al. 1998). Environmentally relevant concentrations of petroleum (used crankcase oil) had no effect on hatching success in *Hyla cinerea*, but decreased larval growth rates (Mahaney 1994). Finally, hatching of *R. pipiens* and *R. clamitans* was unaffected by exposure to polychlorinated biphenyl 126, but newly hatched tadpoles had high mortality (Rosenshield et al. 1999). The egg coat may have provided sufficient protection for the developing embryo (Mahaney 1994; Berrill et al. 1998), or exposure times may have been too short to have a pronounced effect. The effects of contaminant exposure during embryogenesis may not be manifested until after hatching, such as in the mobilization of lipid-bound contaminants during yolk nutrition.

Effects of contaminants on hatchability of amphibian embryos are varied and warrant further investigation. Hatch success of amphibian embryos has been proposed as a sensitive and reliable screening endpoint to assess effects of contaminants (Ankley et al. 1998). Reduced hatching has been demonstrated at contaminated sites, thus population recruitment may be significantly impacted.

Larval Phase

Amphibian larvae are typically fully aquatic, gill-respiring herbivores/omnivores, whereas adults are (semi) terrestrial, air-breathing carnivores (Shi 2000). Metamorphosis is the discrete period of post-embryonic changes in physiology, morphology and biochemistry, resulting in the transformation of non-reproductive structures from the larval form to the adult form (McDiarmid 1994; Henry 2000). It involves massive changes throughout the body, including the regression of larval characteristics (e.g., tail and gill resorption), the transformation of existing tissues and organs to the adult form (e.g., skin, intestine and eyes), and the *de novo* formation of adult structures (e.g., limbs and lungs) (Yoshizato 1989). Such changes facilitate a biphasic life cycle in which the larvae inhabit an ecological niche different from that of the adults. Metamorphic

changes are induced by permissive actions of circulating endogenous thyroid hormones, which are typically low during the early larval phase and surge at metamorphic climax (Shi 2000, see Chapter 3).

Environmental Factors Affecting Larval Growth and Development

Biotic and abiotic factors influence larval development, the most important of which is temperature (Duellman and Trueb 1986; Hayes et al. 1993), but also food quality and availability (Kupferberg et al. 1994; Kupferberg 1997), pond drying (Denver 1997), predators (Wassersug 1997), inter- and intraspecific competition, crowding, and light (Shi 2000). Within physiological limits, higher temperatures generally enhance growth and development (Duellman and Trueb 1986). Exact mechanisms by which environmental factors influence development have not been demonstrated though potential pathways have been identified (Wassersug 1997; Shi 2000). Sensory stimuli such as light and heat perceived by the central nervous system may induce a “hormonal cascade” in the hypothalamus-pituitary-thyroid axis, stimulating development. Environmental stressors induce the release of corticotropin-releasing factor, a hypothalamic hormone that directly activates the thyroid axis to accelerate metamorphosis (Denver 1997). Factors such as temperature, salinity, and pH affect the rate of biochemical reactions, thereby impacting growth and developmental responses. Environmental influence on metamorphosis may be stage-dependant i.e. a given factor may inhibit growth during premetamorphosis and stimulate development during prometamorphosis (Denver 1997).

Ecological Significance of Larval Growth and Development Rates

Rates of growth and development in anuran larvae have important implications for survival. A critical minimum body size must be attained before tadpoles can metamorphose; therefore impairment of growth may delay or completely block transformation (Wilbur and Collins 1973). Greater size is advantageous because larval predation rates decrease with increasing size (predators are often gape-limited), and because avoidance behaviour is improved (Baker and Waights 1993; Baker and Waights 1994). Conversely, small size at metamorphic climax

decreases terrestrial performance, juvenile survival, fecundity, and size at maturity, and increases the time to maturity (Denver 1997). Delayed development makes larvae more susceptible to predation, pond drying in ephemeral waters, and overcrowding (Hayes 2000). Plasticity in growth and development rates can be an ecological advantage for wild tadpoles. Under optimal conditions, development rates decrease while growth rates increase, resulting in larger size and greater overall fitness. Conversely, under suboptimal conditions such as pond drying, it is advantageous to retard growth, and utilize energy reserves for rapid development to expedite transformation. Thus, factors influencing larval development are of great significance for the remainder of the life cycle and ultimately for population sustainability.

Contaminant Effects on Larval Growth and Development

Given the ecological significance of larval development, disruption by environmental contaminants has important implications for the survival of species. Reduced growth occurred in *Rana pipiens* and *R. clamitans* tadpoles exposed to environmentally relevant concentrations of PCB (Rosenshield et al. 1999). Increased time to metamorphosis of *R. pipiens* occurred in association with stormwater detention pond water contaminated with metals, PCBs and PAHs (Bishop et al. 2000). Reduced growth and development occurred in *R. clamitans* tadpoles exposed to environmentally relevant concentrations of ammonia (Jofre and Karasov 1999). Acetochlor, a pre-emergent herbicide, accelerates metamorphosis (Veldhoen and Helbing 2001). Short-term exposure to endosulfan, an organochlorine pesticide, impaired growth of *R. clamitans* and *R. sylvatica* tadpoles (Berrill et al. 1998). *Hyla cinerea* tadpoles exposed to environmentally relevant concentrations of petroleum exhibited reduced growth (Mahaney 1994). Exposure to PCBs caused an increase in length of larval period and a decrease in T₄ levels in *X. laevis* and *R. temporaria* tadpoles (Gutleb et al. 2000). Atrazine was associated with reduced size at metamorphosis and prolonged larval development (Sullivan and Spence 2003). Mechanisms of disruption were not explicitly demonstrated, though central or peripheral modulation of thyroid hormone function is probable (see Chapter 3). Contaminant effects could be detrimental to wild populations (Rosenshield et al. 1999).

Estrogen Effects on Larval Growth and Development

Natural and synthetic estrogens can influence development of anuran larvae (Hayes and Licht 1993; Hayes 2000). Most evidence suggests that steroids depress thyroid status in amphibians, which has also been documented in fish (Cyr and Eales 1996). Common hypothalamic controls exist for both the thyroid and reproductive axes, therefore negative feedback actions caused by steroids or steroid mimics could depress thyroid status and ultimately delay development (Vandorpe and Kuhn 1989). Administration of the natural estrogen 17 β -estradiol reduced circulating TH concentrations in *R. ridibunda*, and similar effects of 17 β -estradiol (E2) administration on plasma TH levels have been reported in teleost fish (Leatherland 1985; Cyr et al. 1988; Vandorpe and Kuhn 1989; Flett and Leatherland 1989). Further, E2 treatment inhibited TH-induced changes in developing *Xenopus laevis* tadpoles (Gray and Janssens 1990; Hayes 1997) and inhibited growth and thyroid hormone-dependent changes in tadpoles of *Bufo* species (Hayes et al. 1993; Hayes 1997). The synthetic estrogen DES (diethylstilbestrol) induced developmental abnormalities in organogenesis, which is likely indicative of thyroidal disruption (Nishimura et al. 1997). Although the amount of hormone administered in these studies generally exceeded physiological levels, the results indicate that estrogen can modulate thyroid function and disrupt larval development in anuran species.

Research Needs and Objectives

The overall objective of the current set of studies is to assess the impacts of environmental estrogen exposure on the early life stages of native anuran species at ELA using whole organism endpoints. The effects of estrogenic contaminants on embryonic and larval stages of these species may have important implications for recruitment and ultimately survival of populations. Tadpoles were exposed to EE2 in the laboratory, *in situ*, and in the wild. The null hypothesis was that EE2 exposure would not inhibit hatching success or growth and development of tadpoles. In the first study, mink and green frog embryos and tadpoles were exposed to EE2 *in situ* from pre-hatch to the mid-larval phase to assess effects on hatch success, growth, and development. In

the second study, mass at various development stages was examined in wild EE2-exposed mink frog tadpoles. Finally, a laboratory study was conducted to expose green frog tadpoles from hatching to the mid larval phase at a range of concentrations, including that used in the lake experiment.

MATERIALS AND METHODS

Lake 260 Estrogen Additions

The epilimnion of Lake 260 was dosed with EE2 (Sigma) from the end of May to the end of October of 2001. EE2 additions and accompanying water chemistry analysis were done according to Palace et al. (2002). Briefly, every Monday, Wednesday and Friday EE2 was dissolved in methanol and mixed with a volume of lake water, and the solution was pumped into the propeller wash of a boat while driving around the lake. The target water column concentration (epilimnion only) was 5 ng/L. Surface water samples were collected weekly (integrated epilimnetic samples from five sites) and the mean EE2 concentration was determined using radioimmunoassay. The amount of EE2 to be added in a given week was based on estimates of the epilimnion volume and existing EE2 concentration from the previous week.

A. In Situ Exposure

Sample Collection

Four green frog egg clutches (G1 to G4) and two mink frog egg clutches (M1 and M2) were collected from reference Lake 442 on 25 June and 4 July 2001, respectively. The eggs were naturally fertilized, and ranged from stage 8 to 12 at time of collection. They were transported to the ELA laboratory in aerated lake water. Each egg clutch was separated into smaller clumps to ensure adequate oxygen supply to developing embryos, and handling of the eggs was kept to a minimum to avoid unnecessary stress. Three subsamples of 200 eggs were taken from each clutch and placed in separate labeled containers filled with lake water. A subsample from each clutch was transported to each of the three study lakes (Lake 260 and reference Lakes 114 and

224) and placed in an individual cage. The green frog egg exposure started on 26 June 2001, and the mink frog egg exposure started on 6 July 2001.

Cage Design

Cages were used to maintain tadpoles on the study lakes. The cages are designed to meet the physiological needs of developing embryos and tadpoles and prevent predation while maintaining the specimens in their native environment (Harris and Bogart 1997, Appendix C). Briefly, white nitex nylon mesh (500 μm pore size) was sewn into a cylinder (60 cm depth, 35 cm diameter) with a permanent bottom and a removable top attached with a continuous strip of Velcro™. Thus, potential predators are excluded, metabolites diffuse out, and water circulates freely. Handling generally causes eggs to lose their natural buoyancy; thus they were placed in a floating inner basket within the cage to simulate their natural position in the water. The inner basket consisted of a pocket of window screen mesh suspended from a 15 x 15 cm unfinished wood frame. The outer cage is suspended from an unfinished wooden frame that floats at the water surface such that the top of cage sits approximately 10 cm above the waterline. This provides access to air for the tadpoles during lung development. Cages were anchored at a near shore site on each lake.

Maintenance and Endpoints

Eggs were monitored daily and when the eggs hatched the tadpoles, unhatched embryos, and egg case remnants were gently tipped from the baskets into the cage. Initially each cage was given 15 g of boiled lettuce and 1.5 g of Tetramin™ flake fish food twice per week. On 25 July rations were increased to 20 g lettuce and 2.0 g fish food every Monday, Wednesday and Friday. At 10 days post-hatch (dph) the tadpoles were counted in each cage to determine hatching success. Hatch rates varied among cages, thus on 9 August tadpoles were culled to 100 individuals per cage to prevent differential density-related crowding effects on growth and development. The culled tadpoles were euthanized by immersion in approximately 1 L of lake water containing 1 ml of 70% ethanol saturated with benzocaine, preserved in Davidson's solution, and the mass and development stage of individuals were determined in the laboratory.

Development stages were determined according to Gosner (1960) throughout. On four sampling dates thereafter (22 August, 5 September, 19 September, 9 October) 10 tadpoles were removed from each cage, preserved and measured. The study was terminated on 9 October and the remaining tadpoles were euthanized and processed for histological analysis (Chapter 4).

On August 1 2001 temperature and dissolved oxygen were measured inside and outside of all cages to determine if the cages had an effect on these parameters.

Statistical Analysis

ANOVA was used to test for significant differences among lakes in green frog hatch success, whereas mink frog hatch data were interpreted without statistical analysis. The hatch success data (proportion of eggs hatched) were transformed by taking the arcsine of the square root of the proportion. A preplanned contrast was used to determine if hatch success on Lake 260 differed from that of the reference lakes. (SAS statistical software, version 8). Statistical significance is at $\alpha = 0.05$ throughout.

ANCOVA was used to assess whether tadpole mass and development stage differed over time among study lakes, i.e. to test for equality of regression slopes among lakes. Each clutch was analyzed separately. Where significant differences were detected, Tukey's multiple comparison procedure was used to test all pairwise combinations of regression slopes for significant differences (Zar 1984).

B. Wild Population

Sample Collection

Wild mink frog tadpoles were collected from Lake 260 and from reference Lakes 442 and 114 on various dates in 2000 and 2001. Green frog tadpoles are also indigenous to these lakes, however the numbers caught were insufficient for this study. Lake 260 was unmanipulated in 2000, and was treated with EE2 in 2001 (season mean concentration of EE2 = 6.1 ± 1.5 ng/L). Tadpoles

were captured using modified Beamish fish trap nets. Nets were typically set near shore at various locations on each lake and checked the following day. Mass and development stage were recorded, and the tadpoles were either released or euthanized for histological analysis (Chapter 4).

Statistical Analysis

ANCOVA was used to test for significant differences among lakes in the mass of tadpoles from stages 26 to 40, i.e. to test for equality of regression coefficients (slopes) when the mass of tadpoles from each lake is plotted as a function of stage. Separate analyses were performed for 2000 and 2001 data. Tukey's multiple comparison procedure was used to test for significant differences in all pairwise combinations of slopes. All stages from hatching (stage 25) to stage 40 were included. Tadpoles beyond stage 40 were excluded from analysis because feeding stops at this point and mass begins to decrease as development proceeds.

C. Laboratory Exposure

Chemicals

17 α -ethynylestradiol (EE2, Schering Pharmaceutical, Germany) and distilled-in-glass grade methanol (99.8% purity, Caledon Laboratories) were used in preparation of stock solutions. Reagent-grade methanol was used for rinsing glassware (Caledon Laboratories). Exposure solutions were prepared using lake water (Lake 240, ELA) for the first 16 weeks of the exposure and dechlorinated City of Winnipeg water for the final 11 weeks. Lake 240 is an unmanipulated reference lake.

Exposure Assay

Four replicates of 6 treatments were used: water control (WC), solvent control (SC, 0.001% methanol), 5, 20, 80, and 1000 ng/L of EE2. The lowest EE2 concentration examined is comparable to those detected in sewage treatment effluents (Ternes et al. 1999), and corresponds to the target water column concentration in the lake exposure study. The highest

EE2 concentration has been shown to induce intersex gonad development in Ranids (C. Mackenzie, pers. comm.).

Exposure solutions were prepared as follows: An EE2 stock solution was prepared by dissolving EE2 in methanol to a final concentration of 100 µg/ml. Exposure solutions were prepared by diluting aliquots of this stock solution to the appropriate final concentration. The 1000 ng/L solution was made by dissolving 10 µl of stock in 1 liter of water, thus the final methanol content was 0.001%. To make the 5 ng/L solution, the previous step was repeated, and a 5 ml aliquot of the 1000 ng/L solution was diluted in 1 liter of water. The 20 and 80 ng/L solutions were prepared in a similar fashion. All glassware and pipette tips were cleaned with reagent grade methanol to prevent EE2 contamination in the solutions. Exposure solutions were replaced every 48 h, and new EE2 stock solutions were prepared every 2 to 4 weeks.

As described in the cage study, fertilized green frog egg clutches were collected from Lake 442. Eggs from clutches G3 and G4 (the two largest clutches) were housed in coolers with aerated host water at ambient temperature, hatched out in the laboratory, and the newly hatched tadpoles were used in the current study. At 7 dph 10 tadpoles from each clutch were combined into glass jars containing 250 ml of exposure solution. Jars were kept in an environmental chamber at 18°C on a 12-h light cycle (natural spectrum light). Tadpoles were fed boiled lettuce *ad libitum* immediately following solution renewals. Aeration was initiated in the jars at 27 dph. To accommodate the growth of tadpoles, the volume of solution in the jars was increased to 400 ml at 83 dph, and at 100 dph the original jars were replaced with 1-liter glass jars with 750 ml of solution. At 119 dph the tadpoles and experimental apparatus were transported to the Freshwater Institute (Winnipeg, Canada), and the experimental protocol was continued using dechlorinated City of Winnipeg water. On one occasion (17 July 2001), duplicate samples of the 5, 20 and 80 ng/L solutions were collected and processed according to Palace et al. 2002 to determine the EE2 concentration in the exposure jars. The 1000 ng/L solutions were not tested to avoid potential contamination of the analytical equipment.

Endpoints and Statistical Analysis

At 196 dph all tadpoles were euthanized, preserved in Davidson's solution, and mass and stage were recorded. Total exposure time was 189 days. One way ANOVA was used to test for differences among treatment groups (pooled replicates) in mass and stage of tadpoles at the end of the exposure. Data from jars with 10 or fewer individuals at the end of the study were excluded from the analysis, because tadpole density affects growth and development, thus differential "crowding effects" are considered to be a confounding factor in data analysis. Dunnett's multiple comparison procedure was used to compare each EE2 treatment group to the solvent control group.

RESULTS

The mean concentration of EE2 in the epilimnion of Lake 260 in 2001 was 6.1 ± 1.5 ng/L (Appendix D). EE2 was below assay detection limits (1 ng/L) in Lake 260 in 2000 (K. Kidd, unpublished data).

A. Caging Exposure*Hatching Success*

There was little or no difference in temperature and dissolved oxygen of surface waters inside and outside the cages (Figure 2.1). Embryos hatched on the fourth exposure day for both species (29 June and 9 July for green and mink frogs, respectively). The mean hatch success of green frog eggs (clutches G1 to G4) was significantly lower on Lake 260 (53.4%) than on Lakes 114 and 224 (77 and 75%, $p=0.02$, Figure 2.2, p.38). The mean EE2 concentration \pm SD during the green frog egg exposure was 6.0 ± 0.6 ng/L. Hatching success was lower on Lake 260 than on the reference lakes for G1, G2, and G4, whereas clutch G3 was consistently low on all lakes (Table 2.1, p.39). The hatching success of mink frog eggs (clutch M1 and M2) was similar across lakes (Figure 2.3, p.38). For clutch M1, 91% of eggs hatched on Lake 260, whereas 87 and

95.5% hatched on Lakes 114 and 224, respectively. For clutch M2, 71% hatched on Lake 260, and 77 and 66% hatched on lakes 114 and 224, respectively. The mean EE2 concentration \pm SD during the mink frog egg exposure was 7.3 ± 0.8 ng/L.

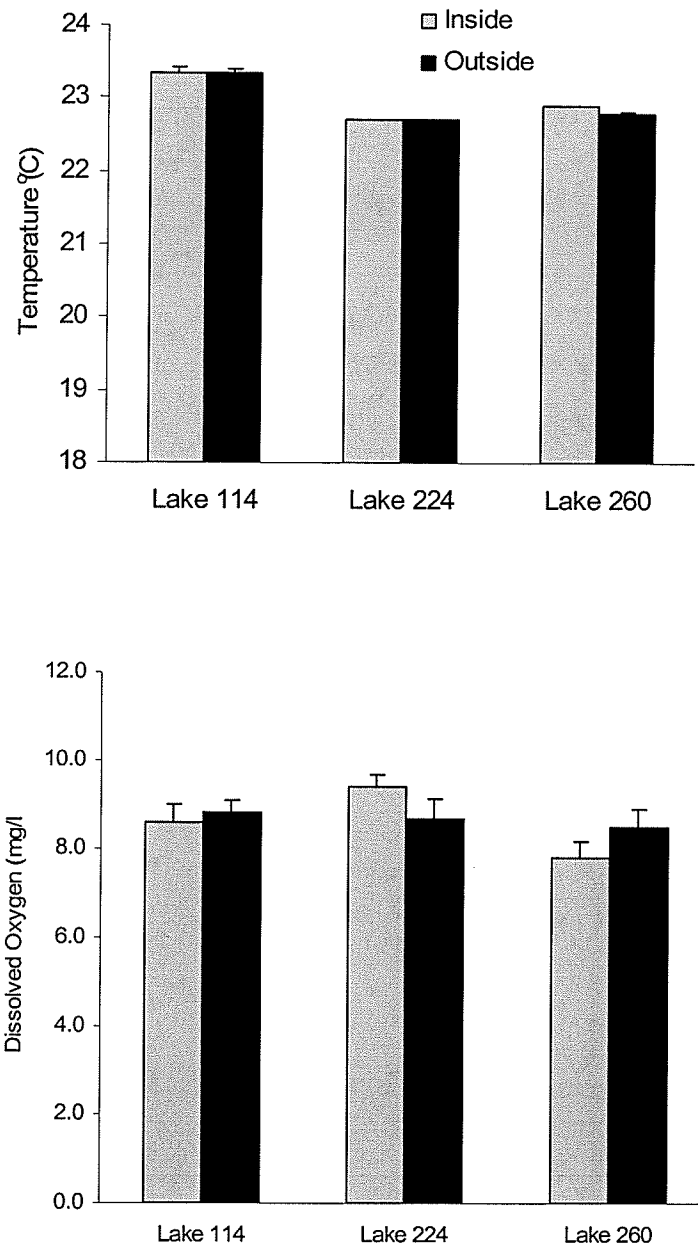


Figure 2.1 Mean (A) temperature and (B) dissolved oxygen concentrations (+SD) inside and outside cages on Lake 260 and on reference lakes, August 1, 2001. n=6.

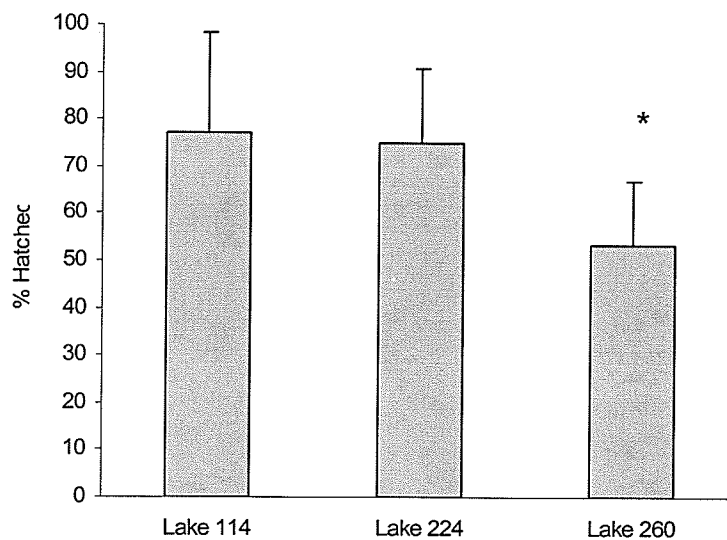


Figure 2.2 Mean hatch success (+SD, n=4) of green frog eggs reared in cages for 4 days on reference lakes and on Lake 260 (mean EE2 concentration \pm SD = 6.0 \pm 0.6 ng/L). Arithmetic means are presented in figure; statistical analysis was performed with arcsine transformed data, *indicates significant difference.

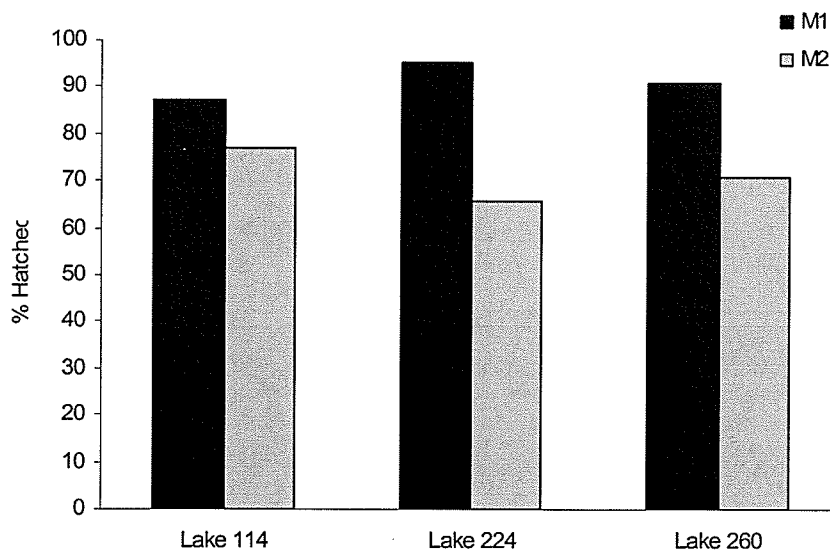


Figure 2.3 Hatch success of mink frog eggs (Clutches M1 and M2) reared in cages for 4 days on reference lakes and on Lake 260 (mean EE2 concentration \pm SD was 7.3 \pm 0.8 ng/L).

Table 2.1 Hatch success of green and mink frog eggs (Clutches G1 to G4, and M1-M2, respectively) reared in cages for 4 days on reference lakes and on Lake 260. Clutches G1 to G4 were exposed to 6.0 ± 0.6 ng/L EE2; Clutch M1 and M2 were exposed 7.3 ± 0.8 ng/L EE2.

Lake	Clutch	Number Hatched (out of 200)	Percent Hatched
114	G1	171	85.5
114	G2	155	77.5
114	G3	95	47.5
114	G4	195	97.5
114	M1	174	87.0
114	M2	154	77.0
224	G1	173	86.5
224	G2	144	72.0
224	G3	108	54.0
224	G4	175	87.5
224	M1	191	95.5
224	M2	132	66.0
260	G1	87	43.5
260	G2	97	48.5
260	G3	96	48.0
260	G4	147	73.5
260	M1	182	91.0
260	M2	142	71.0

Larval Growth and Development Rate

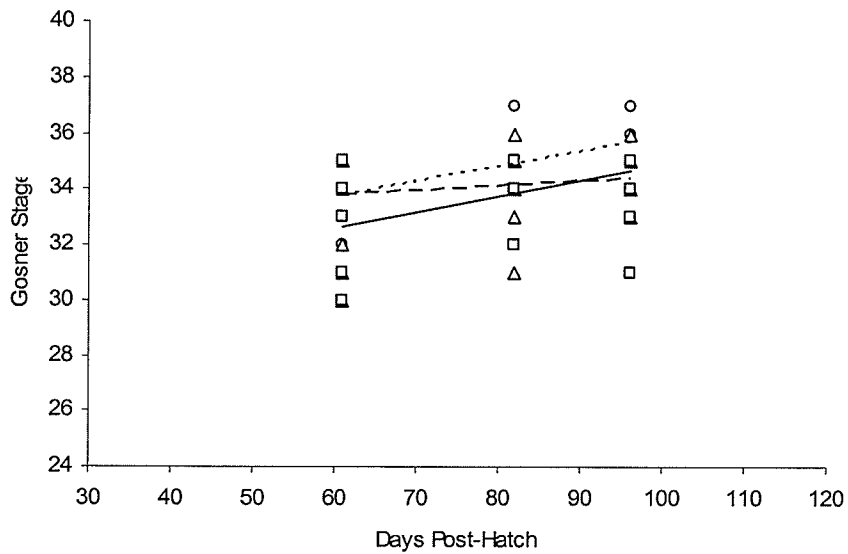
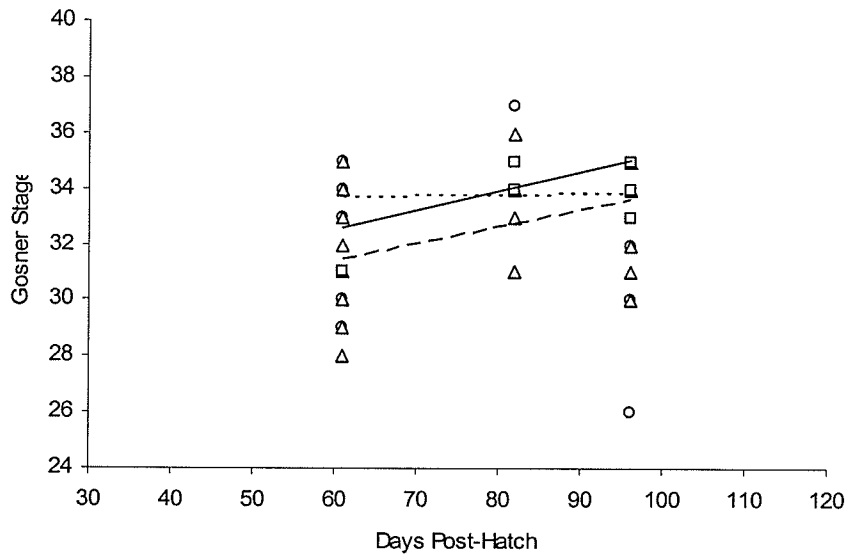
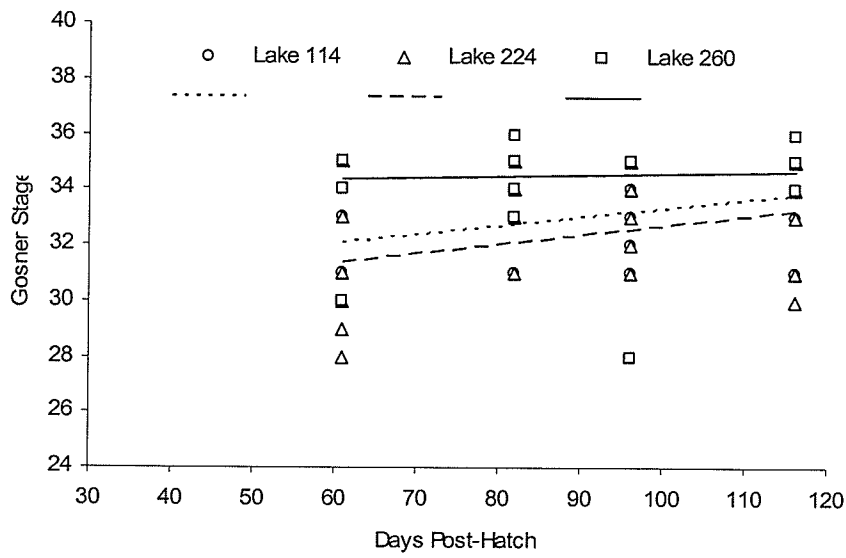
The mass of tadpoles increased over time in all cages (Figure 2.4, p.41). The growth rates of hatchlings from clutches G2, G4 and M1 were significantly different among lakes (ANCOVA, $p=0.0065$, <0.0001 , and 0.0067 , respectively, Table 2.2, p.47). However, pairwise comparisons of regression slopes from these clutches revealed no consistent treatment effect. For example, for clutches G2 and G4, the tadpoles caged in Lake 224 had significantly higher growth rates than those from Lake 114. The growth of hatchlings from clutches G1, G3 and M2 were not

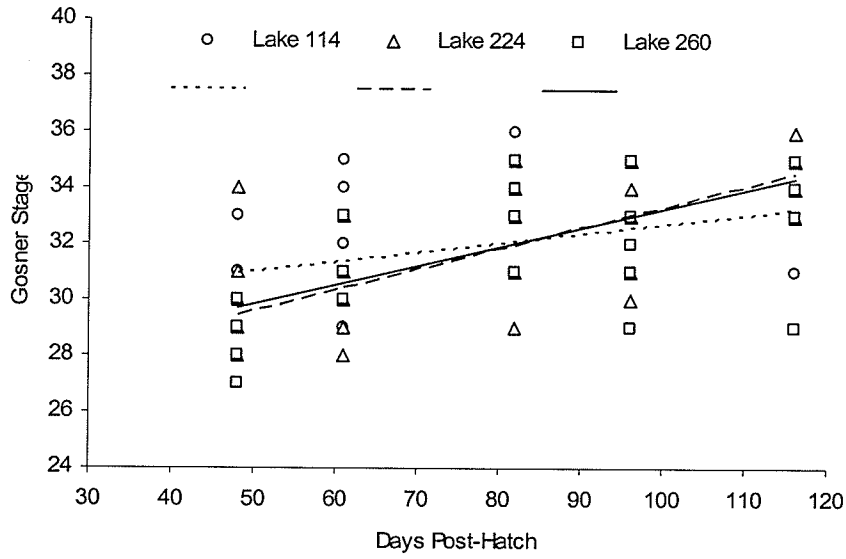
significantly different among lakes (ANCOVA, $p=0.29$, 0.23 , and 0.97 , respectively). Pairwise comparisons of the regression line elevations (i.e. y intercepts) revealed no consistent treatment effect (data not shown). Therefore, in both species there was no consistent treatment effect on growth rate.

Development stage of tadpoles increased over time in all cages (Figure 2.5, p.44). A pattern similar to that of the growth rates was detected. The development rates of hatchlings from clutches G2, G4, and M1 were significantly different among lakes (ANCOVA, $p=0.025$, 0.0087 , and 0.0202). Again, pairwise comparisons of regression slopes revealed no consistent treatment effect, e.g. for clutches G2 and G4, tadpoles caged in Lake 224 had greater development rates than those from Lake 114. The development rate of hatchlings from clutches G1, G3 and M2 were not significantly different among lakes (ANCOVA, $p=0.25$, 0.20 , and 0.84 , respectively), and comparisons of regression line elevations revealed no consistent treatment effect. See Table 2.3 (p.47) for regression line slopes, y intercepts, and correlation coefficients. There was no consistent treatment effect on regression line elevations.

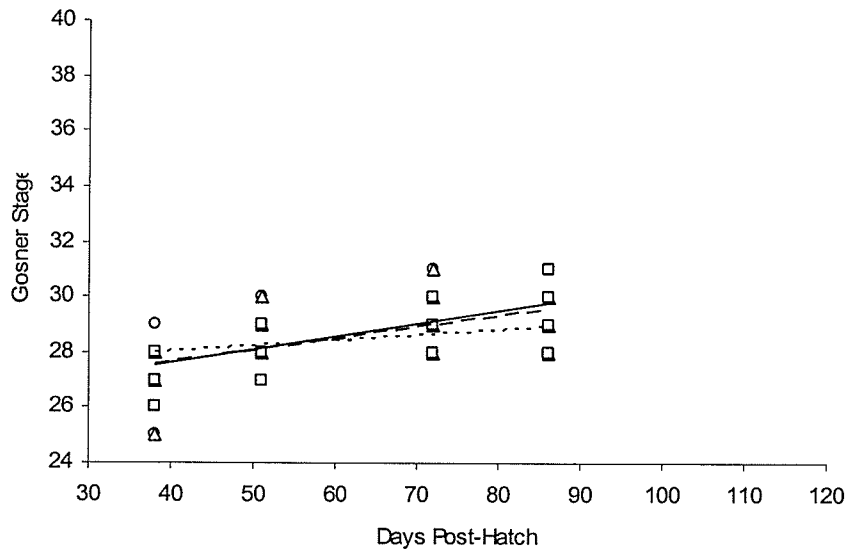
Figure 2.4 Mass of cage-reared tadpoles from reference lakes and from Lake 260 sampled on various dates in 2001 (n=10 per cage per sampling date). Clutches G1 to G4 were green frogs; Clutches M1 and M2 were mink frogs. Regression lines with different letters were significantly different. The season mean concentration of EE2 in Lake 260 was 6.1 ± 1.5 ng/L.

Figure 2.5 Development stage of cage-reared tadpoles from reference lakes and from Lake 260 sampled on various dates in 2001 (n=10 per cage per sampling date). Clutches G1 to G4 were green frogs; Clutches M1 and M2 were mink frogs. Regression lines with different letters were significantly different. The season mean concentration of EE2 in Lake 260 was 6.1 ± 1.5 ng/L.

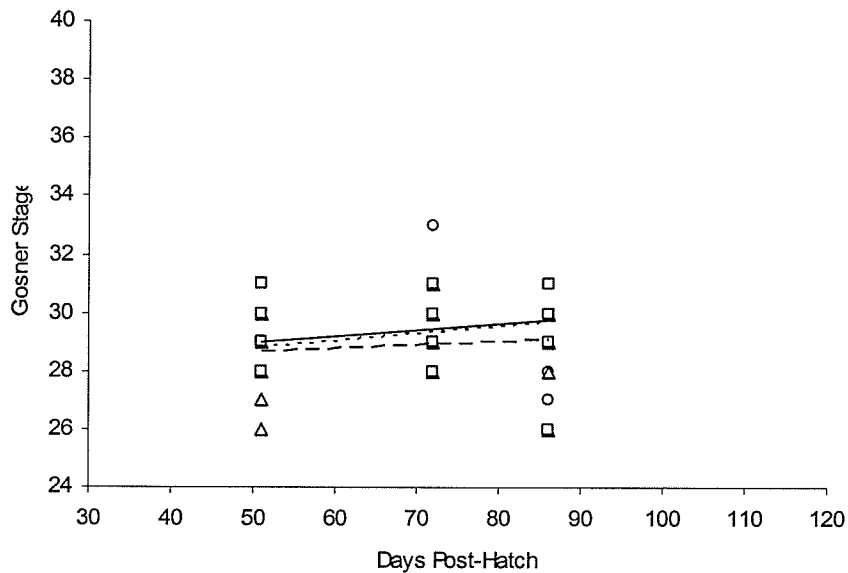




Clutch G4
SD (p=0.0087)



Clutch M1
SD (p=0.0202)



Clutch M2
NSD (p=0.84)

Table 2.2 Slopes, y intercepts, and correlation coefficients for the growth rates of cage-reared tadpoles from Lake 260 and reference Lakes 114 and 224 in 2001. Clutches G1 to G4 were green frogs; Clutches M1 and M2 were mink frogs.

Clutch	Lake 114			Lake 224			Lake 260		
	Slope	Intercept	r^2	Slope	Intercept	r^2	Slope	Intercept	r^2
G1	0.0075	0.2005	0.3066	0.0114	-0.2518	0.5232	0.0099	0.1070	0.4569
G2	0.0062	0.2808	0.102	0.0208	-0.9739	0.6174	0.0112	-0.1638	0.3302
G3	0.0170	-0.3521	0.3208	0.0091	0.0165	0.3530	0.0138	-0.3240	0.5687
G4	0.0087	-0.0646	0.5370	0.0170	-0.6618	0.7814	0.0123	-0.2959	0.6554
M1	0.0089	-0.1142	0.4733	0.0148	-0.4501	0.7634	0.0143	-0.4030	0.7078
M2	0.0118	-0.1591	0.2774	0.0127	-0.3021	0.3865	0.0119	-0.1929	0.3554

Table 2.3 Slopes, y intercepts, and correlation coefficients for the development rates of cage-reared tadpoles from Lake 260 and reference Lakes 114 and 224 in 2001. Clutches G1 to G4 were green frogs; Clutches M1 and M2 were mink frogs.

Clutch	Lake 114			Lake 224			Lake 260		
	Slope	Intercept	r^2	Slope	Intercept	r^2	Slope	Intercept	r^2
G1	0.0319	30.115	0.1371	0.0340	29.286	0.1252	0.0059	34.023	0.0068
G2	0.0041	33.437	0.0007	0.0621	27.636	0.1903	0.0706	28.302	0.3832
G3	0.0574	30.205	0.3172	0.0180	32.640	0.0313	0.0579	29.121	0.2368
G4	0.0594	26.763	0.4496	0.0805	25.243	0.6749	0.0755	25.711	0.5893
M1	0.0164	27.478	0.0938	0.0408	26.050	0.4023	0.0530	25.298	0.4228
M2	0.0259	27.493	0.0799	0.0120	28.029	0.0182	0.0218	27.914	0.0466

B. Wild Population

In 2000, the vast majority of samples were caught in May and June, and some were caught in October. In 2001, reference samples were caught from May through to early July, and Lake 260 samples were caught in June and October in the third, fifth, and twentieth weeks of the EE2 additions. Approximately 60 percent of the Lake 260 samples were caught in the fall. Early stage tadpoles (stage 26 to 31) were relatively infrequent, whereas tadpoles ranging from stage 35 to 37 were most abundant in all lakes.

In 2000 there was no significant difference among lakes in the relationship between mass and developmental stage of mink frog tadpoles ($p=0.35$, Figure 2.6). For example, at stage 37, the mean mass (\pm SD) of tadpoles was 7.7 ± 1.36 g, 7.8 ± 1.44 and 8.4 ± 1.40 g for Lakes 260, 114 and 442, respectively. However there was a significant difference in 2001 ($p=0.02$, Figure 2.6). The slope of the regression line from Lake 260 tadpoles is significantly higher than that of both reference lakes, whereas the two reference lakes did not differ from each other (Table 2.4, p.50). The mean mass of tadpoles at stage 37 was 8.0 ± 0.75 g, 6.7 ± 1.56 and 6.7 ± 1.03 g for Lakes 260, 114 and 442, respectively.

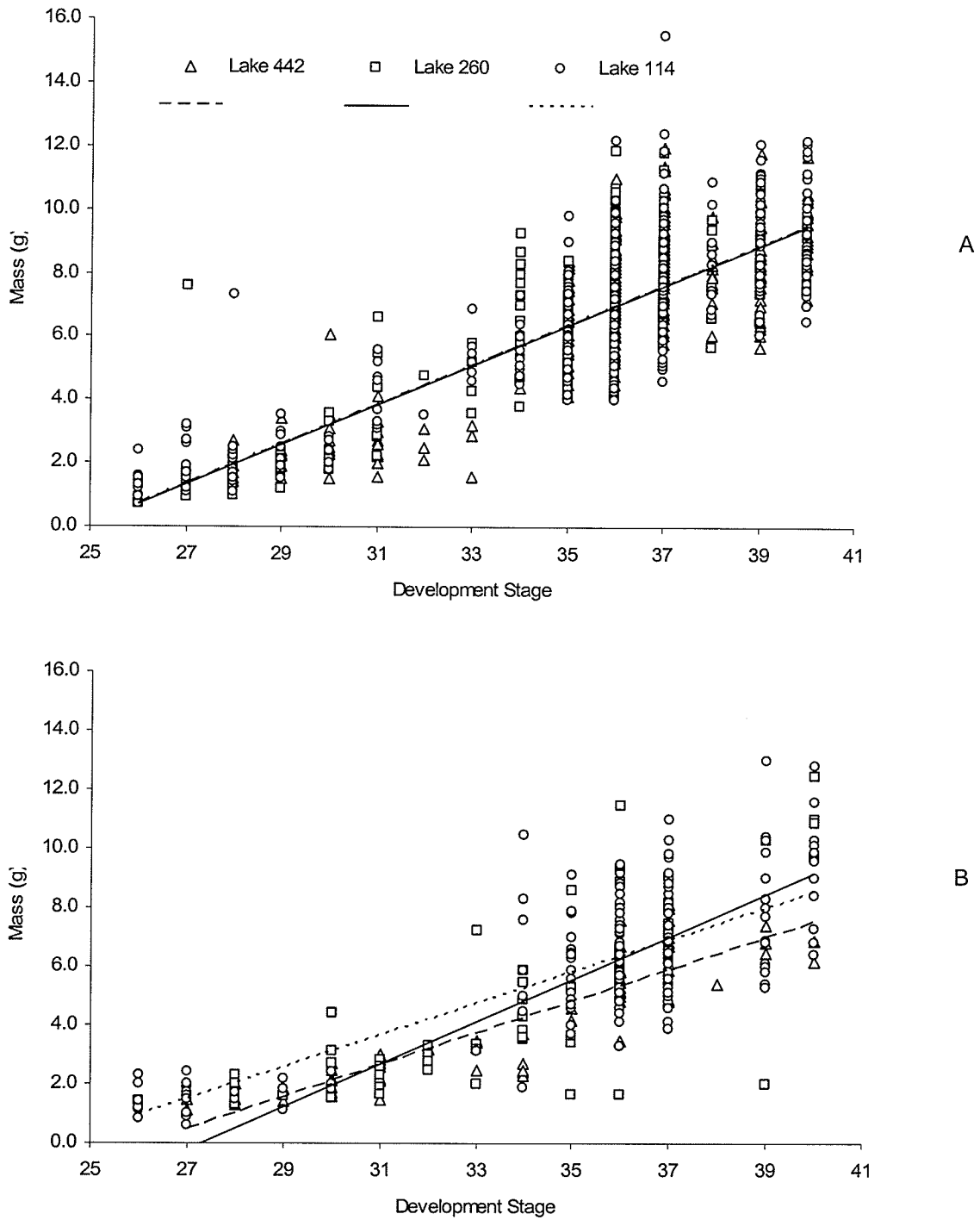


Figure 2.6 Mass of wild mink frog tadpoles from reference lakes and from Lake 260 sampled on various dates in (A) 2000 and (B) 2001. The Lake 260 regression line was significantly different from reference lines in 2001, but not in 2000. The season mean concentration of EE2 in Lake 260 in 2001 was 6.1 ± 1.5 ng/L. Sample size per lake ranged from 71 to 765.

Table 2.4 Slopes, y intercepts, and correlation coefficients for the regression lines (mass vs. development stage) of wild mink frog tadpoles Lake 260 and reference Lakes 114 and 442 in 2000 and 2001.

Year	Lake 114			Lake 442			Lake 260		
	Slope	Intercept	r ²	Slope	Intercept	r ²	Slope	Intercept	r ²
2000	0.6266	-15.576	0.6794	0.6731	-17.128	0.6416	0.6286	-15.636	0.6276
2001	0.5439	-13.214	0.6087	0.5437	-14.255	0.8152	0.7256	-19.819	0.6169

C. Laboratory Exposure

The EE2 concentrations in exposure jars (17 July 2001) were as follows: 5 ng/L= 4.82 and 5.81, 20 ng/L= 9.34 and 10.72, 80 ng/L= 87 and 78 ng/L.

Results of the laboratory exposure experiment are summarized in Table 2.5 (p.52). Mortality occurred in all exposure jars to varying degrees (from 10 to 100%). All water control data were dropped from the statistical analysis because of high mortality (from 55 to 100%). There was a weak inverse relationship between the number of individuals per jar and the mean mass of individuals in the jars. With the exception of replicate 3 for the 5 ng/L treatment, jars with fewer tadpoles had greater masses at the end of the experiment. For example, in the 20 ng/L treatment group the mean mass of larvae for replicate 1 (n=8) was 0.51 g (\pm 0.20) as compared to 0.29 g (\pm 0.16) for replicate 3 (n=15). Further, in the 1000 ng/L treatment group, the mean development stage of tadpoles in replicate 2 (n=7) was higher than that of the other replicates (n=13 to 17).

The mean mass of tadpoles at the end of the experiment ranged from 0.30 g (20 ng/L and 1000 ng/L) to 0.34 g (5ng/L and 80 ng/L). At the end of the exposure there was no significant difference in mean mass of tadpoles among treatment groups ($p=0.62$, Figure 2.7, p.53). The mean development stage of tadpoles at the end of the experiment was 28 in all exposure jars except for replicates 2 and 3 in the solvent control group, which had a mean development stage of 27.

There was a significant difference among treatment groups in mean stage of tadpoles ($p=0.044$, Figure 2.7). Specifically, the mean stage of tadpoles in the 80 and 1000 ng/L treatment groups was significantly higher than the solvent control.

Table 2.5 Mass and development stage of laboratory-reared green frog tadpoles exposed to EE2 (5, 20, 80, and 1000 ng/L) or control solutions for 189 days (starting at 7 dph) in a 48 h static renewal system. Values are replicate means \pm SD.

Treatment	Replicate	Number		
		Surviving (out of 20)	Mass (g)	Stage
Water	1	0		
	2	8	0.44 \pm 0.24	29 \pm 0.8
	3	9	0.43 \pm 0.26	28 \pm 1.1
	4	0		
Solvent	1	12	0.34 \pm 0.20	28 \pm 1.0
	2	15	0.27 \pm 0.17	27 \pm 0.8
	3	11	0.32 \pm 0.21	27 \pm 1.0
	4	5	0.47 \pm 0.15	28 \pm 0.5
5 ng/L	1	11	0.33 \pm 0.15	28 \pm 0.6
	2	11	0.34 \pm 0.11	28 \pm 1.2
	3	7	0.25 \pm 0.12	28 \pm 1.3
	4	0		
20 ng/L	1	8	0.51 \pm 0.20	28 \pm 1.0
	2	13	0.30 \pm 0.21	28 \pm 1.0
	3	15	0.29 \pm 0.16	28 \pm 1.1
	4	8	0.37 \pm 0.23	28 \pm 0.8
80 ng/L	1	17	0.31 \pm 0.20	28 \pm 0.9
	2	15	0.32 \pm 0.19	28 \pm 1.3
	3	16	0.34 \pm 0.23	28 \pm 0.9
	4	14	0.37 \pm 0.22	28 \pm 1.1
1000ng/L	1	17	0.29 \pm 0.17	28 \pm 1.2
	2	7	0.54 \pm 0.21	29 \pm 0.9
	3	16	0.28 \pm 0.20	28 \pm 0.7
	4	13	0.28 \pm 0.11	28 \pm 0.6

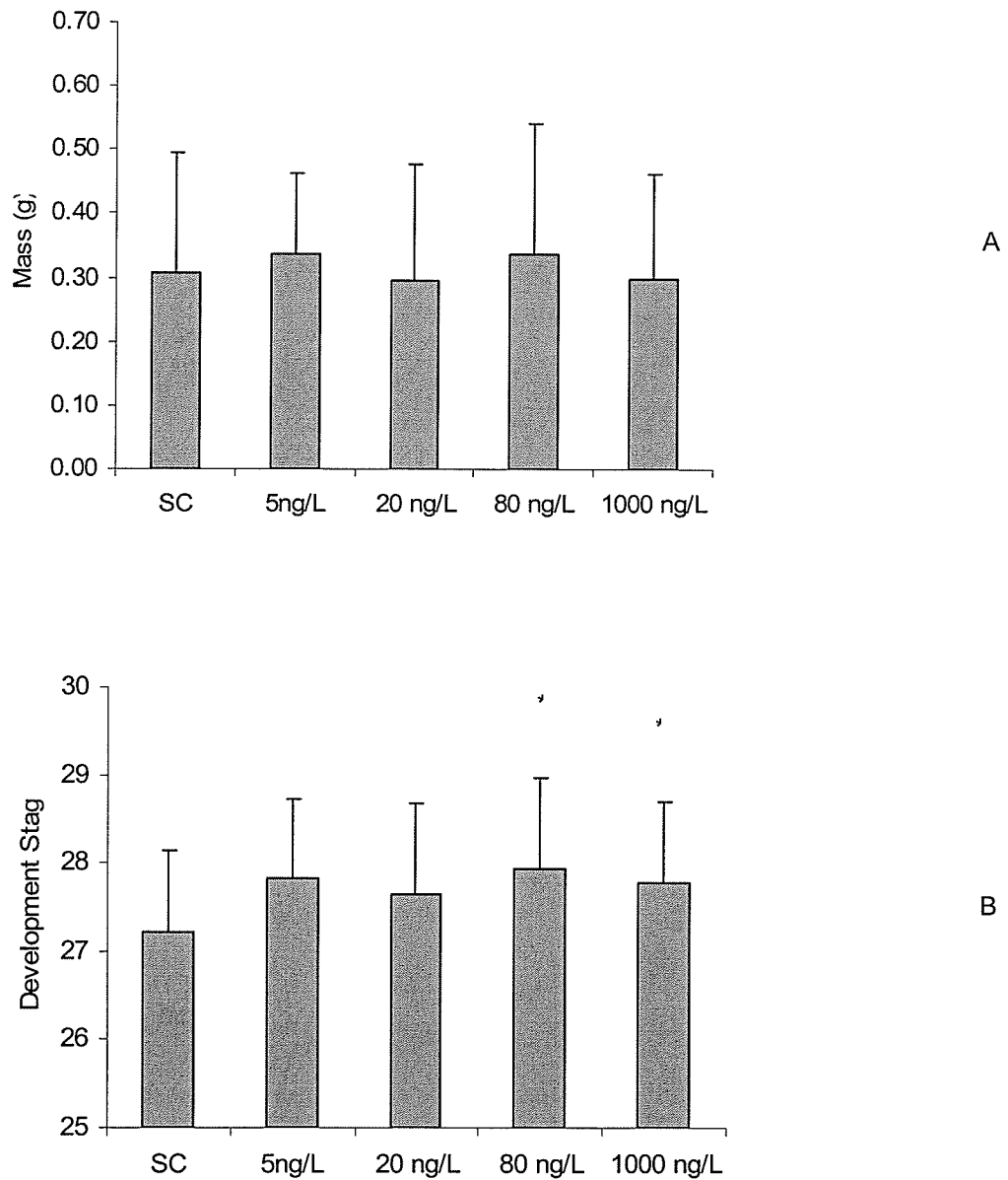


Figure 2.7 (A) Mean mass and (B) mean development stage (+SD) of laboratory-reared green frog tadpoles exposed to EE2 (nominal concentrations of 5, 20, 80, and 1000 ng/L) or solvent control (0.001% methanol; SC) after 189 days exposure starting at 7 dph in a 48 h static renewal system. Treatment means are based on pooled data from exposure jars containing 11 or more surviving individuals. *indicates significantly different from SC.

DISCUSSION

A. Cage Exposure

Hatching success

Hatching success was as high as 97% in reference cages in the current study, and hatch success over 90% has been documented for other *Rana* species in the wild (Duellman and Trueb 1986), thus it appears that the caging study provided a reasonable predictor of hatch rates in the wild. Hatching success was significantly reduced in EE2-exposed green frog eggs, which could ultimately impact recruitment in the wild. It is noteworthy that of the four green frog clutches, three showed marked treatment responses while the fourth was relatively uniform across treatments. In *Rana* larvae, survival is generally less than 10% under natural conditions (Duellman and Trueb 1986). Thus, despite relatively large clutch sizes, reduced recruitment from the embryonic to the larval phase could greatly impact overall population sustainability. In contrast, hatching success in mink frog eggs appeared to be unaffected by EE2 exposure, although this observation is based on only two clutches. The reduced hatching in green frogs and apparent lack of effect in mink frogs likely reflects a species-specific difference in sensitivity.

The nature of the jelly layer surrounding anuran embryos is critical in determining the sensitivity to perturbations (Laposata and Dunson 2000). The number of layers, complexity, and physical and chemical characteristics of the layers vary among species, and incur varying levels of protection (Carey and Bryant 1995). Mechanisms of embryonic disruption by xenoestrogens have not been reported for amphibians, but given the lack of a role for steroid hormones in early embryonic development, it is not likely that endocrine modulation has occurred (Hayes 2000). Embryo death may have occurred relatively early in the exposure, or embryos may have been viable throughout but were unable to escape the jelly layer at hatching. Recording development stage throughout the exposure would clarify this point, however, in this study eggs were not handled or removed (to avoid stress), and as such could not be observed in detail. Reduced hatching in green frogs may have resulted from a direct effect of EE2, such as a biochemical interference with hatching

enzymes (Laposata and Dunson 2000), although this mechanism has not been demonstrated for xenoestrogens. Alternately, EE2, which binds readily to organic matter, may have permeated the egg coat and induced subtle physical or chemical changes in the nature of the jelly. Given the role of the jelly layer in gas exchange (passage of oxygen and metabolites), resistance to pathogens, thermoregulation, and UV resistance (Beebee 1996), such changes may be detrimental. Again, contaminant effects on the jelly layer specifically have received little attention. In the current study, the EE2 exposure was started after oviposition/fertilization of the eggs, i.e. the egg coat was fully formed. In the wild however, EE2 could also affect eggs and sperm during breeding, and a pulse of contaminants may be taken up as the jelly layer swells with water during egg coat formation (Larsson and Thuren 1987). Finally, numerous biological, chemical, and physical factors may act interactively in determining hatch success (Laposata and Dunson 2000).

Larval Growth and Development

Little is known about the function of steroidal hormones in early larval development of amphibians; thus, the effects of environmental estrogens on these processes remain to be clearly elucidated. A significant difference in tadpole growth rate among lakes was detected for two of the four green frog clutches and one of the two mink frog clutches. In each case, the most rapid growth occurred in Lake 224 samples, and the slowest growth occurred in Lake 114 samples. The same pattern occurred across lakes for development rates. Therefore, there was no effect of EE2 on growth or development rates of either species. Similarly, where no significant difference in growth or development rate was detected among lakes, there was no treatment-related effect on the elevation of the slopes. This indicates that EE2 did not impact the development stage or mass of tadpoles at the outset of the exposure. Thus, based on these results, EE2 did not impact the growth or development of caged tadpoles of either species.

Despite the fact that all tadpoles in a given cage were from the same brood and treated uniformly throughout the experiment, there were striking differences in size and development stage of individuals within and among clutches on any given date. As an example, development stages

and masses in clutch G1 at 61 dph ranged from 30 to 35 and from 0.51 to 0.98 g, respectively. A sample size of ten individuals/clutch/date may therefore be too low to account for the natural range of variability in growth and development. Rosenshield et al. (1999) detected major differences between clutches but within treatment groups of Ranid species in morphometric and behavioural parameters, and proposed that high inter-brood genetic variation is an important factor in population survival. Although it has not been examined, the same may hold true for genetic variation within broods.

Further, the timeframe of this study may be too narrow to detect effects of EE2 on growth and development. On average, tadpoles only progressed through one to five development stages from the first to the last sampling dates used in the analyses, thus a relatively narrow window of the larval phase was examined. Impacts may be more readily assessed in this type of study using other Ranid species, in which tadpoles transform into adults in a single growing season.

B. Wild Population

In 2000, prior to EE2 additions, there was no significant difference among lakes in the size of wild mink frog tadpoles at a given stage. However, EE2-exposed tadpoles tended to be larger than non-exposed tadpoles in reference lakes, particularly at later stages of development (stage 36 and 37). Comparisons between years for each lake reveal that the slope of the mass/stage regression line for Lake 260 was steeper in 2001 than in 2000, whereas reference slopes were shallower in 2001 than in 2000. Thus, larger tadpoles were coincident with EE2 exposure in this study. EE2 in Lake 260 may have directly enhanced growth of mink frog larvae. A single study reports an enhancing effect of 17 β -estradiol (endogenous estrogen) and diethylstilbestrol (synthetic estrogen) on a TH-induced developmental change (i.e. the rate of decrease in length) in *Bufo bufo* tadpoles, although change in mass was not assessed (Frieden and Nalle 1955). Conversely, the vast majority of reports indicate that larval anurans exposed to estrogen exhibit either no effect or an inhibition of both growth and development (Hayes 1997). There is a lack of mechanistic experimental data demonstrating the enhancement of growth and development

through estrogen exposure. It is unlikely that there are any adverse effects associated with increased size of tadpoles in Lake 260. Generally, greater size is beneficial with respect to increased physiological fitness, reproductive output, and predator avoidance (Denver 1997).

A second possibility is that EE2 enhanced growth of wild tadpoles indirectly by inhibiting their development. Estrogen exposure has been shown to antagonize thyroid hormone-induced developmental changes and decrease development rates (Vandorpe and Kuhn 1989; Gray and Janssens 1990; Hayes 1997; Nishimura et al. 1997). It has been suggested that larvae of some anuran species exhibit plasticity in growth and development rates, and that retarded development enhances growth, and vice versa (Denver 1997). If this type of mechanism were to occur in mink frog tadpoles, it is possible that EE2 could prolong development stages (possibly by inhibiting the thyroid axis), resulting in increased foraging, and ultimately larger tadpoles at any given stage. However, developmental plasticity, an adaptive defense mechanism to avoid pond drying or density effects, is likely of significance for species inhabiting small ephemeral waters, and would not be expected in overwintering larvae in permanent waters. Because mink frogs exhibit relatively long and asynchronous larval development, it is necessary to track individuals to determine conclusively whether EE2 affects development rate in wild tadpoles. Further, no impacts of EE2 on the thyroid axis at metamorphic climax were observed (Chapter 3).

Factors other than EE2 exposure could account for among-lake differences in tadpole mass observed in 2001. Temperature is the most important abiotic factor affecting growth and development rates in tadpoles (Duellman and Trueb 1986). Temperature and degree-day profiles for each study lake did not differ markedly between 2000 and 2001 (Appendix E). In 2001, the mean surface temperatures in Lake 260 were comparable to those of the reference lakes throughout the exposure. Degree-days were consistently slightly lower on Lake 260 than on reference lakes, which would more likely be associated with reduced growth (due to lower metabolic rates) than enhanced growth.

C. Laboratory Exposure

Green frog tadpoles exposed to high concentrations of EE2 for several months were more developmentally advanced than those exposed at lower concentrations and controls. Estrogen has previously been shown to interact with the anuran thyroid system and impact development. However, estrogens typically inhibit development or have no effect, and few enhancement effects have been reported (Hayes 1997). For example, Frieden and Naile (1955) demonstrated that 17 β -estradiol (endogenous estrogen) and diethylstilbestrol (synthetic estrogen) enhance a thyroid hormone-induced developmental change in *Bufo bufo* tadpoles (i.e. percent decrease in length). Also, exposure to discharge from swine, dairy and chicken feedlot operations stimulated metamorphosis (i.e. reduced the time to tail resorption) in *Xenopus laevis* tadpoles, and significantly increased in plasma vitellogenin and estradiol levels (D. Janz, University of Saskatchewan, pers. comm.), indicating a potential estrogenic thyroid enhancing effect.

The enhanced development of tadpoles in this study may have been induced by factors other than the experimental treatment. At the end of the exposure, tadpole densities were higher in the 80 and 1000 ng/L exposure jars (13 to 17 per jar) than in the other treatment groups (11 to 15 per jar, Table 2.2, p.47). The increased development rate may have been induced by a crowding effect, i.e. a stress response may have been invoked which increased thyroid status, accelerating development to escape suboptimal conditions.

The highest exposure concentration in this study is far higher than the highest reported values of EE2 levels in waterways. A single study reports median EE2 concentrations in U.S. waters of 73 ng/L (Kolpin et al. 2002), thus the second highest concentration may be considered environmentally relevant. The density of tadpoles in exposure jars in this study are likely higher than those encountered in nature and, as a result, do not likely reflect the natural growth and development rates seen in natural conditions (Cecil and Just 1979; Duellman and Trueb 1986). The biological significance of increased development rate in this study is unknown. Differences

between treatment means are very slight. Overall, environmentally relevant concentrations of EE2 showed no consistent effect on growth and development.

The series of studies presented in this chapter have yielded varied results. The cage exposure demonstrates that there are differences between two closely related Ranid species in the sensitivity of embryos to estrogen exposure. Previous studies indicate that the embryonic phase of amphibian development is less sensitive to various pesticides than the early larval phase (Berrill et al. 1994; Berrill et al. 1995; Berrill et al. 1998; Pauli et al. 1999). Results of the current study indicate the opposite may be true for estrogenic contaminants, though the growth and development results varied. The lab exposure indicates that estrogen significantly increases development at high concentrations, whereas a growth effect was detected in the wild population at ecologically relevant concentrations. This is in accordance with previous studies of amphibian contaminant exposure in which greater sensitivity was detected in field studies than in corresponding laboratory studies (Hatch and Burton 1998; Laposata and Dunson 2000). Overall, these results indicate that xenoestrogens can affect early amphibian development. Treatment-induced effects may or may not be immediately deleterious to the intact organism, but on a broader level they demonstrate that this class of pollutant can modulate important life processes.

Chapter 3: Effects of 17 α -ethynylestradiol on tissue thyroid hormone concentrations in cage-reared green frog (*Rana clamitans*) tadpoles at metamorphic climax

INTRODUCTION

Anuran Thyroid Hormone Dynamics

Most changes that occur in anuran metamorphosis are induced by TH, though other hormones, such as prolactin, adrenal corticoids and sex steroids can also influence development (Hayes 2000; Henry 2000). Circulating endogenous TH levels are low during the early larval phase (premetamorphosis) which is characterized by rapid growth and little morphological change. Plasma TH levels begin to increase during the mid-larval phase (prometamorphosis) which is characterized by reduced growth and more rapid metamorphic changes, particularly in hindlimb development. A surge of TH occurs at the end of the larval phase (metamorphic climax) at which time rapid changes occur. After metamorphosis, TH levels drop and remain low throughout the adult phase (Mondou and Kaltenbach 1979; Shi 2000). The regulation of thyroid hormone levels throughout the larval phase is critical; different tissues are responsive at different times. In experimental manipulations, blocking or inhibiting the surge of TH at metamorphic climax prevents transformation into the adult stage (Shi 2000).

Thyroid function in amphibians is comprised of a dynamic system of regulatory mechanisms involving the central hypothalamus – pituitary – thyroid (H-P-T) axis and the peripheral tissues. Environmental stimuli are relayed to the hypothalamus via the central nervous system, resulting in the secretion of neuropeptides that act on the pituitary. In larvae, corticotropin – releasing hormone is the principal neuropeptide influencing the thyroid axis (Denver and Licht 1989; Denver 1993). The pituitary, stimulated by CRH, secretes thyroid - stimulating hormone into the plasma. Subsequent stimulation of the thyroid gland results in the synthesis and secretion of iodinated thyronines, mainly thyroxine (3,5,3',5'-tetraiodothyronine; T₄), and negligible amounts of 3,5,3'-triiodothyronine (T₃), which circulate in the plasma (Shi 2000). In the bloodstream, the

majority of TH is bound to transthyretin, a thyroid hormone-binding protein which facilitates transport to the peripheral target tissues (Yamauchi et al. 2000).

Monodeiodinase enzymes in various target tissues catalyze the stepwise removal of iodide from iodothyronines. The result may be activation or deactivation of the substrate molecule, depending on the form of deiodinase involved (Eales 1985). T_4 can be converted to T_3 , the physiologically active form of TH, or reverse T_3 (rT_3), an inactive form. In amphibians, the biological activity of T_3 is ten times greater than that of T_4 (Galton 1988a). T_3 and rT_3 can be further metabolized by deiodinase activity, and ultimately the metabolites are excreted or recycled (Galton 1988a). Thus, normal development is affected by the tissue-specific activity of these enzymes as well as ontogenetic changes in their relative amounts.

Circulating TH enter the target tissue cells and interact with cytosolic binding proteins, which may function in intracellular metabolism or transport of TH to the nucleus (Yamauchi and Tata 1997). TH bind reversibly to the nuclear TH receptor (TR). Both T_3 and T_4 bind the TR, but T_3 binding affinity is 5 to 10 times greater than that for T_4 . The TR, part of the superfamily of nuclear hormone receptors, forms heterodimers with retinoic acid receptors (RXRs) (Shi 2000). The hormone-receptor complex acts as a ligand-dependant transcription factor, resulting in the formation of new mRNAs and ultimately proteins that are required for most metamorphic changes (Galton 1988b).

Virtually all tadpole tissues undergo some form of TH-induced change. These tissue-specific changes occur at different points in development, and the sequence of events is consistent among species, e.g. hindlimb development precedes forelimb development, which precedes tail resorption. Given that all tissues are exposed to the same plasma TH concentration at any given time and that TH-induced changes occur at different times, it is clear that regulatory mechanisms are crucial in controlling and coordinating metamorphosis (Becker et al. 1997).

Thyroid Disruption in Anurans

Laboratory manipulations have shown that alteration of circulating TH levels in developing tadpoles is detrimental. Treatment with excess TH prior to metamorphic climax results in rapid, uncoordinated development and eventually death (Galton 1988b). Alternately, reducing TH action through thyroidectomy or chemical thyroid inhibitors/blockers results in non-transforming larvae, or delayed rate of development (Shi 2000). Delayed development in wild tadpoles can result in reduced fitness and increased susceptibility to predation (see Chapter 2; Werner 1986). Thus, agents that inhibit amphibian thyroid function may impact the viability of populations in the wild.

There is mounting evidence that contaminant exposure inhibits larval development in anurans (see preceding chapter for effects on growth and development rates), and there is increasing concern that the viability of wild populations may be compromised as a result. Environmental contaminants such as steroids, retinoids, halogenated biphenyls, and chlorinated hydrocarbons can disrupt thyroid economy in vertebrate species (Burkhardt et al. 1998). Environmentally relevant concentrations of ammonium perchlorate, a fuel component detected in western U.S. surface waters, inhibited the TH-dependant processes of forelimb emergence, tail resorption, and hindlimb development, and reduced tissue T_4 concentrations in *Xenopus laevis* larvae (Goleman et al. 2002). *Xenopus* and *Rana* species, when exposed to PCBs, exhibited increased mortality, prolonged larval period, increased eye and tail deformities, and reduced tissue T_4 concentrations (Gutleb et al. 2000). An inhibited rate of tail resorption occurred in *Xenopus* tadpoles exposed to pond water samples from sites in Minnesota and Vermont, USA at which striking numbers of malformations were observed in indigenous amphibians (Fort et al. 1999a). A mixture of pesticides and other anthropogenic organic compounds were present at the sites. The effects were reversible through the administration of T_4 in some cases but not others, indicating that a variety of endocrine disruptions may have been involved. Further, native anurans at the Minnesota sites showed delayed or incomplete maturation consistent with thyroid dysfunction (Fort et al. 1999b). Because larval TH dynamics are affected by several regulatory mechanisms,

there are various potential contaminant interactions that could occur. In light of the current trends in declining amphibian populations, these impacts warrant further investigation.

Estrogen – Thyroid Interactions

Natural and synthetic sex steroids and contaminants with steroidal activity have been detected in aquatic environments (Allen et al. 1999). These substances can interact with the anuran TH system, and can have inhibitory or otherwise deleterious effects on development. E2 treatment in adult *R. ridibunda* reduced plasma T₃ and T₄, reduced T₃/T₄ ratios, and decreased 5'deiodinase activity in the kidney (Vandorpe and Kuhn 1989). E2 treatment in *X. laevis* tadpoles antagonized the metamorphic effects of T₃ on mass, cranial dimensions, and gut length *in vivo* (Gray and Janssens 1990). Notably these effects were not demonstrated *in vitro*, indicating that the inhibition occurred at a more central site, possibly affecting the release of TH. Jacobs et al. (1988) demonstrated that luteinizing hormone-releasing hormone (LHRH) increases plasma T₄ in amphibians. The presence of excess estradiol could induce a negative feedback on LHRH resulting in decreased TH synthesis or secretion. Thus, disruption can occur in both the peripheral tissues and in the central thyroid axis.

Other inhibitory effects of E2 on tadpole development have been demonstrated, though the modes of action are not understood. Hayes et al. (1993) reports that treatment of *Bufo boreas* tadpoles with E2 reduced snout-to-vent length of metamorphs. In *X. laevis*, E2 treatment inhibited tail resorption, a process known to occur under TH stimulation (Hayes 1997). E2 also decreased the number of mucous glands but had no effect on granular gland development in the skin of *B. boreas* metamorphs (Hayes 1997). This likely indicates thyroid disruption at some level, because development of mucous glands is TH-dependent, whereas development of granular glands is independent of TH. Both E2 and the synthetic estrogen DES can delay cranial development and organogenesis in the digestive and nervous systems of *X. laevis* tadpoles (Nishimura et al. 1997). DES can bind to transthyretin with similar affinity to T₃, thereby increasing the amount of free T₃ in the plasma, and potentially disrupting the free/bound hormone dynamics (Yamauchi et al. 2000).

Given that estrogenic contaminants are present in aquatic environments, and that they can disrupt thyroid status through various endocrine pathways, a greater understanding of the effects of such contaminants on amphibian thyroid status in natural populations is crucial.

Research Needs and Objectives

The thyroid hormone system controls essentially all aspects of larval development in anurans (Hayes 2000). As indicated in the previous chapter, contaminants can inhibit growth and development in anuran larvae, which may be in turn detrimental to populations. There is therefore a need to elucidate interactions of contaminants with the anuran thyroid system. The main objective of the current study was to determine if an environmentally relevant exposure to the xenoestrogen EE2 during larval development of Ranids inhibits thyroid status at metamorphic climax, as indicated by the concentration of in the body tissues. The null hypothesis was that EE2 exposure would not reduce tissue T_3 and T_4 concentrations. Metamorphic climax was targeted because it is a critical phase in development during which rapid morphological changes occur.

METHODS AND MATERIALS

Cage Exposure

A preliminary trial was conducted in 2000 to obtain estimates of body tissue TH concentrations prior to EE2 exposure, and to test the efficacy of the tadpole tissue digestion and hormone extraction methods for use in a radioimmunoassay. Lake 260 was dosed with EE2 in 2001, and was unmanipulated in previous years. In 2000 and 2001, tadpoles at stage 35 to 37 were collected from reference Lake 114 and allocated to cages in near shore areas of Lakes 114, 224 and 260. Mink frog tadpoles, which were readily caught in spring of 2000, were to be used in both years of the study allowing for comparisons between pre-exposure data (2000) and EE2-exposure data (2001) from Lake 260. However, insufficient numbers of mink frog tadpoles were caught in 2001. Therefore, the background study in 2000 was conducted using mink frog tadpoles and the exposure study in 2001 was conducted using green frog tadpoles. Measuring circulating

TH levels was problematic in this study due to difficulty in collecting sufficiently large plasma samples from tadpoles in the field thus body tissue homogenates were used.

Mink frog tadpoles were placed in cages on 20 May 2000 (15 tadpoles per cage, 5 cages per lake). Every second day each cage received 15 g of boiled lettuce and 1.5 g of Tetramin™ flaked fish food. Time of feeding varied throughout the study. Once per week cages were cleared of excess food and scrubbed to eliminate algal fouling. Tadpoles were sampled between July 14 (week 8) and August 7 (week 11). Where possible, two tadpoles per cage from each of stages 43 and 45 were sampled. These stages were chosen to bracket the theoretical peak of plasma TH (plasma T₄ levels begin to surge at stage 43 in green frog tadpoles, a closely related species, Weil 1986).

Green frog tadpoles were placed in cages on 22 June 2001. Due to low catches, one cage was used per lake (15 tadpoles per cage), and tadpoles were sampled at stage 43 only. Feeding and cleaning was conducted as above. Tadpoles were sampled between July 5 (week 2) and July 16 (week 4). Final sample sizes were n=9 for Lakes 260 and 224, and n=13 for Lake 114. The mean concentration of EE2 in Lake 260 during this period was 6.1 ± 0.8 ng/L.

Sample Processing

Tadpoles were removed from the cages and euthanized. Each tadpole was given a unique identification number and dissected on site. To ensure that only extrathyroidal hormone was measured in the assay, the thyroid gland was excluded from each carcass by removing the lower jaw. In addition, the liver and remnant of the tail were also removed for use in separate enzyme analysis. The four tissue components (tail, liver, jaw, and body) were placed in separate Whirlpac™ bags, immediately frozen on dry ice, and the mass of each tissue sample was recorded. Samples were taken between 12:00 and 17:00 to standardize for daily rhythms in TH secretion from the thyroid gland.

Tissue Digestion and Thyroid Hormone Extraction

Thyroid hormones were extracted from the tadpole body tissues based on the methods of Plohman (2000) and Fok (1988). The frozen body tissue (with lower jaw, liver, and tail removed) was diced with a razor blade. The sample was homogenized in 1 ml of phosphate buffer (Na_2HPO_4 , 15.15 g/L, 1mM propylthiouracil, pH 7.5) per gram of tissue using a Sorvall™ Omnimixer. The homogenate was placed in a labeled vial, and any remaining homogenate was rinsed from the blade and chamber into the vial with phosphate buffer. Homogenates were individually weighed and frozen.

To digest the samples, the homogenates were thawed and vortexed, and approximately 3 g of each homogenate was dispensed into a test tube, the exact mass of which was recorded. Each tube received 7.5 mg of pronase and 2.5 mg of collagenase to digest the tissues. Test tubes were covered with Parafilm™ and placed in a 37°C water bath for 18 hrs. Digestion was considered complete when tissues easily broke apart upon stirring with a glass rod. Digested samples were vortexed with 5 ml of ethanolic ammonia (99:1 vol/vol), covered and refrigerated for 24 hr. The tubes were centrifuged at 1420 g for 5 min at 4°C. Supernatants were drawn off and transferred to clean tubes. The pellets were vortexed with another 5 ml aliquot of ethanolic ammonia, re-centrifuged, and the supernatants were again drawn off and combined with the first supernatant. This step was performed a third time, and pellets were then discarded. The supernatants were placed in a 37°C water bath and evaporated to a volume of approximately 10 ml. The supernatants were added to separatory funnels containing 45 ml of chloroform. Fifteen ml of 2N NH_4OH was swirled in the test tubes to rinse out remaining supernatant and added to the separatory funnels. The funnels were shaken for 5 min with a Burrell™ Wrist Action Shaker, and set aside for 1 h to allow the chloroform and aqueous fractions to separate. The chloroform layer was discarded and the upper layer containing the TH was transferred to a test tube. The samples were evaporated to dryness and reconstituted in 1ml of 0.1 N NaOH.

Additional samples were prepared to determine the efficiency of the digestion and extraction process. Radiolabeled T_4 was added to these samples prior to digestion, and the initial radioactivity (counts per minute, cpm) was recorded. The final radioactivity of the reconstituted samples was recorded. The final cpm divided by initial cpm was taken as the extraction efficiency, and all assay results were adjusted using the mean extraction efficiency from these samples.

Radioimmunoassay Overview

The radioimmunoassay (RIA) is a competitive binding technique used to measure the concentration of TH (either T_3 or T_4) in experimental samples. It is based on the competition between radiolabeled TH and unlabeled "cold" TH to bind to an antibody. A series of gel columns is prepared, and each column receives a set amount of labeled TH and antibody. The columns then receive varying known quantities of cold TH (i.e. standards) or an aliquot of an experimental sample (containing an unknown amount of endogenous cold TH). The cold and labeled TH on the columns then compete to bind to the antibody. The more cold hormone is present, the less labeled hormone is bound to the antibody. The amount of antibody-bound labeled hormone from each column is counted by a gamma detector. The values from the standard columns are used to plot a standard curve (TH standard concentration vs. cpm of radioactivity), and the concentration of TH in the experimental samples is interpolated from this curve.

If the cold TH concentration in the experimental samples is greater than that of the highest standard, the original samples must be diluted and the assay repeated such that the results fall within the range of concentrations of the standard curve.

Radioimmunoassay Procedure

The concentration of T_3 and T_4 in the tadpole body tissue extracts was determined by RIA, following the procedures of Brown and Eales (1977). For the 2000 preliminary study, the assays were conducted to determine the approximate range of hormone concentrations in the tissues. For those samples that fell outside the standard curve, further dilutions and assays were not

conducted due to time constraints. The 2001 samples were repeatedly diluted and analyzed such that the results were within the standard curve.

Columns for the RIA were prepared by suspending G-25 Sephadex™ in double distilled (dd) H₂O (60 g/L) and dispensing 5 ml into each column. Gels were allowed to settle, and a plastic disk was placed on the surface. For storage, the columns were filled with 0.1 N NaOH and sealed at both ends. Three columns were used for each standard concentration. Single columns were used for the 2000 samples, whereas triplicate columns were used for the 2001 samples. Assays were conducted as follows:

1. Remove tips from columns and drain the 0.1 N NaOH to waste.
2. To condition the columns, elute with 3 ml barbital buffer (12.15 g sodium barbital/L ddH₂O, pH 8.6) for T₄ assay or phosphate buffer (14.21 g Na₂HPO₄ and 11.17 g Na₂EDTA/L ddH₂O, pH 7.4) for T₃ assay. Drain to waste, and replace tips.
3. Add 100 µl of radiolabeled solution to each column (0.1 N NaOH containing labeled T₄ or T₃, approximately 10,000 cpm per 100 µl)
4. Add 100 µl of radiolabeled solution (as above) to 3 collecting tubes. Place in gamma detector. (The mean cpm of these tubes is taken as the "total counts recoverable" or TCR for the assay)
5. Add 100 µl of TH standards to the appropriate columns. Standards are T₃ or T₄ in 0.1 N NaOH at the following concentrations: 20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, and 0.078 ng/ml.
6. Add 100 µl of the samples to the appropriate columns.
7. Swirl columns to mix.
8. Remove tips from columns to allow the hormone to drain onto the gel.
9. Elute columns with 3.5 ml of barbital buffer for T₄ assay or phosphate buffer for T₃ assay. Drain to waste.
10. Transfer the columns to their respective collecting tubes.

11. Add 0.5 ml barbital buffer containing T_4 antibody or 0.75 ml phosphate buffer containing T_3 antibody to each column
12. Incubate for 16 hours.
13. Elute all columns with 3.5 ml barbital buffer for T_4 assay or phosphate buffer for T_3 assay.
14. Place all collecting tubes in a gamma detector to determine radioactivity (cpm) for each.

For each assay, three extra columns were used to determine the amount of radioiodide contamination (NSB/I-) and non-specific binding (NSB). The columns were given an aliquot of labeled TH, eluted with buffer, and the eluents were collected and their radioactivity was measured. Labeled TH remains on the column, whereas labeled iodide dissociated from the TH passes through the column in the eluent. This value (NSB/I-) was subtracted from the TCR. After the incubation period, these columns were again eluted with buffer (without antibody), and the radioactivity of the eluents was counted. No antibody was present; therefore the unbound labeled TH remains on the column, whereas labeled TH bound to impurities passes through the column in the eluent. This value (NSB) was subtracted from the standard and sample results.

Statistical Analysis

No statistical analysis was performed on data from 2000. For 2001 data, ANOVA was used to test for significant differences among lakes in the mean T_3 and T_4 concentrations in body tissues, and in the mean T_3 to T_4 ratio (SAS statistical software, version 8). The ratio data (total moles of T_3 : total moles of T_4) were log transformed to satisfy the assumptions of ANOVA.

RESULTS

2000 Preliminary Study

Body tissue TH concentrations of stage 43 and 45 mink frog tadpoles are summarized in Tables 3.1 (p.71) and 3.2 (p.72), respectively. For T_3 and T_4 , the number of values that fell within the bounds of the standard curve were 18 and 11 out of 24, respectively for stage 43 samples, and 23 and 5 out of 30, respectively, for stage 45 samples. The results show considerable variation in

T_3 and T_4 content at both developmental stages. Of the values that fell within the bounds of the assay standard curve, T_3 concentrations ranged from 1.1 to 61.4 ng/g in stage 43 tadpoles, and from 0.8 to 86.9 ng/g in stage 45 tadpoles. T_4 values that fell within the bounds of the standard curve range from 1.6 to 35.3 ng/g for stage 43 and from 5.6 to 100.7 ng/g for stage 45. The remainder of the values for both T_3 and T_4 were too high to quantify. The majority of the T_3 values fell within the bounds of the standard curve, whereas the majority of the T_4 values were too high to detect, which indicates, in all likelihood, that at these stages endogenous T_4 levels are generally higher than those of T_3 . Further, within a given lake, samples from earlier dates typically had lower T_4 concentrations (i.e. those that fell within the standards), whereas hormone concentrations in later samples were consistently too high to measure. Thus, it is possible that not only the developmental stage, but also the time required to reach that developmental stage may affect tissue thyroid hormone concentrations in this species. Based on this observation, tadpoles were sampled over a narrower window of time in 2001 (12 days) than in 2000 (25 days).

Table 3.1 Concentrations of triiodothyronine (T_3) and thyroxine (T_4) in body tissues (excluding lower jaw, liver, and tail) of cage-reared mink frog tadpoles at stage 43. Tadpoles were reared in cages on unmanipulated study lakes for 8 to 11 weeks in 2000. -- indicates that the hormone concentration was too high to accurately measure with the associated standard curve.

Sample #	Lake	Collection Date (2000)	Hormone Concentration (ng/g)	
			T_3	T_4
4	114	14 July	48.4	20.7
5	114	14 July	--	9.4
6	114	14 July	21.6	4.4
1	114	14 July	--	27.2
2	114	14 July	--	31.3
3	114	14 July	--	35.3
24	114	21 July	36.1	--
35	114	24 July	49.0	--
36	114	24 July	17.1	--
39	114	28 July	22.0	--
25	224	21 July	37.9	--
28	224	21 July	25.1	--
37	224	28 July	32.4	--
55	224	7 August	53.2	--
56	224	7 August	22.6	--
8	260	14 July	2.0	2.3
9	260	14 July	--	15.0
10	260	16 July	--	21.2
11	260	16 July	1.8	1.6
13	260	16 July	1.1	5.4
18	260	18 July	61.4	--
19	260	18 July	46.1	--
20	260	21 July	31.8	--
33	260	24 July	23.5	--

Table 3.2 Concentrations of triiodothyronine (T₃) and thyroxine (T₄) in body tissues (excluding lower jaw, liver, and tail) of cage-reared mink frog tadpoles at stage 45. Tadpoles were reared in cages on unmanipulated study lakes for 8 to 11 weeks in 2000. -- indicates that the hormone concentration was too high to accurately measure with the associated standard curve.

Sample #	Lake	Collection Date (2000)	Hormone Concentration (ng/g)	
			T ₃	T ₄
7	114	14 July	--	10.5
14	114	16 July	2.3	2.2
15	114	16 July	1.3	--
16	114	16 July	10.9	47.8
17	114	18 July	51.1	--
21	114	21 July	11.8	100.7
23	114	21 July	36.8	--
34	114	24 July	--	--
42	114	1 August	--	--
50	114	3 August	27.6	--
26	224	21 July	25.2	--
38	224	28 July	45.7	--
41	224	1 August	47.9	--
40	224	1 August	--	--
46	224	3 August	38.0	--
47	224	3 August	31.4	--
48	224	3 August	34.2	--
49	224	3 August	20.9	--
44	224	3 August	86.9	--
45	224	3 August	--	--
12	260	16 July	0.8	5.6
29	260	24 July	33.3	--
30	260	24 July	27.8	--
31	260	24 July	34.6	--
32	260	24 July	--	--
43	260	1 August	38.4	--
51	260	3 August	52.9	--
52	260	3 August	--	--
53	260	3 August	29.0	--
54	260	3 August	42.1	--

2001 Exposure Study

There was no significant difference in the mean tissue concentrations of T_3 ($p=0.35$) or T_4 ($p=0.73$) in metamorphic green frog (*R. clamitans*) tadpoles from EE2-treated and reference lakes. The mean T_3 concentration in tadpoles from Lakes 114 and 224 were 1391 ± 492.1 and 1763 ± 949.9 ng/g, respectively, whereas that of Lake 260 was 1340 ± 565.9 ng/g (Figure 3.1). The mean T_4 concentration in tadpoles from Lakes 114 and 224 were 176 ± 66.3 ng/g and 197.8 ± 63.6 , respectively, whereas that of Lake 260 was 206 ± 141.8 ng/g (Figure 3.1). The mean T_3 to T_4 ratio (total pmoles) was slightly lower on Lake 260 (8.8 ± 2.4) than on Lake 114 (9.7 ± 1.3) and Lake 224 (10.6 ± 3.8), but the difference was not significant ($p=0.26$) (Figure 3.2). Exposure times in the cages ranged from 14 to 25 days. The coefficient of variation in T_3 and T_4 assays (using nine replicates of a single sample) was 9.7% and 7.0%, respectively.

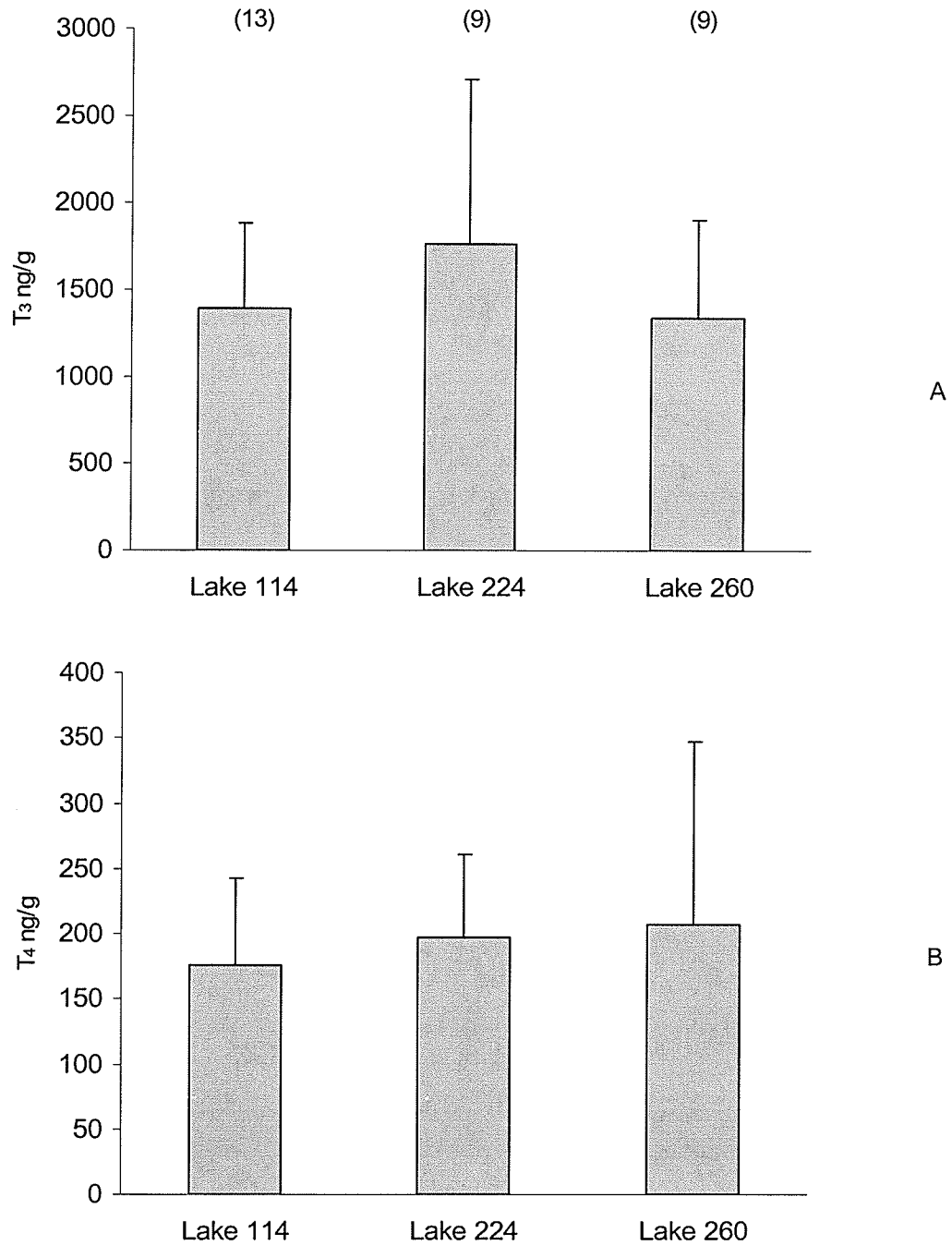


Figure 3.1 Mean concentration (+SD) of (A) triiodothyronine (T₃) and (B) thyroxine (T₄) in body tissues (excluding lower jaw, liver, and tail) of cage-reared green frog tadpoles at stage 43. Tadpoles were reared in cages on Lake 260 and reference lakes for 14 to 25 days in 2001. Mean EE2 concentration was 6.1 ± 0.8 ng/L. Sample sizes are above bars. No significant differences were observed across lakes for T₃ ($p=0.35$) or T₄ ($p=0.73$).

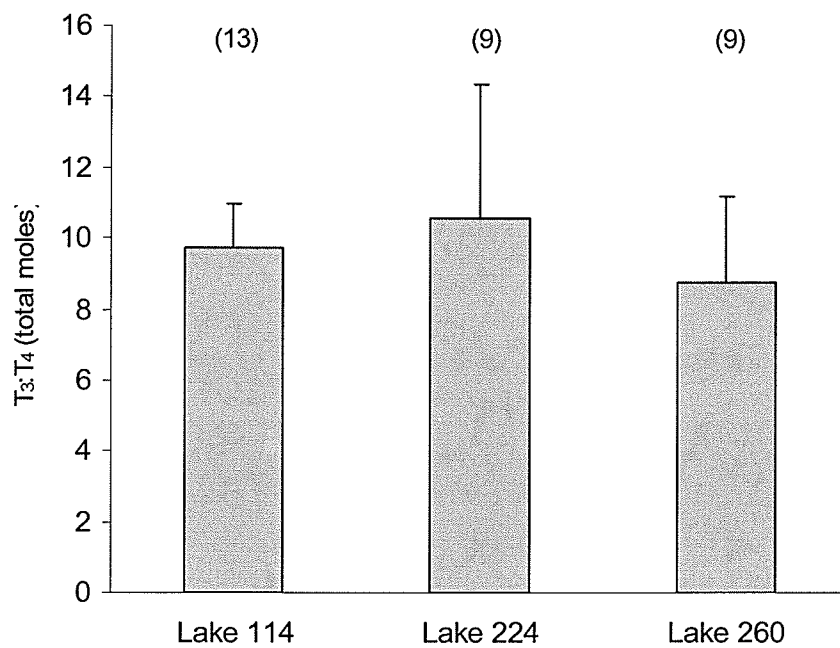


Figure 3.2 Mean ratio (+SD) of T₃ to T₄ (total moles of hormone) in body tissues of cage-reared green frog tadpoles at stage 43. Lower jaw, liver, and tail were excluded from the tissue samples. Tadpoles were reared in cages on Lake 260 and reference lakes for 14 to 25 days in 2001. Mean EE2 concentration was 6.1 ± 0.8 ng/L. Values are adjusted for extraction efficiency. Sample sizes are above bars. No significant differences were observed across lakes ($p=0.26$).

DISCUSSION

Tissue concentrations of thyroid hormones in spontaneously metamorphosing mink and green frog tadpoles from reference habitats have not been previously reported, though plasma thyroxine concentrations have been assessed in wild-caught *R. clamitans* tadpoles (Weil 1986). There was pronounced variation in TH content of individuals within a given stage in the current study, as was previously reported by Weil (1986). The concentrations detected in the current study are higher than those previously reported for other species. Whole body thyroid hormone concentrations have been reported in the low ng/g range for *Xenopus laevis* tadpoles and metamorphs (0.21 to 1.95 ng/g for T₄, 0.79 ng/g for T₃) (Gancedo et al. 1997; Goleman et al. 2002). Similarly, peak whole-body thyroid hormone concentrations during ontogenesis in the toad *Bufo marinus* were 8.4 and 5.3 ng/g for T₄ and T₃ (Weber et al. 1994). In these studies, tissue homogenates were rinsed with alcohol to extract TH. In the current study, the homogenates were enzymatically digested prior to TH extraction. TH may have been sequestered to intracellular storage sites in tissues (such as microsomes), and liberated by the digestion process, thus accounting for the relatively high hormone concentrations in these samples. In addition, the presence of ectopic thyroid (thyroid follicles found in areas other than the thyroid gland) could contribute to high tissue TH concentrations, particularly T₄, the main storage product of the follicles. Ectopic thyroid tissue is present in numerous fish taxa, but has not been reported in amphibians (Woodhead and Bryant 1982).

The tadpole tissue samples were prepared with the lower jaw, liver, and tail removed. The thyroid hormone content measured in the samples is thus the sum of the plasma and extrathyroidal tissue pools of free, protein-bound, and receptor-bound hormone, exclusive of the tail and liver. Therefore, several different potential pools of hormone were measured at once in these assays, and inputs and outputs of these pools are affected by various mechanisms. Overall, numerous regulatory points in the thyroid axis factors contributed to the total amount of thyroid hormones detected in the assays, and these are discussed below.

The total T_4 content in the tissue samples is a function of the inputs and outputs at the time of sampling. T_4 input is a function of synthesis and secretion rates from the thyroid, which are in turn influenced by iodide availability and thyroidal uptake, and enzymatic processes in hormone biosynthesis and secretion (Hanaoka et al. 1973). Perchlorate has been shown to inhibit iodide uptake, reducing the amount available for TH hormone synthesis, potentially contributing to reduced body T_4 concentrations in tadpoles, but an analogous effect has not been demonstrated for estrogens (Goleman et al. 2002). T_4 output from the tissues examined is dependent upon metabolism rates in the peripheral tissues, i.e. deiodination to T_3 via 5'D activity, and to rT_3 via 5D activity (Galton 1988b). Estradiol treatment has been shown to reduce kidney 5'-deiodinase activity and plasma $T_3:T_4$ ratio in adult Ranids, and it is feasible that EE2 could induce the same effect in tadpoles (Vandorpe and Kuhn 1989). In the current study, the mean T_4 concentration is slightly higher in the EE2-treated samples than reference samples. If this were a treatment-induced accumulation attributable to reduced 5'D activity, there would likely be a concomitant decrease in T_3 concentration and the $T_3:T_4$ ratio. The mean $T_3:T_4$ ratio is lower in Lake 260 samples than in reference samples, but the difference is slight and more likely represents natural variation in the data than a treatment effect.

Similarly, the T_3 content in the samples is a function of the inputs and outputs at the time of sampling. T_3 is produced through deiodination of the prohormone T_4 , though some can be secreted directly from the thyroid, thus factors affecting the T_4 levels in tissue samples will also influence T_3 inputs (Shi 2000). T_3 removal from the tissues is dependent upon the rate of T_3 conversion to T_2 (diiodothyronine). Deiodinase activity is tissue specific in anuran tadpoles (Galton and Hiebert 1987). Disruption of deiodinase activity or other peripheral effects would therefore likely occur in a tissue-specific manner, and may not be detectable in a whole body tissue sample. Alternately, it is possible that if disruption occurred at more than one point, the cumulative effect could be either complementary or compensatory. There are numerous regulatory points that could have direct or indirect effects on the tissue TH concentrations in the

samples used in this study, and a detailed examination of responses in specific peripheral tissues would be beneficial.

Overall, the results indicate that short-term exposure to a single potent estrogen mimic at an environmentally relevant dose does not significantly alter the concentration of T₃, T₄, or the T₃:T₄ ratio in body tissues of green frogs at metamorphic climax. Based on these results, the tissue responses that typically occur at metamorphic climax are likely able to occur uninhibited at this level of EE2 exposure. However, the length of EE2 exposure was relatively short (15 to 22 days), and it occurred exclusively in the late larval stages. Prolonged exposure throughout the embryonic and larval stages (as would occur in contaminated environments) may yield some form of thyroid disruption. In addition, species-specific differences in sensitivity to contaminants have been previously demonstrated (Berrill et al. 1994; Berrill et al. 1995; Henry 2000), thus other native anurans may be more susceptible to environmental thyroid disruption than those in the current study. Finally, wild anurans are exposed to multiple estrogenic compounds, and it is possible that at low concentrations these may act additively or synergistically to produce a more pronounced estrogenic effect than that of a single contaminant, though this notion remains controversial (Palmer et al. 1998; Bergeron et al. 1999; Kortenkamp and Altenburger 1999).

The current study represents the first known research effort to address the effects of a single estrogenic contaminant *in situ* on the anuran thyroid axis. Further, it is one of few studies in which endocrine endpoints were assessed in an *in situ* exposure. Further research is required to demonstrate mechanisms of thyroid disruption in anurans, and ultimately the impact of thyroid disruption on population viability in wild anurans.

Chapter 4: Effects of 17 α -ethynylestradiol on gonad development in green frog (*Rana clamitans*) and mink frog (*R. septentrionalis*) tadpoles

INTRODUCTION

Contaminants can induce reproductive defects in aquatic wildlife which may ultimately compromise viability of populations (Mihaich et al. 1999). In amphibians, the process of sex differentiation, in which the primordial gonad develops into an ovary or a testis, can be disrupted by chemical exposure (Hayes 1998; Kloas and Lutz 1999; Ohtani et al. 2000; Ohtani et al. 2001). Sex hormones play a crucial role in gonad differentiation and other aspects of reproductive development (Wallace et al. 1999); therefore, hormonally active environmental contaminants are of grave concern. A histological analysis was conducted to determine effects of EE2 exposure on gonad development in wild and cage-reared tadpoles from the ELA lake exposure study.

Amphibian Gonad Development

The majority of amphibian species are gonochoristic, i.e. male and female gonads are found in separate individuals, and all species studied to date have a genetic mechanism of sex determination (Hayes 1999; Wallace et al. 1999). Despite being genetically predetermined, the sexes are morphologically indistinct in early development. The primordial or "bipotential" gonad consists of primordial germ cells, which are destined to become gametes, and somatic cells. At some point in development primary sex differentiation occurs. This is the actual tissue development process involved in the formation of testes or ovaries from the bipotential gonad; the timing and pattern of which vary markedly among species (Hayes 1998).

Three general patterns of gonad differentiation have been identified in all amphibians studied to date. In the "differentiated" species, individuals exhibit bipotential gonads up to a particular developmental stage, at which point the gonads differentiate into either testes or ovaries. In "semidifferentiated" species, the bipotential gonad undergoes ovarian development in all individuals despite genotypic sex, and in genetic males the ovarian tissue eventually regresses

and forms testes. Thus, part of the population exhibits a natural transitory period. In “undifferentiated” species, the bipotential gonad in females develops into ovaries, and in males it remains undifferentiated until later developmental stages, thus male differentiation lags behind female differentiation (Saidapur et al. 2001). Closely related species do not necessarily exhibit the same patterns, and different patterns have been documented for a single species (Gramapurohit et al. 2000).

The factors controlling the induction, timing, and pattern of amphibian gonad differentiation remain unclear; however, sex steroids likely direct all aspects of reproductive development (Hayes 1998). The bipotential gonad in most amphibians consists of an outer cortex and an inner medulla. Proliferation of the cortex with medullary regression results in ovary formation, and the opposite process (cortex regression and medulla proliferation) results in testis formation (Hayes 1999). Witschi (1934) proposed that chemical cues regulate this process, i.e. in females a substance is secreted that inhibits medulla development and promotes cortex development, whereas in males a substance is secreted that has the opposite effect. Although no such endogenous chemicals have been clearly identified, sex steroids have been implicated (Hayes 1998). However, it remains unclear whether sex hormones are the cause or an early consequence of gonad differentiation (Petrini and Zaccanti 1998).

Hayes (1998) compiled results from studies of the effects of steroid treatment on primary sex differentiation in amphibians and made several observations. Most importantly, there is ample evidence that steroid exposure can cause intersex and sex reversal in various amphibian species, though methodologies varied widely among studies. He noted that chemically unrelated steroids may have similar results, and that a single steroid may have both masculinizing and feminizing effects in different species and at different doses. There were no consistent patterns of response in related species. The mechanism of anuran sexual differentiation and the role of steroids therein remain to be clearly demonstrated (Ankley et al. 1998).

Contaminant Effects on Amphibian Reproductive Development

Chemical disruption of gonad differentiation can occur to varying degrees in amphibians. Intersex gonads consisting of testicular and ovarian tissue interspersed in the same gonad have been reported (Sower et al. 2000), as well as lateral hermaphrodites (one ovary and one testis), and multiple gonads (Hayes et al. 2002). Complete functional development of the inappropriate gonad type (sex reversal) and skewed sex ratios in wild populations have been documented (Hayes 1998; Reeder et al. 1998). Spontaneous functional sex reversal has been reported in unmanipulated laboratory specimens, but the prevalence of this phenomenon in nature is not known (Grafe and Linsenmair 1989).

Several studies have demonstrated that hormonally active xenobiotics can disrupt amphibian gonad development. Kloas et al. (1999) reported that exposure of *Xenopus laevis* tadpoles to nonylphenol, octylphenol, bisphenol A, and butylhydroxyanisol, all of which are estrogenic contaminants, resulted in a higher percentage of female phenotypes relative to controls in a laboratory study. Reeder et al. (1998) examined wild Cricket frogs (*Acris crepitans*) from polluted and unpolluted sites, and detected a relationship between sex ratio reversal and PCB/PCDF contamination (contaminated sites favoured males, natural populations are skewed toward females). In the same study, increased occurrence of intersex was detected in the presence of atrazine, the most heavily used herbicide in North America. *Xenopus laevis* tadpoles exposed to ecologically relevant concentrations of atrazine during gonad differentiation exhibit hermaphroditism and multiple gonads, as well as reduced testicular volume, numbers of spermatogonial cell nests and nurse cells (Hayes et al. 2002, Tavera-Mendoza et al. 2002). A small percentage of genotypic male *Rana rugosa* tadpoles exposed to styrene monomer and trimer, styrene compounds exuded from packaging materials into foods, exhibited ovarian tissue development (Ohtani et al. 2001). Dibutyl phthalate, a phthalate ester commonly used as a plasticizer in polyvinyl chloride resins, also induced an estrogenic effect in genetic male tadpoles of *R. rugosa*, resulting in intersex and full sex reversal (Ohtani et al. 2000).

Environmental chemicals also impact aspects of reproductive development other than gonad differentiation. Reduced gonad size in response to contaminants can be an indicator of potential reproductive impairment and population-level effects (Ankley et al. 1998). Reduced testicular volume due to atrazine exposure has been observed in *Xenopus laevis* tadpoles (Tavera-Mendoza et al. 2002). Similarly, oocyte development can be inhibited by estrogenic substances (Pickford and Morris 1999), and could be used in screening estrogenicity of compounds (Ankley et al. 1998). Steroid treatment has been shown to disrupt vocal sac development and dorsal coloration in a sexually dichromatic species coincident with gonad differentiation (Hayes and Menezes 1999), thus hormonally active contaminants may be disruptive as well. DDT and DDE had antiestrogenic and estrogenic effects, respectively, on gonaduct development of larval salamanders (*Ambystoma tigrinum*) (Clark et al. 1998).

Research Needs and Objectives

Environmental chemicals can disrupt sexual differentiation in amphibians (Kloas et al. 1999). Ultimately, the disruption of reproductive development may compromise the survival of populations (McNabb et al. 1999; Hayes 2000). Reproductive endpoints such as gonadosomatic index (the ratio of gonad weight to body weight), number of gametes, stages of eggs in ovaries, sex ratios and sex reversal in tadpoles, and germinal vesicle breakdown could be used in the detection of estrogenic or androgenic contaminants (Ankley et al. 1999; Kloas et al. 1999)

As part of the ELA research effort, two histological studies were undertaken to assess the impacts of ecologically relevant EE2 exposure on gonad development in tadpoles of two native anuran species. Little information has been published on the pattern of gonad development and the impacts of contaminants in these two species. In both studies tadpoles were collected from the EE2-treated lake (Lake 260) and from unmanipulated reference lakes, and the histological appearance of the gonads was contrasted between sites. In the first study, mink frog (*R. septentrionalis*) tadpoles were captured at Lake 260 and two reference lakes in 2000 and 2001 to assess the effects of EE2 exposure on sex ratios and incidence of intersex in the wild population.

In the second study, green frog (*R. clamitans melanota*) and mink frog (*R. septentrionalis*) eggs collected from a reference site in 2001 were held in cages on Lake 260 and two reference lakes. The hatchlings were maintained until the mid-larval stage to assess the effect of continual early life stage EE2 exposure on sex ratios and incidence of intersex. In addition, gonad size and oocyte development stages were examined in green frog tadpoles. The null hypothesis of the studies was that EE2 exposure would not disrupt aspects of gonad development in tadpoles.

MATERIALS AND METHODS

Sample Collection

Wild Population Study: Wild mink frog tadpoles were collected from Lake 260 and from reference Lakes 442 and 114 on various dates in the spring and summer 2000 and 2001. Tadpoles were captured using modified Beamish fish trap nets, which were set in near shore areas and checked the following day. In 2000, samples were caught on sampling dates from May to August. In 2001, reference lake samples were caught in early July, and EE2-exposed samples were caught in June and October in the third, fifth, and twentieth weeks of the whole-lake experiment. Sample sizes were as follows in 2000 and 2001, respectively: Lake 114 n = 62 and 10, Lake 442 n = 32 and 30, Lake 260 n = 77 and 64. Tadpoles ranged from stage 28 (premetamorphosis, hind limb growth) to 46 (completion of metamorphosis).

Lakes 442 and 114 were used as reference lakes because they had consistent catches of mink frog tadpoles throughout both sampling seasons. Samples from Lake 260 in 2000 serve as references because the lake was unmanipulated prior to the start of the estrogen additions in 2001. Green frog tadpoles are also indigenous to these lakes, but were captured in insufficient numbers to support histological study.

Cage Study: In 2001, mink and green frog tadpoles were reared in cages as outlined in Chapter 2. Briefly, four green frog and two mink frog egg clutches were collected from a reference site, and subsamples of each clutch were raised in individual cages on Lakes 260, 114, and 224. After

hatching, a feeding and cleaning protocol was initiated, and the tadpoles were maintained in the cages up to 119 and 109 dph (greens and minks, respectively). At the end of the exposure, the tadpoles were sacrificed for histological preparation. Mean EE2 concentration in Lake 260 during the study period was 5.8 ± 1.4 ng/L. The mean developmental stages (\pm SD) of the samples were as follows: green frogs Lake 114: 34 ± 1.9 , Lake 224: 33 ± 1.8 , Lake 260: 34 ± 2.1 , mink frogs Lake 114: 29 ± 1.3 , Lake 224: 29 ± 1.4 , Lake 260: 31 ± 2.0 .

Sample Preparation

Tadpoles were euthanized, then each individual was given a unique identification number, and mass, snout-to-vent length, total length, and developmental stage were recorded. A mid-ventral incision was made through the body wall of each individual to expose the internal organs. Tadpoles were then placed in Bouin's tissue fixative (10-12 individuals per liter) for 3 to 4 days and then transferred to 70% ethanol for storage. The kidney – gonad – fat body complex was dissected out of the body cavity, and most of the fat body was removed. A transverse cut was made through the kidney complex posterior to the gonad, and the posterior portion was discarded. The remaining kidney-gonad tissue sample was placed in a labeled histocassette, put through a butanol series (Fisher MVP I Tissue Processor), and embedded in paraffin oriented for transverse sectioning. Serial sections ($7 \mu\text{m}$) were made through the kidney-gonad complex, working anteriorly. The first set of 8-10 consecutive sections was taken at the posterior tip of the gonad and mounted on a glass microscope slide, and then three more sets were taken at $350 \mu\text{m}$ intervals. Slides were stained with Harris' haematoxylin and eosin (Edwards 1967).

Microscopy

In both studies all gonad sections were examined under a Leitz compound microscope, and each sample was classified as male, female, or intersex. An intersex gonad was considered one in which both male and female tissues are present in the gonad.

Gonad Transverse Section Area Measurements

It was not possible to weigh the gonads in this study without destroying the sample for histology. Thus, to compare gonad sizes among lakes, transverse-sectional area of gonads was examined. Caged green frog males and females at stage 35 were used, because they were most plentiful. Mink frogs were not examined because there were not enough samples at any given stage to allow comparisons between lakes for either the wild or caged samples. Left and right testis/ovary area was measured on a single section from the second set of sections from each gonad, and the two values were summed. Area measurements were performed using a Zeiss Photomicroscope III, a Sony™ digital video camera, and Northern Eclipse™ version 6.0 software. ANOVA was used to test for significant differences in mean male or female gonad transverse section area among lakes (SAS statistical software, version 8).

Oocyte Measurements and Development Stage Classification

The size and stage of oocytes were examined in the female green frog tadpoles at stage 35 from the caging study. Only those oocytes in which the complete nuclear membrane of the cell was distinctly visible were examined. The first measurement was taken across the cell in the longest orientation, a second measurement was taken perpendicular to the first at the widest point. The mean of these two values was taken as the oocyte diameter. The oocyte was then assigned a stage according to the following criteria (modified from Takashima and Hibiya 1995). Chromatin-nucleolar stage oocytes have clear (non-staining) cytoplasm and a densely stained nucleus that occupies 80% or more of the total cell area. Early perinucleolar stage oocytes possess darkly stained cytoplasm, a nucleus with a granular appearance containing several scattered distinct nucleoli. Late perinucleolar stage oocytes are the same as the previous stage with half or more of the nucleoli in contact with the nuclear membrane. Measurements were performed using a compound light microscope with a desktop image projection unit, a digitizer and Sigmascan™ Image software (version 1). The proportion of oocytes at each stage was determined for each individual, and ANOVA was used to test for significance differences among lakes, using an arcsine transformation of the proportions (SAS statistical software, version 8). The mean diameter

of oocytes at each stage was determined for each individual, and ANOVA was used to test for significant differences among lakes ($\alpha=0.05$).

RESULTS

The pattern of gonad differentiation and sex ratios of mink and green frog tadpoles has not been previously reported. In the current study, mink frog tadpoles ranging from stage 26 to 46 and green frog tadpoles ranging from stage 25 to 37 were sampled from cages and from the wild at pristine reference sites. All specimens had clearly differentiated gonads, and there were no signs of natural intersex or transitory periods at any stage. It therefore appears that in both species, primary sex differentiation naturally occurs early in development (i.e. premetamorphosis, Figures 4.1 and 4.2, p.88, 89). Testicular tissue appears to develop directly from the indifferent tissue without the appearance of ovarian-like tissue, thus mink frogs exhibit the differentiated pattern of gonad development, as has been reported for other Ranid species (Iwasawa and Kobayashi 1976).

Sex Ratios

Sex ratios of wild mink frog tadpoles are summarized in Figure 4.3 (p.90, note: samples from various capture dates are pooled for each lake). In Lake 260, 55 and 48% of wild-caught tadpoles were female in 2000 (n=77) and 2001 (n=64), respectively. In the reference lakes, the sex ratios of wild-caught samples varied among lakes and among years. Lake 442 samples were skewed toward females in both years (59% and 67% in 2000 and 2001, n=32 and 30), whereas in Lake 114 50% and 40% of tadpoles were females in 2000 and 2001 (n=62 and 10).

Sex ratios of cage-reared tadpoles are summarized in Figure 4.4 (p.91 note: samples from the various clutches are pooled for each species). In the green frog tadpoles, the proportion of females was slightly lower in Lake 260 cages (47%) than in the reference lake cages (54% and 51% for Lake 114 and 224). In the mink frog tadpoles, 56% were female in Lake 260 cages, and 51 and 57% were female in Lakes 114 and 224 cages.

Incidence of Intersex

In the wild population study, there were no intersex mink frog tadpoles found in the reference lakes or in Lake 260 in 2000. However, some gonadal abnormalities were detected in EE2-exposed samples in 2001. Of the 64 EE2-exposed mink frog tadpoles examined from Lake 260 in 2001, one was intersex, and a second (classified as a male) showed abnormal gonad development. The intersex individual (stage 33) showed a distinct region containing only oocytes at the posterior end of one testis, while the remainder of the gonad had the appearance of normal male testis (Figure 4.5, p.93). In the second individual, there was no evidence of intersex; however, one testis had the typical male appearance, and the other was almost completely devoid of germ cells. Both individuals were caught on week 20 of the whole-lake exposure. It is also noteworthy that a green frog tadpole (an incidental catch from the same set of samples) was intersex; however, reference samples from wild populations were unavailable.

In the cage study, there were no intersex green frog tadpoles in the reference or EE2-treated groups. There were no intersex mink frog tadpoles in the reference lakes cages (total n=85); however, one of 18 EE2-exposed mink frog tadpoles was intersex. This individual had typical testicular structure throughout most of the gonad, except one testis contained a distinct region of oocytes at the posterior end (Figure 4.6, p.94).

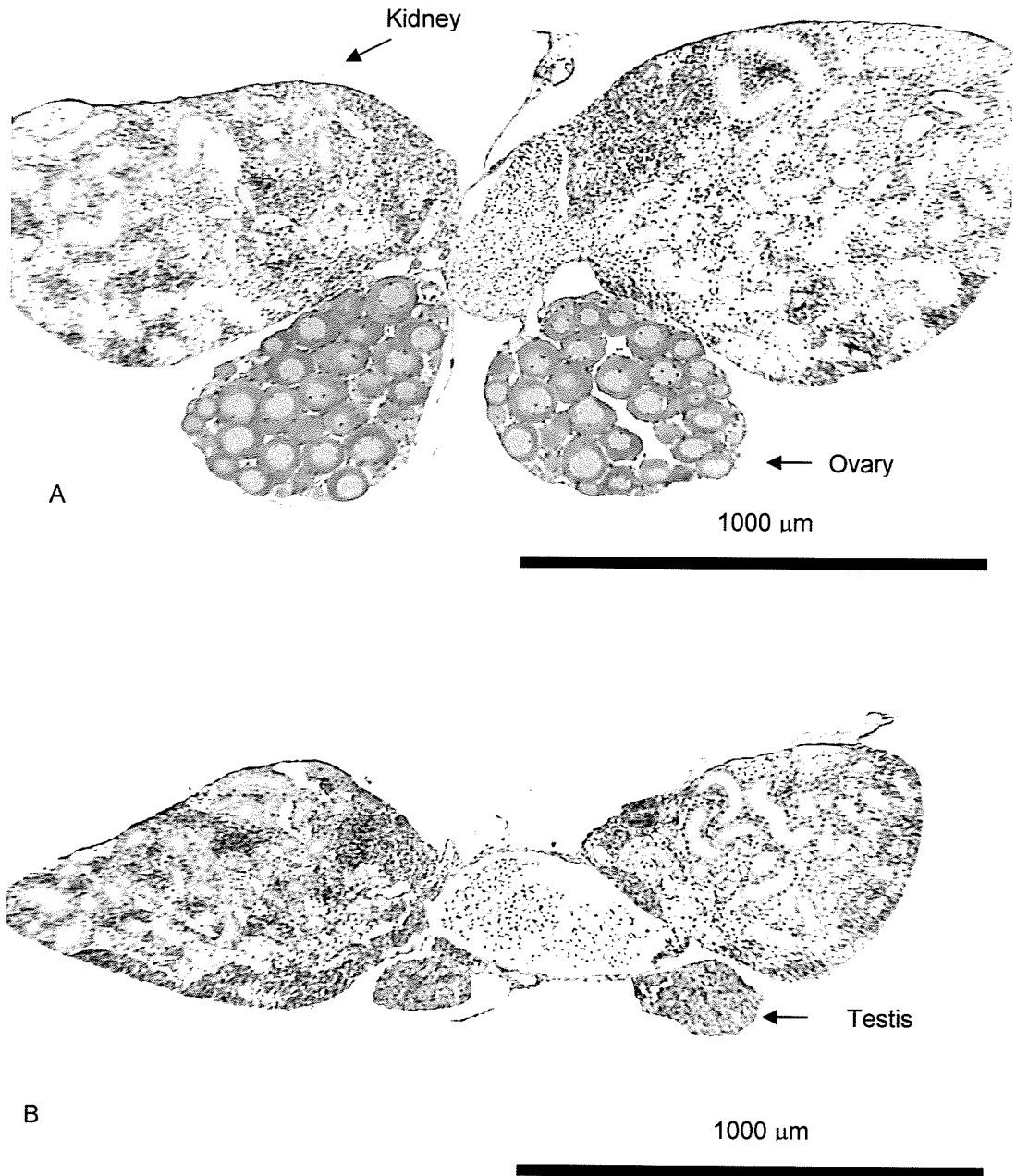


Figure 4.1 Photomicrographs of transverse sections through the kidney-gonad complex of mink frog tadpoles collected at reference sites, showing typical (A) ovarian and (B) testicular early development.

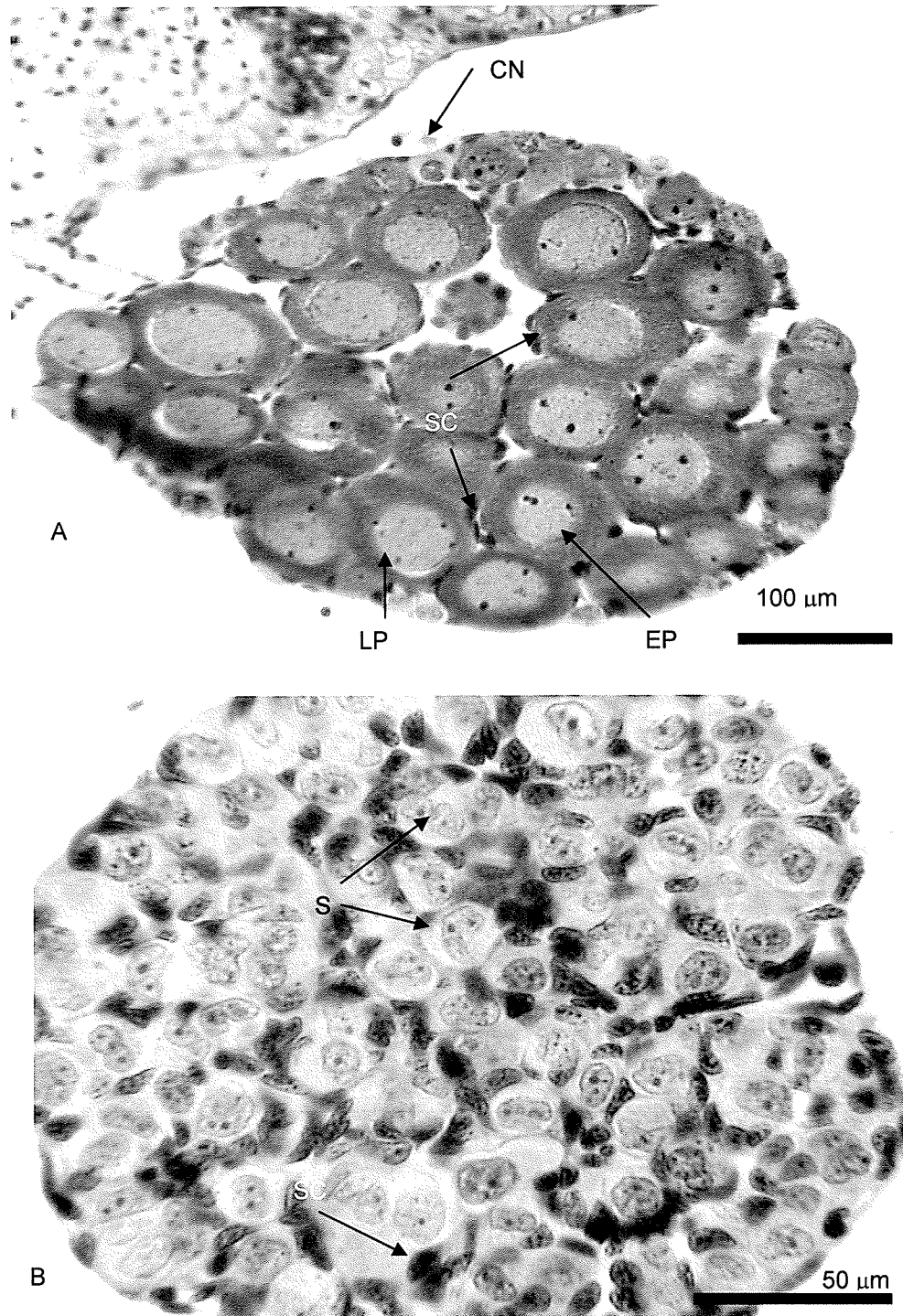


Figure 4.2 Photomicrographs of transverse sections through the gonad of mink frog tadpoles collected at reference sites. (A) Ovary. Primary growth phase oocytes (CN= chromatin-nucleolar, EP= early perinucleolar, LP= late perinucleolar) interspersed with dark-stained somatic cells (SC). (B) Testis. Proliferating spermatogonia interspersed with somatic cells (S= polymorphonuclear spermatogonia, SC= somatic cells).

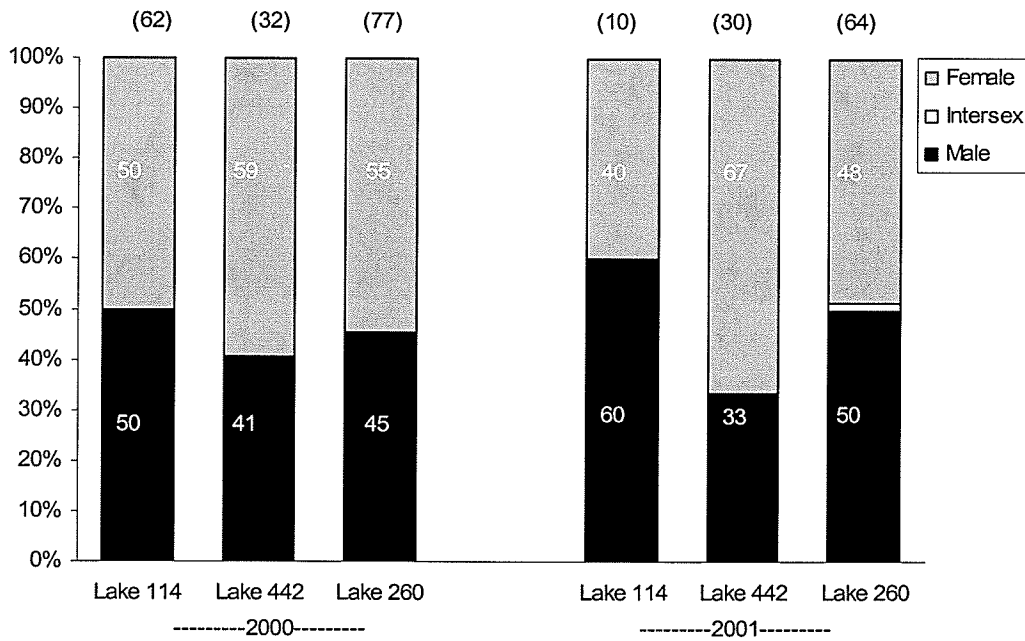


Figure 4.3 Gonadal sex of wild mink frog tadpoles from Lake 260 and from reference lakes in 2000 and 2001. The season mean concentration of EE2 in Lake 260 in 2001 was 6.1 ± 1.5 ng/L. Samples ranged from stage 28 to 46, and were caught on various dates. Sample sizes are above bars.

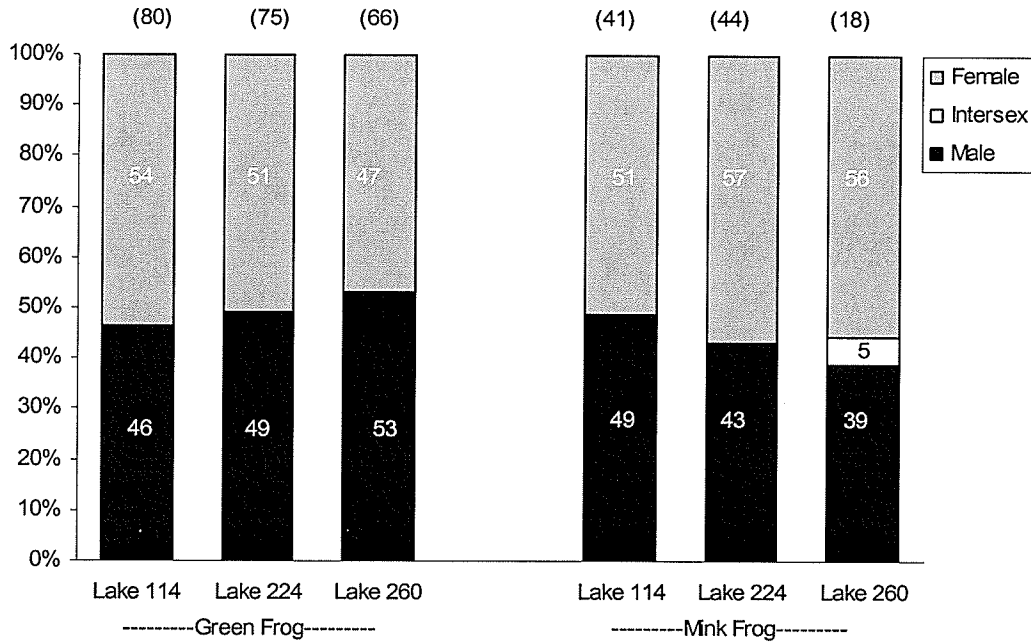


Figure 4.4 Gonadal sex of cage-reared green and mink frog tadpoles from Lake 260 and from reference Lakes in 2001. Exposure was from 4 days pre-hatch to 119 and 109 dph (greens and minks, respectively). Mean EE2 concentration in Lake 260 was 5.8 ± 1.4 ng/L. Sample sizes are above bars.

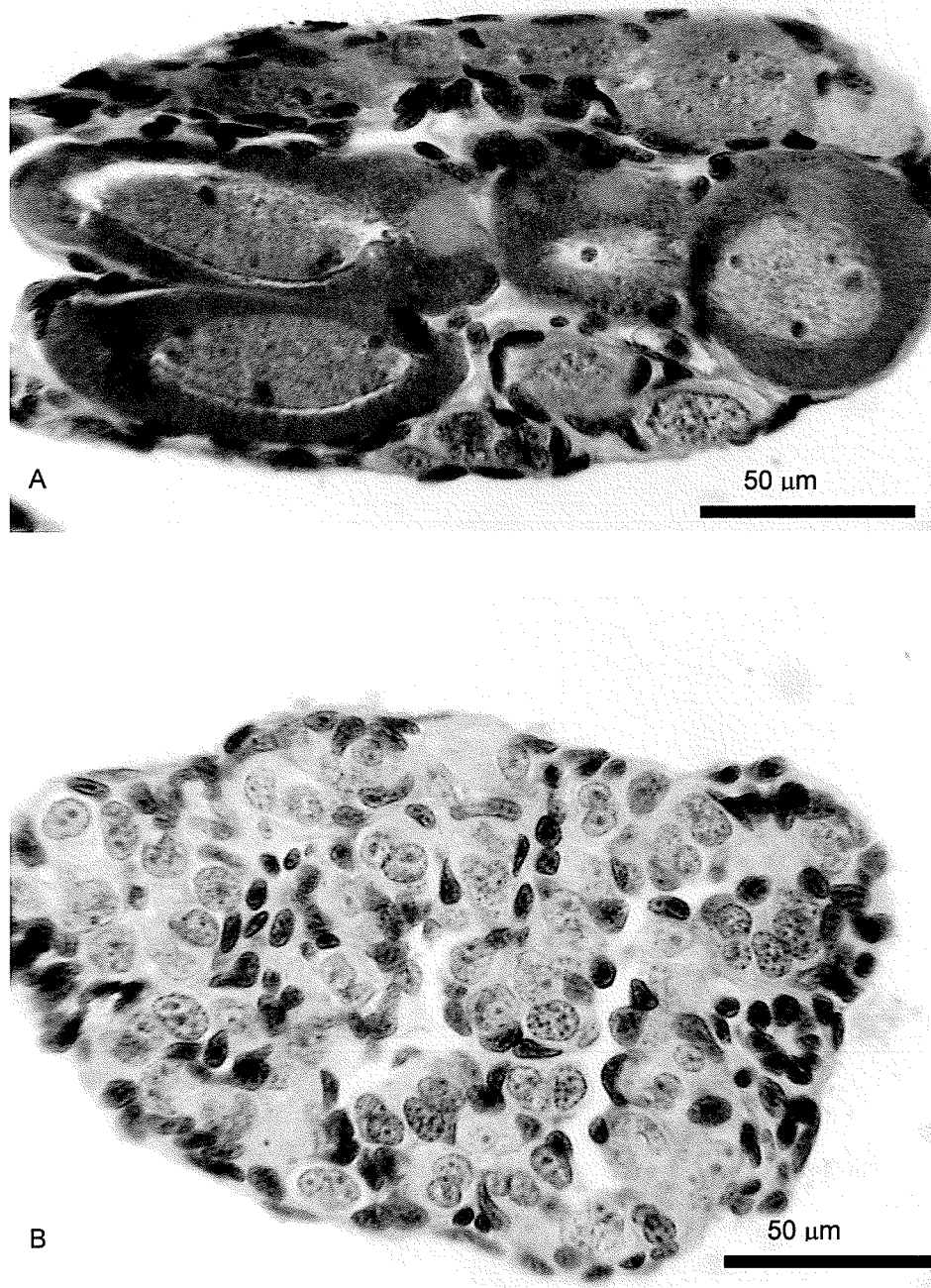


Figure 4.5 Photomicrographs of transverse sections through the gonad of an intersex wild mink frog tadpole from Lake 260. (A) Posterior section showing well developed primary oocytes at various stages of development. (B) Anterior section showing testicular development seen throughout the remainder of the gonad.

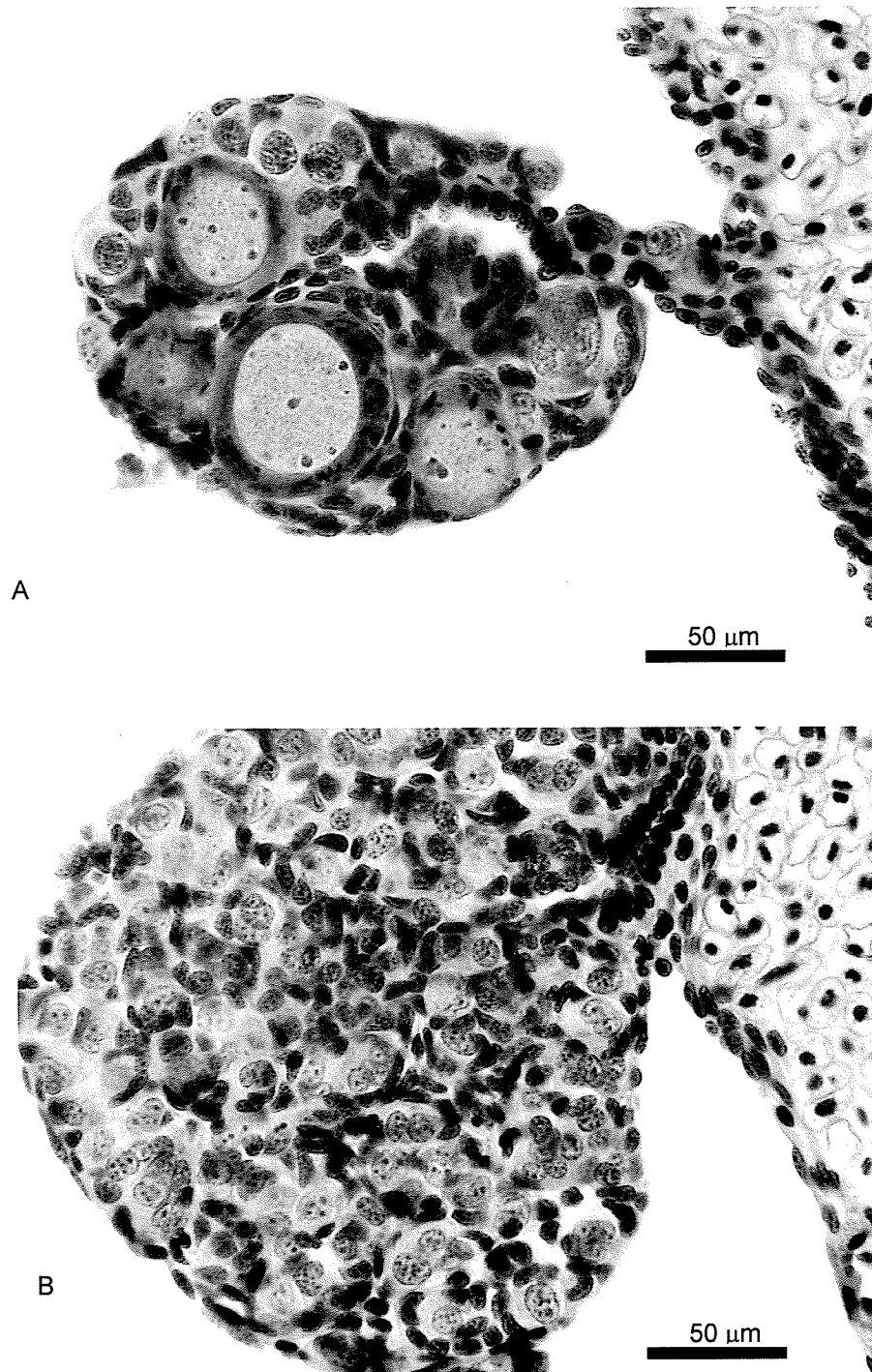


Figure 4.6 Photomicrographs of transverse sections through the gonad of an intersex cage-reared mink frog tadpole from Lake 260. (A) Posterior section showing well developed primary oocytes at various stages of development and a rudimentary ovarian cavity. (B) Anterior section showing testicular development seen throughout the remainder of the gonad.

Gonad Transverse Section Area Measurements

Mean transverse sectional areas of gonads in cage-reared green frog tadpoles (stage 35) are summarized in Table 4.1 (p.96). The mean area of ovaries in Lake 260 tadpoles was $3.59 \times 10^5 \mu\text{m}^2$, which was slightly greater than that of the reference lake tadpoles (3.55 and $3.41 \times 10^5 \mu\text{m}^2$ for lakes 114 and 224, respectively); however, the difference was not significant ($p=0.92$). Mean area of testes was slightly lower in EE2-exposed tadpoles ($3.00 \times 10^4 \mu\text{m}^2$) than in those from Lake 114 ($3.25 \times 10^4 \mu\text{m}^2$), again the difference was not significant ($p=0.52$). There were an insufficient number of stage 35 males from Lake 224 to support analysis.

Oocyte Measurements and Development Stage Classification

The number of oocytes measured and staged per female ranged from 66 to 173. The mean diameter and proportion of oocytes at the three development stages are summarized in Table 4.2 (p.96).

Lake 260 tadpoles had a slightly higher mean proportion of oocytes in the chromatin-nucleolar stage (0.23), relative to those from the reference lakes (0.20 and 0.22 for Lakes 114 and 224, respectively). Further, Lake 260 tadpoles had a slightly lower mean proportion of oocytes in the early perinucleolar phase (0.24) than reference tadpoles (0.25 and 0.30 for Lakes 114 and 224, respectively). Finally, the mean proportion of oocytes in the late perinucleolar phase was greatest in Lake 114 tadpoles (0.55), followed by those from Lake 260 (0.53) and Lake 224 (0.48). Overall there was no significant difference among lakes in the mean proportion of oocytes at the chromatin-nucleolar ($p=0.47$), early perinucleolar ($p=0.13$) or late perinucleolar stage ($p=0.25$).

There was a significant difference in mean diameter of chromatin-nucleolar stage oocytes across lakes ($p<0.0001$), i.e. each lake was significantly different from the other two. The mean diameter was highest in Lake 114 tadpoles (21.60 μm), followed by those in Lake 260 and 224 (20.35 and 18.77 μm , respectively). There was also a significant difference in mean diameter of early

perinucleolar stage oocytes across lakes ($p < 0.0001$), i.e Lake 224 was significantly different from the other two. The mean diameter was highest in Lake 260 tadpoles ($40.53 \mu\text{m}$), followed by those from Lake 114 and 224 (38.34 and $35.64 \mu\text{m}$, respectively). There was no significant difference across lakes in the mean diameter of late perinucleolar stage oocytes. Again, the mean diameter was highest for Lake 260 tadpoles ($63.71 \mu\text{m}$), followed by those from Lakes 114 and 224 (62.84 and $59.83 \mu\text{m}$). Overall, there was no treatment effect on the mean diameter of oocytes at the chromatin-nucleolar, early perinucleolar, or late perinucleolar stage.

Table 4.1 Mean transverse section area (\pm SD) of gonads in cage-reared green frog tadpoles (stage 35) from Lake 260 and from reference lakes. There were an insufficient number of males from Lake 224 to support analysis. Mean EE2 concentration = 5.8 ± 1.4 ng/L. Exposure was 119 d, from 4 days prehatch. Sample sizes are in brackets. Treatment groups with different letters are significantly different.

Lake	Testes Area (μm^2) \pm SD $\times 10^4$	Ovaries Area (μm^2) \pm SD $\times 10^5$
114	3.25 ± 0.66 (8) ^A	3.55 ± 0.95 (12) ^A
224	---	3.41 ± 1.32 (12) ^A
260	3.00 ± 0.97 (14) ^A	3.59 ± 1.11 (12) ^A

Table 4.2 Mean proportion and diameter (μm) of oocytes (\pm SD) at various developmental stages in cage-reared green frog (*R. clamitans*) tadpoles from Lake 260 and from reference lakes. Mean EE2 concentration = 5.8 ± 1.4 ng/L, exposure time = 119 d, from 4 days prehatch. n=12 for each group. Treatment groups with different letters are significantly different.

Lake	Chromatin-Nucleolar		Early Perinucleolar		Late Perinucleolar	
	Proportion	Diameter	Proportion	Diameter	Proportion	Diameter
114	0.20 ± 0.06	21.60 ± 0.80 ^A	0.25 ± 0.06	38.34 ± 2.72 ^A	0.55 ± 0.09	62.84 ± 5.13 ^A
224	0.22 ± 0.08	18.77 ± 0.94 ^B	0.30 ± 0.09	35.64 ± 1.62 ^B	0.48 ± 0.10	59.83 ± 8.59 ^A
260	0.23 ± 0.06	20.35 ± 1.45 ^C	0.24 ± 0.06	40.53 ± 2.10 ^A	0.53 ± 0.08	63.71 ± 5.34 ^A

DISCUSSION

Sex Ratios

There was no discernable effect of EE2 on sex ratios in wild or caged tadpoles. Previously, under laboratory conditions, exposure to environmentally relevant concentrations of ammonium perchlorate in *Xenopus laevis* induced a skewed sex ratio favouring females (Goleman et al. 2002). Functional, spontaneous sex reversal has been reported in captive reed frogs (*Hyperolius viridiflavus*); however, the occurrence of sex reversal in undisturbed anuran populations is poorly documented, and has not been reported for mink or green frogs. A single field study has examined the potential influence of environmental contaminants on sex ratios in wild amphibians. Reeder et al. (1998) report a relationship between the presence of PCB/PCDF point sources and sex ratio reversal (favouring males) in cricket frogs (*Acris crepitans*). In the present study, there was considerable variability in the sex ratios among reference lakes and between years within each reference lake. The estrogen concentration may have been too low to induce full reversal of gonadal sex in these species. Alternately, given that the sex ratio reversal reported by Reeder et al. (1998) was detected in juvenile and adult frogs, impacts may occur later in the life cycle after longer exposure. Ultimately, to interpret results of this nature from contaminated field sites, more information is required on natural sex ratios of native species and their year-to-year variability.

Intersex

Intersex gonads and hermaphroditism are known to occur spontaneously, though infrequently in amphibians (Grafe and Linsenmair 1989; Sullivan et al. 1996). In this study; intersex gonads occurred exclusively in EE2-exposed mink frog tadpoles whereas no abnormal gonads were detected in a total of 211 wild and 85 caged reference samples. This represents an estrogenic effect (albeit low incidence) in males during early gonad development that was detected in other species in the ELA study. Four of 11 male pearl dace (*Semotilus margarita*) sampled from Lake 260 in September 2001 were intersex (testes contained oocytes; R. E. Evans; personal communication); and male fathead minnows (*Pimephales prominales*) showed widespread fibrosis and disrupted germ cell development (Palace et al. 2002). The incidence of intersex in the

Lake 260 (1.6% in the wild and 5.6% in the cages) is similar to that found in wild adult cricket frogs (*A. crepitans*) at sites with multiple contaminants (3.6; 2.5; and 2.6% over three consecutive years; Reeder et al. 1998).

It is not known what degree of disruption in the larval phase is sufficient to reduce adult fecundity. Ovotestes have been observed in wild anurans; including scattered oocytes in otherwise normal testes; and active spermatogenesis has been documented in testes containing occasional oocytes (Sullivan et al. 1996; Reeder et al. 1998). Therefore, a slightly feminized male may be able to successfully mate despite the presence of female gonad tissue. Further, should intersex result in reproductive impairment of individuals, the frequency in a population that will impact overall recruitment is unknown. Thus the implications of contaminant-induced intersexuality for amphibian populations remain unclear. Gonad development is assessed by autopsy; therefore, it is not known how anomalies during the larval or juvenile stages affects reproductive output in the adult stage. However, intersex rate in E2-exposed *R. catesbiana* tadpoles increased with duration of exposure (Chang et al. 1996); thus, chronic exposure at contaminated sites may lead to significant disruption in adults, particularly in species with a long larval period.

The mechanism by which testicular and ovarian tissue is produced in the same gonad cannot be determined on the basis of the current study. Disruption was seen exclusively in the posterior portion of the gonad in this study. The same pattern of disruption was reported in *R. rugosa* tadpoles exposed to an estrogenic phthalate (Ohtani 2000); thus, a common estrogenic mechanism may have occurred. Small detectable amounts of endogenous steroids in early larval development could regulate the proliferation and differentiation of germ cells (Petrini and Zaccanti 1998). Chang et al. (1996) documented the induction of oocyte development by E2 administration in otherwise male tadpole gonads. Experimental evidence indicates that administration of estradiol results in increased estradiol concentration in the ovary; thus, environmental estrogens may accumulate in the gonad as well (Vandorpe and Kuhn 1989). EE2 may bind estrogen receptors in the gonad, inducing aromatase activity that results in conversion of endogenous

androgen to estrogen and subsequent tissue feminization. Aromatase and 5 α -reductase likely play key roles in ovarian and testicular development, respectively (Miyata and Kubo 2000; Saidapur et al. 2001). It has been determined that the primordial germ cells in amphibians have the potential to develop into either germ cell type, and it is the gonad that determines the fate of germ cell development, not vice versa. Germ cell development is asynchronous in amphibians, and is likely influenced by sex steroids. Thus, it is possible that the initial (genetic) determination induced male differentiation in the intersex specimens, with occasional primordial germ cells being induced into the oocyte development pathway by EE2.

The Lake 260 estrogen additions were repeated in 2002, and tadpole gonad histology analyses were repeated (these data are not part of this thesis). No wild intersex mink frog tadpoles were detected in Lake 260 (n=99). However, the majority of the samples were taken in the spring, prior to initiation of the EE2 additions; therefore, relatively few samples were analyzed from fall catches (n=17). In addition, five mink frog egg clutches were subsampled and reared in cages on Lakes 260 and 224. The incidence of intersex was 12.5% (n=56) for Lake 260, and 0% for Lake 224 (n=55). It is therefore apparent that disruption occurred again in 2002 in the amended lake (personal observation).

Gonad Area

Reduced gonad size in response to environmental chemicals can be an indicator of potential reproductive impairment and population-level effects in vertebrates (Ankley et al. 1998). Reduced gonadosomatic has been observed in fish from polluted sites (Jobling et al. 1998). Reduced testicular volume due to atrazine exposure has been observed in *Xenopus laevis* tadpoles (Tavera-Mendoza et al. 2002). Atrazine has been shown to induce aromatase activity, thereby converting androgens to estrogens, resulting in inhibited testicular development and enhanced ovarian development (Tavera-Mendoza et al. 2002). Atrazine impairs testicular development in mammals by interfering with testosterone and dihydroxytestosterone (DHT) receptor interactions and by inhibiting 5 α -reductase activity, i.e. conversion of testosterone to DHT, the more potent

androgen (Tavera-Mendoza et al. 2002). In this study, testicular transverse section area was slightly lower in EE2-exposed males than in controls, though the difference was not significant. This could simply represent natural variability, or it could be an EE2-induced inhibition. Impacts on gonad size would likely be more evident in later stages when individuals reach reproductive maturity.

Oocyte Size and Stage

The size and stages of oocytes in ovaries of wildlife are differentially sensitive to sex steroids, and can be used to assess exposure to environmental estrogens (Ankley et al. 1998). Oocyte maturation, the final phase of oogenesis in mature females, is inhibited by the estrogenic pesticide methoxychlor, though natural and synthetic estrogens have varying results (Baulieu et al. 1978; Pickford and Morris 1999). In this study, the proportions of early stage oocytes in tadpoles were not affected by the whole-lake EE2 exposure. Further, there was no consistent treatment effect of EE2 exposure on the size of oocytes at any given stage. Oocytes in the early stage of development may be refractory to the effects of environmental estrogens, whereas vitellogenic oocytes with surrounding follicle cells in mature females are likely more susceptible to hormonal exposure, thus, impacts may be evident in reproductively mature females.

Ultimately, any form of reproductive alteration associated with hormonally active contaminants in wild anurans should be cause for alarm. Any effect, however subtle, represents a disruption of normal physiological function, and is indicative of potential disruption at other regulatory points.

Chapter 5: Conclusion

Summary

Amphibians are a crucial component of healthy ecosystems. They are both predator and prey, typically occupy both aquatic and terrestrial habitats in their life cycle, and contribute significant vertebrate biomass in many systems (Stebbins and Cohen 1995). Globally, amphibian populations have been declining for several decades (Houlahan et al. 2000). Although factors affecting amphibian viability undoubtedly impact other wildlife, it is commonly reported that amphibians are particularly sensitive to harmful environmental effects relative to other taxa, due to their permeable skin, biphasic life history, and unprotected embryonic stage (Greenhouse 1976; Cooke 1981; Vitt et al. 1990; Wyman 1990; Blaustein 1994; Pollett and Bendell-Young 2000); thus, amphibians are effective early indicators of environmental degradation which can affect other taxa (Marian et al. 1983; Duellman and Trueb 1986; Grant and Licht 1995; Hayes 1998). Toxicants, including substances which disrupt thyroidal and reproductive function, may ultimately compromise the sustainability of wild populations (Carey and Bryant 1995). The current study serves to elucidate developmental effects of a potent endocrine disruptor on *Ranid* embryos and tadpoles in their native habitat using an ecologically relevant exposure system.

The specific objectives were to determine if EE2 interfered with critical aspects of early development:

- (i) hatching success
- (ii) timing of larval growth and development
- (iii) thyroid hormone content in body tissues at metamorphic climax
- (iv) gonad differentiation

Endpoints were examined at both the embryonic and larval stages, and at biochemical, tissue, and whole organism levels of organization. Further, organisms were exposed in laboratory, *in situ*, and in the wild.

The results of the various EE2 exposures are as follows:

- (i) Hatch success was reduced in green frog eggs, but not in mink frog eggs exposed *in situ*
- (ii) There was no consistent effect on tadpole growth or development
 - Laboratory exposure – green frog tadpoles were developmentally advanced at high EE2 concentrations, but not at ecologically relevant concentrations
 - *In situ* exposure – green and mink frog tadpole growth and development rates were significantly different among lakes in some cases; the differences were not treatment-related
 - Wild exposure – the increase in mass with development was greater in EE2-exposed mink frog tadpoles, than in reference samples (2001); no difference was detected prior to exposure (2000)
- (iii) The concentration of T₃ and T₄ in body tissues of metamorphic green frogs was not affected (the mean T₃ concentration and T₃:T₄ ratio were lower in Lake 260 samples than in reference samples, but differences were not significant)
- (iv) Gonad development was disrupted in mink frogs, but not green frogs
 - Sex ratios of both species varied between years and between lakes without treatment related pattern, in the wild and *in situ*
 - Intersex gonads occurred infrequently in mink frog tadpoles exposed to EE2 *in situ* and in the wild, but not in unexposed samples; none occurred in caged green frog tadpoles. (Note: no wild green frog tadpoles were examined; however, a single incidental catch exhibited an intersex gonad)

Relevance & Implications

Synthetic estrogens have been detected in various waterways around the world in the low ng/L range of concentrations (Table 1.1, p.5). “Whether the concentrations of estrogens determined in the lower ng/L range are sufficiently high to cause environmental endocrine effects has to be explored in further investigations” (Ternes et al. 1999). Exposure to EE2 in this range resulted in some reproductive disruption in native anurans at the ELA, whereas no effect on thyroid status

was detected. Very few studies exist on whether contaminants impact amphibian reproduction in the wild (Carey and Bryant 1995; Clark et al. 1998).

The current study has demonstrated effects of an environmental estrogen on two aspects of reproduction. Reduced hatching success could impair the ability of populations to recover from natural or human-induced decline events. The occurrence of intersex or maldeveloped gonads in tadpoles represents an early and potentially disruptive developmental insult. With chronic exposure in a polluted environment, gonad development may be significantly compromised, thereby inhibiting reproductive ability. It is possible that EE2 could affect the reproductive axis at other regulatory points. Endogenous estrogens have similar/identical effects in all vertebrates (Sumpter et al. 1997); therefore, this study holds relevance for other taxa. Hormonal regulation of development is the most highly conserved feature throughout the vertebrates (Tata 1996).

Overall, responses to EE2 exposure varied with different levels of organization, species, life history stage, and exposure method, thus highlighting the importance of examining multiple endpoints when determining the endocrine activity or toxicity of environmental contaminants. Endocrine function in vertebrates is intricate and is regulated by numerous mechanisms, and as such is susceptible to disruption at multiple endpoints. Previous studies have examined endocrine disrupting effects at multiple levels of organization (Kloas et al. 1999; Lutz and Kloas 1999; Goleman et al. 2002), life stages (Berrill et al. 1994; Berrill et al. 1998; Bishop et al. 2000), and exposure methods (Materna et al. 1995; Hatch and Burton 1998). Results varied within these studies; therefore, a lack of response in a single endpoint/life stage/exposure cannot confirm a lack of endocrine activity.

In this study, there was no consistent treatment effect on thyroid activity, either at the biochemical or whole organism level. This may indicate that these species are refractory to estrogen-thyroid interactions, or conversely, that the thyroid system is able to adequately compensate for subtle disruptions. A more complete assessment of central and peripheral thyroid hormone dynamics is

required. Assessment of thyroid status in the current study was reliant upon a single endpoint, i.e. tissue TH concentration. Given that the thyroid system is regulated at multiple points, a suite of several assays would provide a more thorough assessment.

Finally, this study has presented some novel information on the development of mink and green frog tadpoles irrespective of contaminant effects. This appears to be the first study of gonad differentiation, sex ratios, and incidence of intersex in larval mink frogs from reference habitats. Generally, more information on the modes and timing of gonad differentiation in native species, such as was generated herein, would aid interpreting field study results and in predicting responses of amphibians to environmental contaminants.

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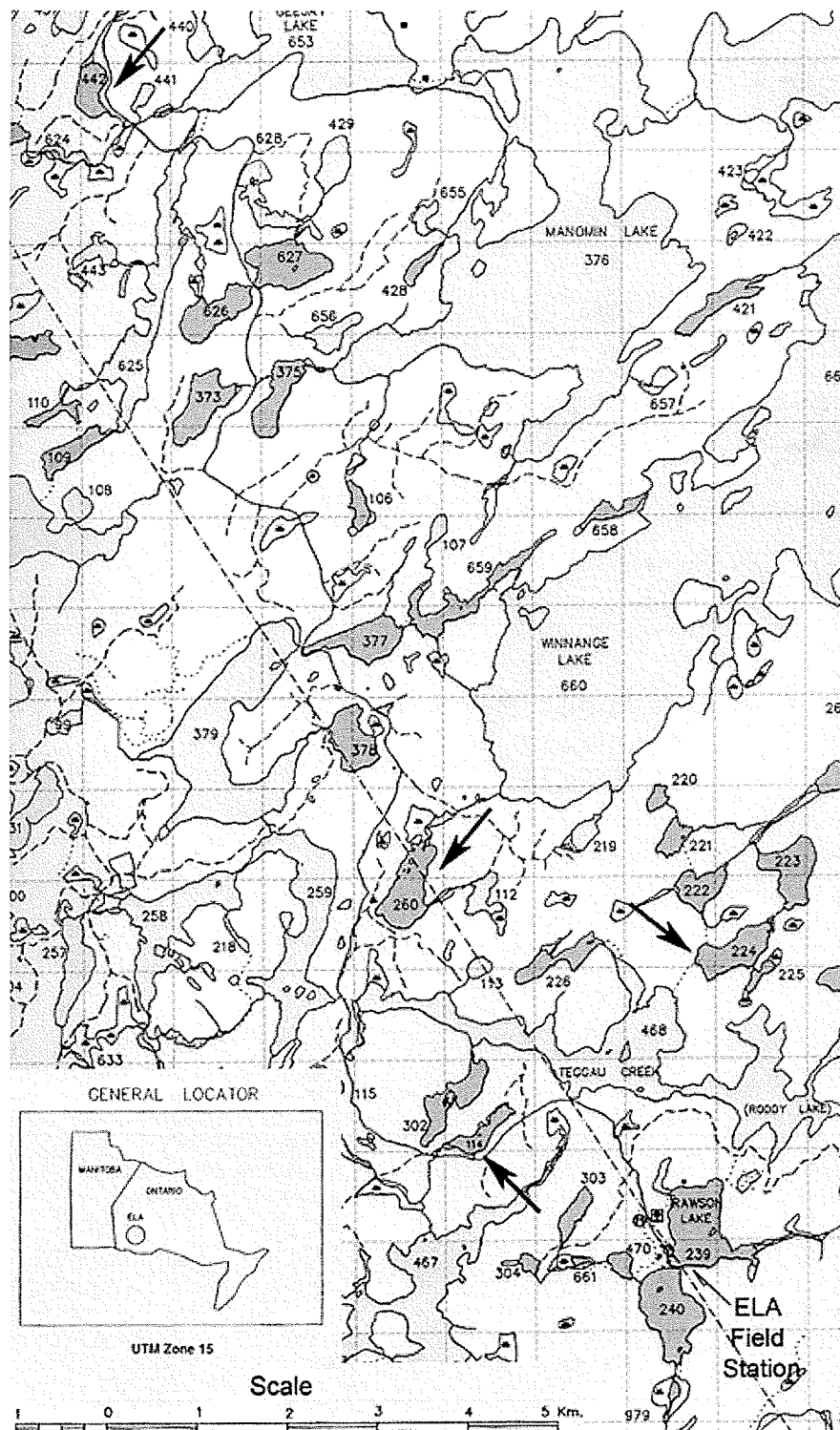
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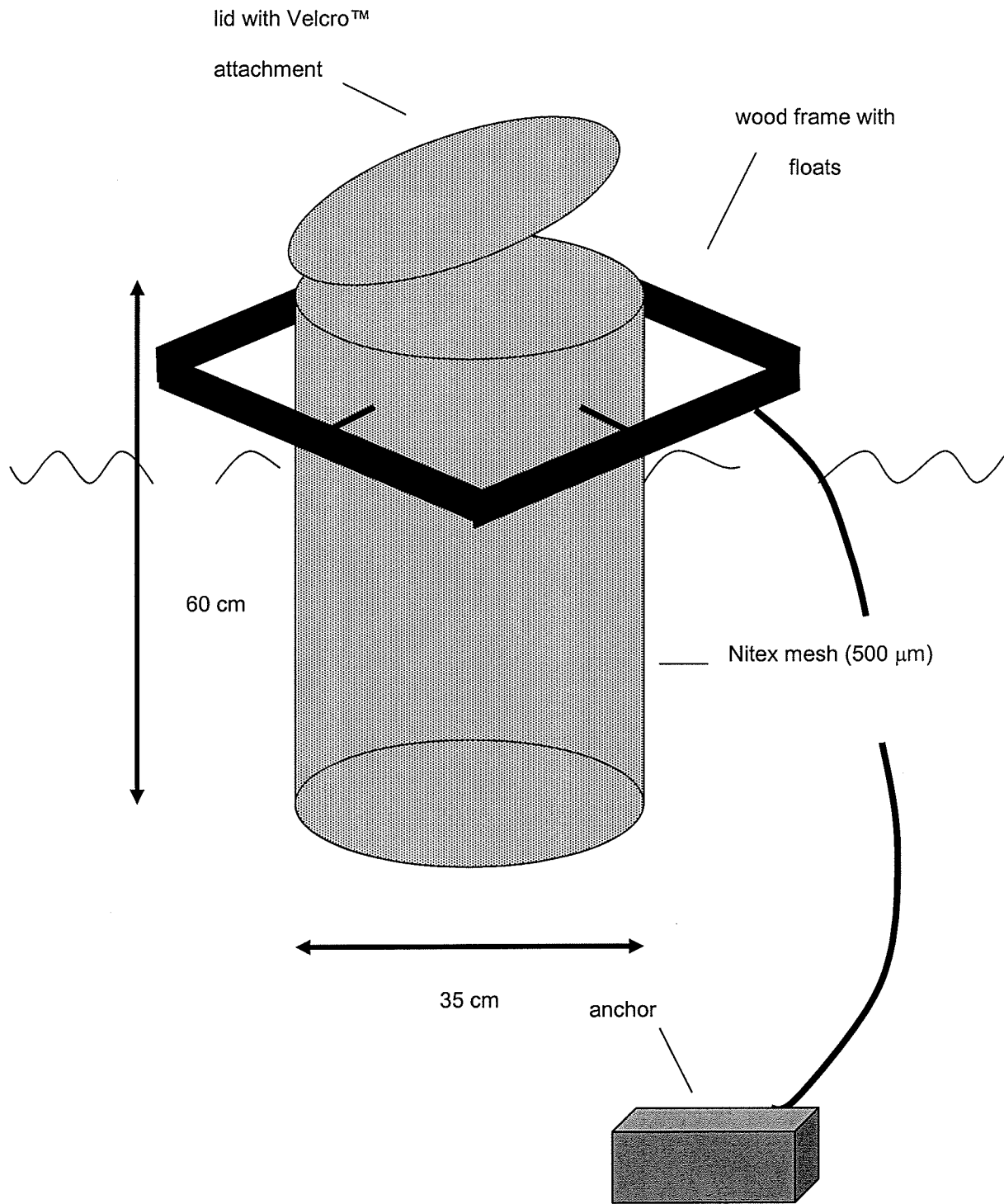
Appendices



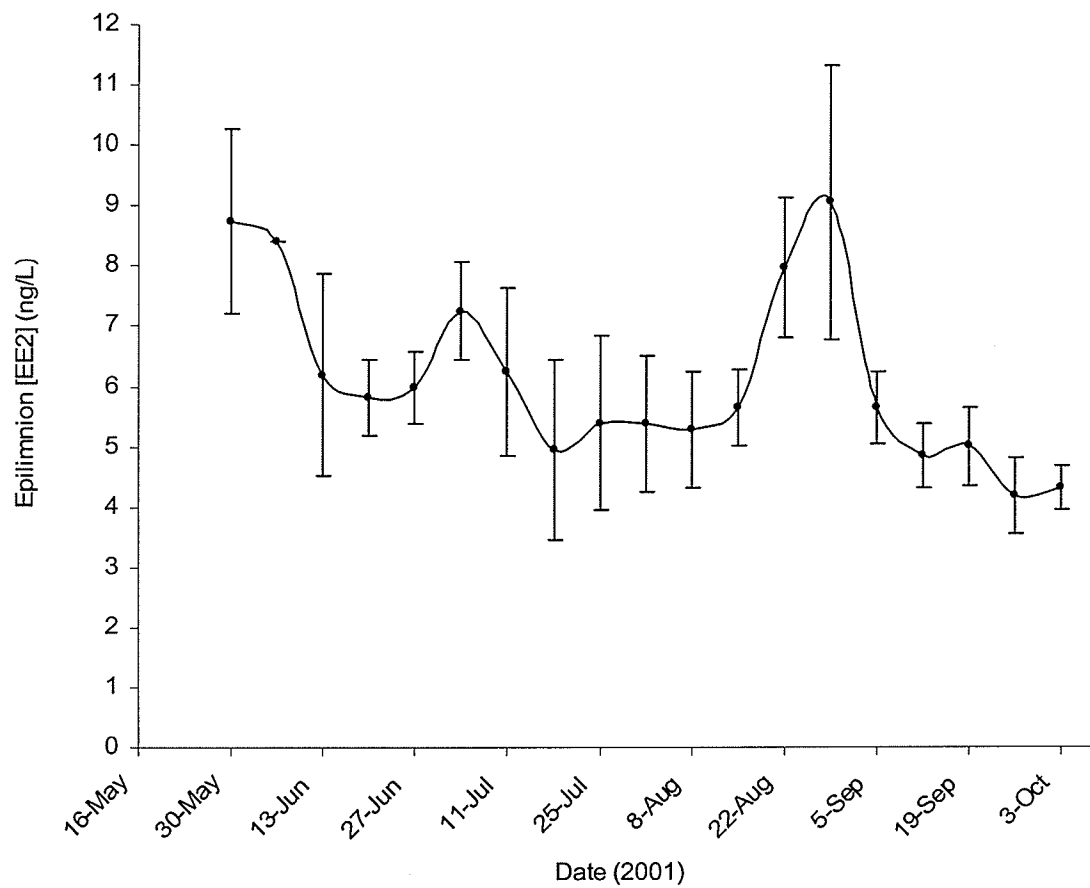
Appendix A Map of the Experimental Lakes Area (Ontario, Canada). Arrows indicate study lakes. (Field Station UTM coordinates: UTM Zone 15, 5501000 N, 447500E).

Appendix B Physical, chemical, and biological characteristics of study lakes at the Experimental Lakes Area. (ELA Long Term Environmental Reference Database, 1983-1993, May to October). Dissolved organic carbon and chlorophyll a values are geometric means (S. Kasian, personal communication).

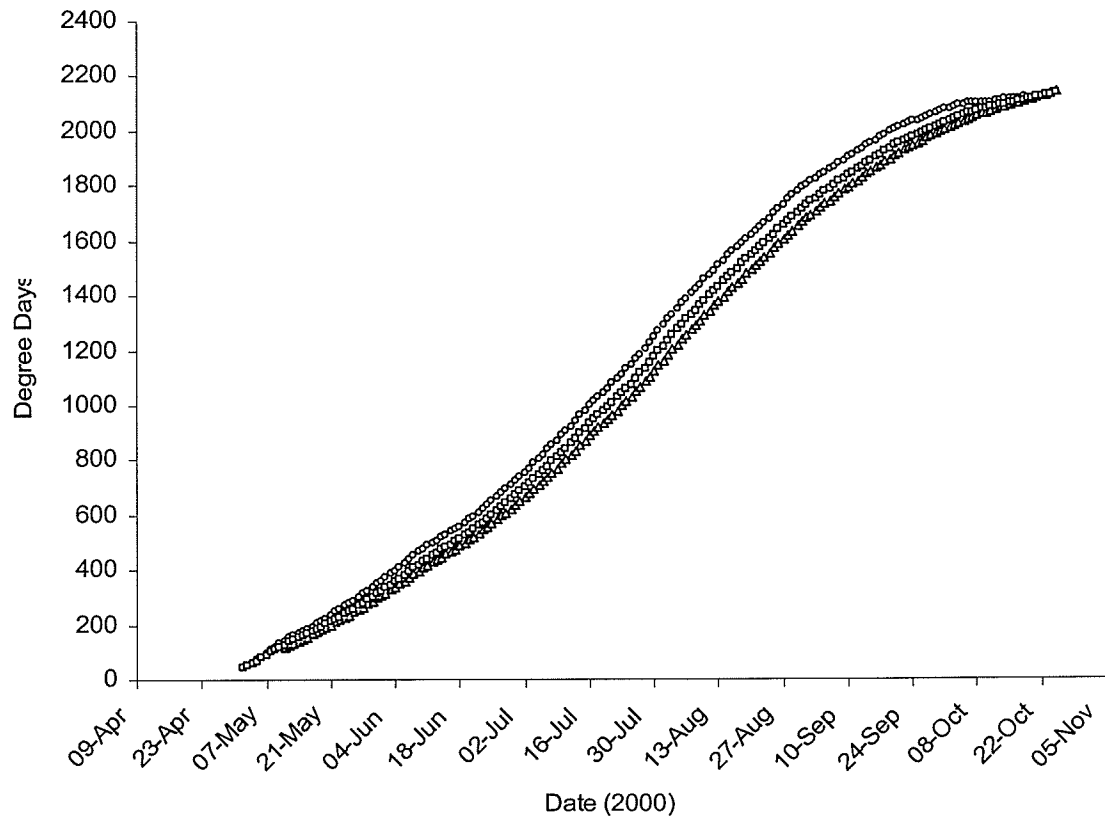
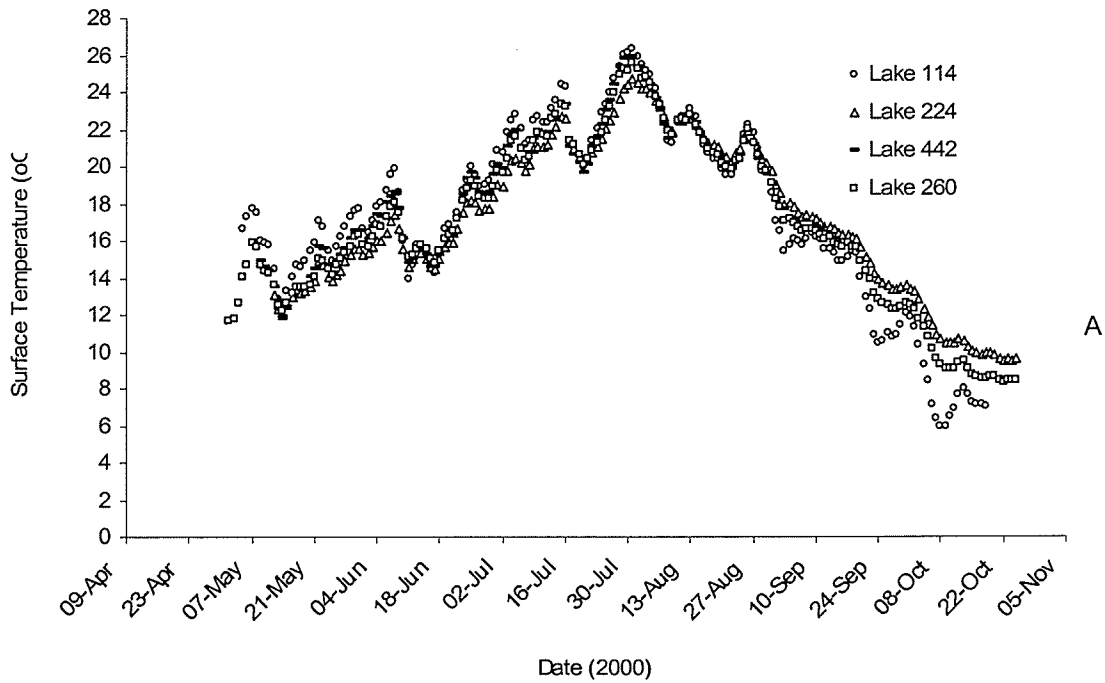
Lake	Lake Order	Area (ha)	Maximum Depth (m)	Volume ($\times 10^5 \text{m}^3$)	Dissolved Organic Carbon ($\mu\text{m/L}$)	Chlorophyll a	pH	Large Fish Species Present
114	1	12.1	5.0	2.07	597	4.1	6.17	none
224	2	25.9	27.4	30.05	245	1.0	6.89	lake trout, white sucker
260	2	32.8	14.0	17.54	459	1.8	7.20	lake trout, white sucker
442	2	16.0	17.8	13.03	557	2.2	7.44	lake trout, white sucker



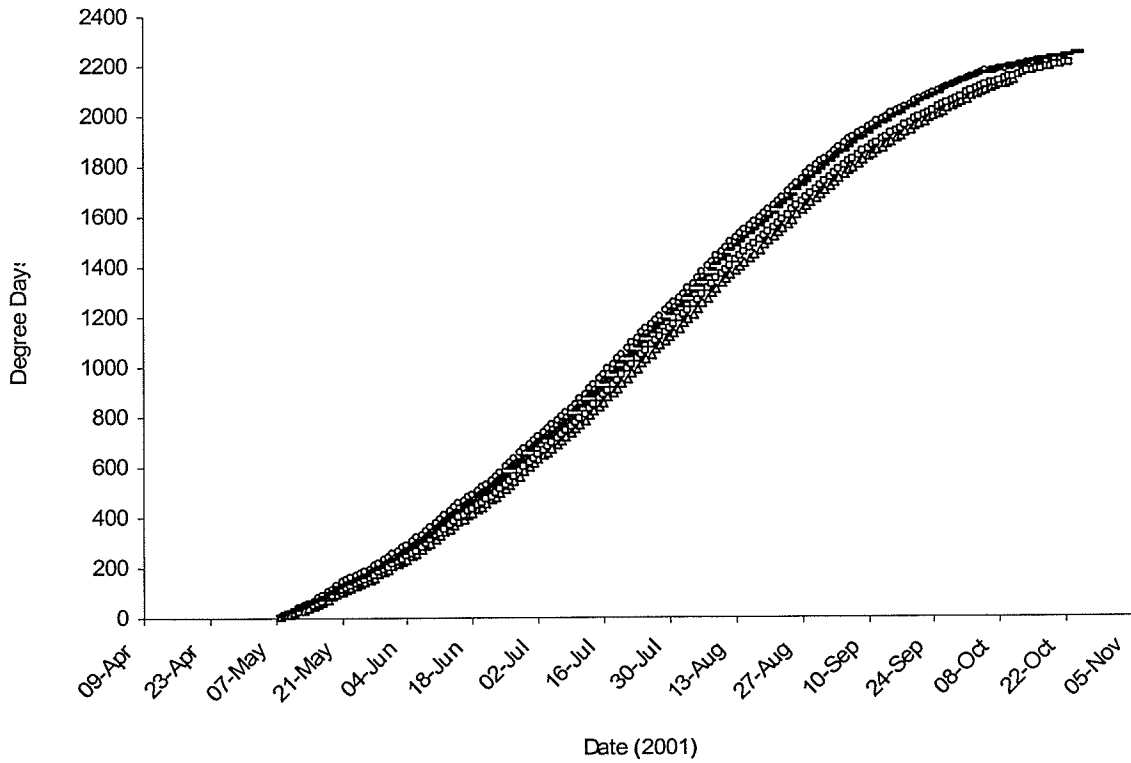
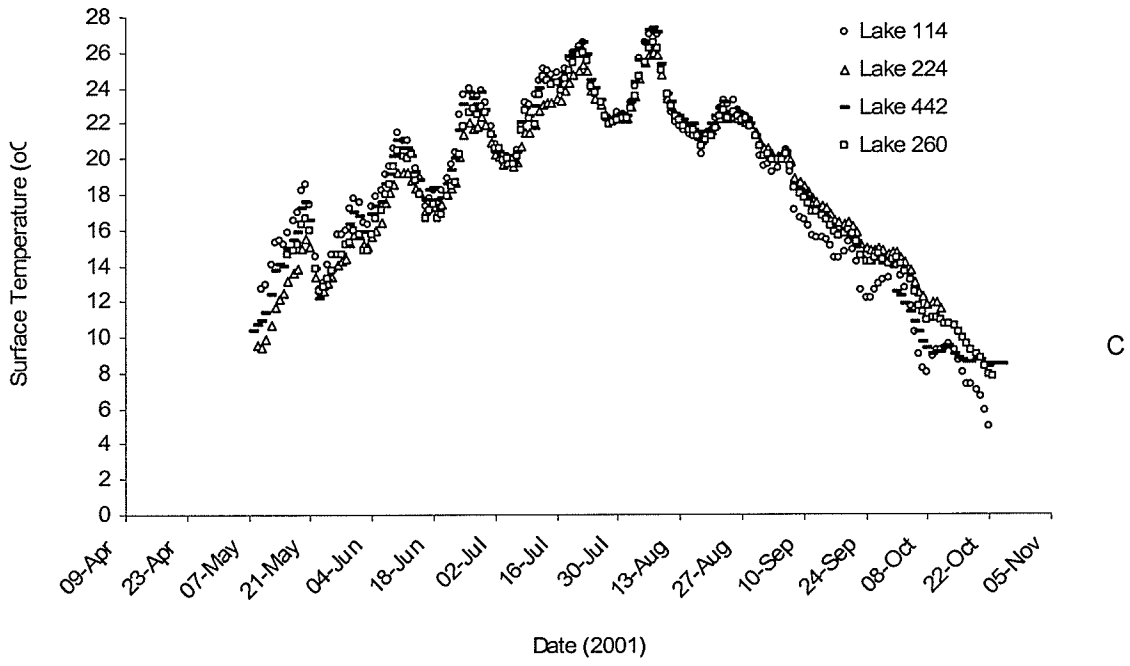
Appendix C Diagram of cage design used in study lakes (adapted from Harris and Bogart 1997).



Appendix D Mean concentration of EE2 (\pm SD) in the epilimnion of Lake 260 during additions in 2001. Each data point is the mean of ten integrated epilimnetic samples taken at five sampling sites. Mean epilimnetic depth was 4.3 m during additions. Season mean = 6.1 ± 1.5 ng/L. (Palace et al. 2002). EE2 was not detected in Lake 260 in 2000. Reference lakes were not tested for EE2 content.



Appendix E Temperature and degree day profiles for study lakes in 2000 (A and B) and 2001 (C and D). Temperature data points are the daily mean of hourly readings.



Thyroid Hormone Results – Sample Calculation

Sample #1, T4 assay result: 48.2 ng/ml

48.2 ng/ml ÷ 0.789 extraction efficiency = 61.1 ng/ml

Mass of tadpole body tissue: 2.14 g

Final mass of homogenate (i.e. tissue + buffer): 13.70 g

Mass of homogenate digested for TH extraction: 3.08 g

$(2.14 \text{ g} / 13.70 \text{ g}) \times 3.08 \text{ g} = \underline{0.48 \text{ g}}$ of tissue digested

Therefore,

$61.1 \text{ ng/ml} / 0.48 \text{ g/ml} = \underline{127.3 \text{ ng/g}}$

Appendix F Sample calculation of thyroid hormone content in tadpole tissues.

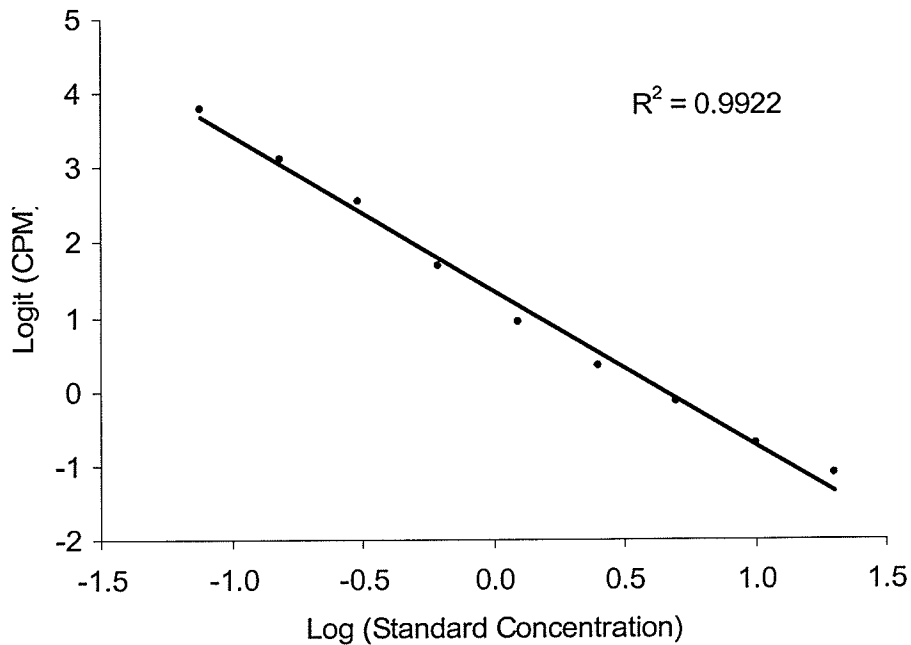
Assay Date: March 25, 02
 Antibody Dilution: 600
 Antibody Binding: 69.28%
 Detection Limit: 0.1009 ng/ml

	Rep1	Rep2	Rep3	Average
NSB I-	1195.5	1211.0	1204.5	1203.7
NSB	104.4	109.1	85.8	99.8

	Rep1	Rep2	Rep3	Average	Corrected
TCR	10661.6	10678.2	10813.6	10717.8	9514.1

Std (ng/ml)	Rep1	Rep2	Rep3	Average	Corrected	Logit	Log [std]
20	1476.9	1638.2	2077.1	1730.7	1631.0	-1.112	1.301
10	2206.5	2241.7	2405.9	2284.7	2184.9	-0.702	1.000
5	3183.8	3212.1	3048.5	3148.1	3048.4	-0.150	0.699
2.5	3679.6	4118.9	4006.0	3934.8	3835.1	0.330	0.398
1.25	4830.1	4735.7	4932.8	4832.9	4733.1	0.935	0.097
0.613	5710.7	5635.3	5671.1	5672.4	5572.6	1.699	-0.213
0.306	6124.1	6343.4	6173.5	6213.7	6113.9	2.549	-0.514
0.153	6171.3	6542.0	6518.3	6410.5	6310.8	3.112	-0.815
0.077	6446.3	6536.1	6649.1	6543.8	6444.1	3.776	-1.116
0	6626.9	6547.3	6900.1	6691.4	6591.7		

RIA Standard Curve



Slope of standard curve	-1.9919
y intercept of standard curve	1.29465

Sample #	Dilution	Rep1	Rep2	Rep3	Average	Corrected	Logit	[T4] (ng/ml)
1	0.05	4348.7	4234.8	4178.9	4254.1	4154.4	0.533	48.2
2	0.05	4693.2	4794.1	4765.3	4750.9	4651.1	0.874	32.5
3	0.05	4206.9	3814.1	4045.3	4022.1	3922.3	0.385	57.2
4	0.05	4650.6	4226.1	4233.9	4370.2	4270.4	0.610	44.1
5	0.05	3500.9	3562.6	3908.7	3657.4	3557.6	0.159	74.3
6	0.05	4387.5	4960.0	4491.2	4612.9	4513.1	0.775	36.5
7	0.05	3778.4	4253.0	4104.8	4045.4	3945.6	0.400	56.3
8	0.05	3768.4	4182.7	4147.2	4032.8	3933.0	0.392	56.8
9	0.05	4214.1	4256.0	4255.0	4241.7	4141.9	0.525	48.7
10	0.05	3557.8	3672.5	3575.6	3602.0	3502.2	0.125	77.3
11	0.05	4532.8	4224.4	4454.9	4404.0	4304.3	0.632	43.0
12	0.05	4306.1	3971.7	4442.5	4240.1	4140.3	0.524	48.7
13	0.05	2829.8	2835.1	2575.6	2746.8	2647.1	-0.399	141.7
14	0.05	4408.8	4113.5	4256.5	4259.6	4159.8	0.537	48.0
15	0.05	3939.5	4478.6	4501.7	4306.6	4206.8	0.568	46.3
16	0.05	3786.0	3878.4	3702.7	3789.0	3689.3	0.240	67.7
20	0.05	3886.7	4108.9	4237.4	4077.7	3977.9	0.420	55.0
21	0.05	4045.3	4518.6	4380.1	4314.7	4214.9	0.573	46.1
22	0.05	3538.9	3606.5	3789.8	3645.1	3545.3	0.152	75.0
23	0.05	4205.2	4052.7	3907.9	4055.3	3955.5	0.406	55.9
24	0.05	4049.4	3915.1	4235.6	4066.7	3966.9	0.413	55.4
25	0.05	4310.0	4442.5	3941.2	4231.2	4131.5	0.518	49.1
26	0.05	4441.2	4524.1	4697.0	4554.1	4454.3	0.734	38.2
30	0.05	3987.5	4512.2	4148.4	4216.0	4116.3	0.509	49.6
31	0.05	3941.2	4403.1	4139.5	4161.3	4061.5	0.473	51.7
40	0.05	4708.4	4774.0	4903.9	4795.4	4695.7	0.907	31.3
41	0.05	3867.6	4206.0	4375.2	4149.6	4049.8	0.466	52.1
42	0.05	3521.6	3475.8	3385.9	3461.1	3361.3	0.040	85.3
45	0.05	4355.5	4372.9	4154.3	4294.2	4194.5	0.559	46.8
46	0.05	4033.3	3497.6	3988.7	3839.9	3740.1	0.271	65.3
50	0.05	4136.6	3969.1	3726.8	3944.2	3844.4	0.336	60.6

Appendix G Sample data output from a thyroxine radioimmunoassay.