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Natacha Suzanne Hogan
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Biology)
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Department of Biology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Developmental Regulation and Estrogenic Endocrine Disruption of Metamorphosis in the Northern Leopard Frog, *Rana pipiens*

TITRE DE LA THÈSE / TITLE OF THESIS

Vance Trudeau
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

David Lean
CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Marc Ekker

Lou Guillette

Mark Forbes

John Lewis

Kathleen Gilmour

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Developmental regulation and estrogenic endocrine disruption of metamorphosis in the Northern leopard frog, *Rana pipiens*

by

Natacha Suzanne Hogan

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DEDICATION

To my parents, my sisters, and my Matthew - for everything.

ABSTRACT

Amphibian metamorphosis is a dramatic larvae-to-adult transformation regulated by temporal changes in thyroid hormones (TH) as well as interactions with other hormone systems. This developmental process is extremely responsive to changes in environmental conditions and susceptible to aquatic contaminants that interfere with hormone-dependent processes. While many of these endocrine disrupting compounds (EDCs) have been studied for their estrogenic effects on reproduction, the plasticity of amphibian metamorphosis provides a unique model for examining the thyroid-mediated effects of developmental exposure to estrogenic EDCs.

The first objective of this thesis was to assess the toxicological response and developmental effects of estrogenic EDCs during the larval life-stages of amphibian development. After establishing baseline toxicity data for the test species, *Rana pipiens*, a developmental exposure was conducted to determine the estrogen-sensitivity of distinct periods during metamorphosis. Stage-specific exposures altered tadpole growth parameters and resulted in lasting effects such as delayed metamorphosis and female-biased sex ratios. Therefore, the second objective was to identify potential mechanisms by which xenoestrogens alter TH-dependent metamorphosis using a targeted gene expression approach. Development of multiplex and simplex real-time PCR assays and subsequent analysis established that thyroid- and estrogen-responsive transcripts in the tadpole brain are differentially regulated by exogenous TH and upon exposure to an estrogenic EDC. A short-term "challenge" assay confirmed that estrogenic exposure suppresses and in some cases blocks the ability of TH to induce the expression of target genes involved in mediating tissue-specific TH sensitivity during metamorphosis. The

results of this research 1) provide evidence of cross-regulation between thyroid and estrogen-regulated genes during amphibian development and 2) indicate that developmental exposure to estrogenic EDCs may affect normal TH-responsiveness necessary for proper timing of metamorphosis.

RESUMÉ

La métamorphose des amphibiens est une transformation drastique d'un stade larvaire à l'état adulte qui est contrôlée par une séquence temporelle de changements dans les niveaux d'hormone thyroïdienne ainsi que par l'interaction avec d'autres systèmes hormonaux. Ce processus de développement est extrêmement sensible aux changements dans les conditions environnementales et aux contaminants aquatiques qui interfèrent avec les processus hormonaux. Tandis que plusieurs de ces perturbateurs endocriniens ont été étudiés pour leurs effets oestrogéniques en reproduction, la plasticité de la métamorphose des amphibiens représente un modèle unique pour l'étude des effets sur les processus contrôlés par la thyroïde lors d'une exposition aux xénoestrogènes au cours du développement.

Le premier objectif de cette thèse était d'évaluer les effets toxicologiques et sur le développement d'une exposition aux xénoestrogènes chez les têtards. Après avoir établit le seuil de toxicité pour l'espèce testée, *Rana pipiens*, une exposition à l'œstrogène a été effectuée afin de déterminer la sensibilité des différents stades de développement lors de la métamorphose. L'exposition à des stades spécifiques a altéré les paramètres de croissance des têtards et entraîné des effets durables tels que des délais dans la métamorphose et un rapport des sexes favorisant les femelles. Suite à ces résultats, le second objectif était d'identifier les mécanismes potentiels par lesquels les xénoestrogènes altèrent la métamorphose contrôlée par l'hormone thyroïdienne en utilisant une approche d'expression génique ciblée. Le développement d'essais multiplex et simplex de PCR en temps réel et les analyses subséquentes ont établit que les transcrits sensibles à la thyroïde et l'œstrogène dans le cerveau des têtards sont régulés de façons

différentes par l'hormone thyroïdienne exogène et suite à une exposition à un xénoestrogène. Une exposition à l'œstrogène de courte durée peut supprimer l'expression de gènes induits par l'hormone thyroïdienne qui sont impliqués dans la médiation de la sensibilité de tissus spécifiques à l'hormone thyroïdienne au cours de la métamorphose. Les résultats de ces études 1) apportent des évidences d'une interaction entre les gènes sous le contrôle de la thyroïde et de l'œstrogène au cours du développement des amphibiens et 2) indiquent qu'une exposition à des xénoestrogènes au cours du développement peut affecter la réponse normale à l'hormone thyroïdienne qui est nécessaire pour un synchronisme approprié de la métamorphose.

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ABBREVIATIONS

ACTH	adrenocorticotropin
APEO	alkylphenol polyethoxylates
CRF	corticotropin releasing factor
D2	iodothyronine deiodinase type II
D3	iodothyronine deiodinase type III
DNA	deoxyribonucleic acid
DTM	days to metamorphosis
E2	estradiol
EDC	endocrine disrupting compounds
EE2	17α-ethinylestradiol
ER	estrogen receptor
ERα	estrogen receptor alpha
ERE	estrogen response element
EtOH	ethanol
LC_{50}	lethal concentration 50%
mRNA	messenger ribonucleic acid
NP	nonylphenol
OP	octylphenol
PRL	prolactin
RNA	ribonucleic acid
PCR	polymerase chain reaction
RXR	retinoid X receptor
SVL	snout vent length
STP	sewage treatment plant
T	testosterone
T3	triiodothyronine
T4	thyroxine
TH	thyroid hormone
TR	thyroid hormone receptor
TRα	thyroid hormone receptor alpha
TRβ	thyroid hormone receptor beta
TRE	thyroid response element
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone

CHAPTER 1

General Introduction

"Metamorphosis is a time of great revolution – commonly it is a dramatic transition to a new life in a new place in a new body"

Jan A. Pechenik

1.1 Motivation

Hormones systematically transform an aquatic, free-swimming tadpole into a terrestrial frog. The morphological and physiological changes associated with this metamorphosis are regulated by temporal changes in thyroid hormone action as well as interactions with other hormone systems. However, this developmental process is also susceptible to disruption by aquatic contaminants that interfere with hormone-dependent processes and have emerged as a threat to the health of wildlife species worldwide. Although popular and scientific attention has been drawn to their reproductive effects, these endocrine disrupting contaminants can also disrupt normal thyroid system function. Amphibian metamorphosis provides a unique system for studying the morphological and physiological effects of contaminant exposure on early vertebrate development. Moreover, the thyroid hormone-dependent events associated with metamorphosis are well-understood and offer a solid foundation for assessing contaminant-induced disruption of normal endocrine function.

1.2 Amphibian metamorphosis

Metamorphosis is a period of significant morphological and biochemical change where an organism alters its mode of living. Almost all chordates undergo some type of metamorphic transformation during their life-cycle; early mammalian organogenesis is developmentally very comparable to amphibian metamorphosis (Tata, 1993). Amphibians are the classical vertebrate example for this life-history process. While all three classes of amphibians (anurans, urodeles, and caecilians) have species which undergo metamorphosis, the anurans have been the most widely studied group. This is primarily due to the dramatic nature of their metamorphosis and their relative ease of use in laboratory research.

There are several staging tables and systems used to classify the distinct periods of amphibian metamorphosis. Most of them are based on major morphological changes that are common across species. According to Etkin (1968), amphibian metamorphosis can be separated into three distinct periods: premetamorphosis, prometamorphosis and metamorphic climax (Figure 1.1). The period of premetamorphosis encompasses embryonic and early larval development and includes major transformations such as the emergence of the hindlimb buds followed by formation of the foot paddle. Prometamorphosis is characterised by rapid hindlimb growth and digit differentiation. Metamorphic climax is the final and most dramatic period of metamorphosis where forelimbs emerge and tail resorption occurs as the tadpole becomes a juvenile frog. These developmental periods are also associated with diverse hormonal changes that mediate the progression of metamorphosis.

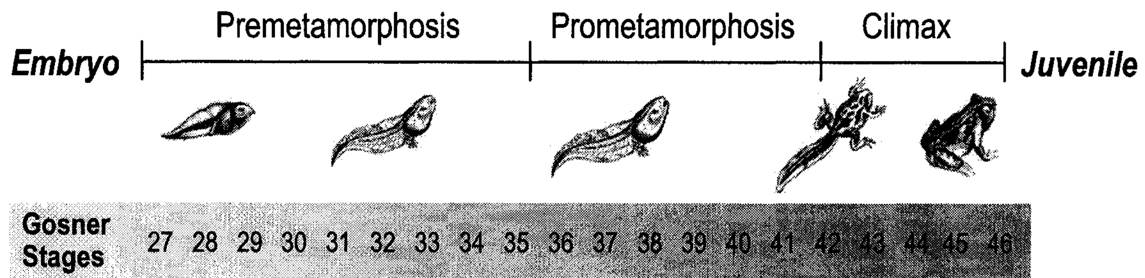

Figure 1.1 The larval development of the anuran amphibian. The general terminology proposed by Etkin (1968) divides metamorphosis into three periods: premetamorphosis, prometamorphosis and metamorphic climax. The staging table by Gosner (1960) assigns specific numbers according to detailed morphological changes and is the system used throughout this thesis for developmental assessment of the test species, *Rana pipiens*.

1.2.1 Hormonal control of metamorphosis

Hormones orchestrate the diverse morphological and physiological changes that occur during metamorphosis. The importance of the thyroid axis and thyroid hormones (TH) to metamorphosis has been long recognized and is currently the most well-characterised vertebrate model for thyroid hormone-dependent development. The neuroendocrine regulation of the thyroid axis, together with tissue-specific TH action, direct the systematic morphological transformations in a developing tadpole. Synthesis of TH during tadpole development is regulated by complex interactions between neuroendocrine control mechanisms and hormone feedback signals (Denver, 1998b). This endocrine pathway is referred to as the brain-pituitary-thyroid axis (Figure 1.2). The

hormonal control of this system is detailed in several excellent reviews (Kikuyama *et al.*, 1993; Shi, 2000; Denver *et al.*, 2002) and is briefly summarized below.

The hypothalamus transduces sensory information from the central nervous system (CNS) into the release of neurohormones from the hypothalamus. These hormones travel through portal vessels to the anterior pituitary gland where they stimulate the release of pituitary hormones. In amphibians, two pituitary hormones, thyrotropin (TSH) and adrenocorticotropin (ACTH), are responsible for the release of TH from the thyroid gland and the release of corticosteroids (CORT) from the interrenal glands, respectively (Kikuyama *et al.*, 1993). In mammals, the primary neurohormonal regulator of TSH secretion is thyrotropin-releasing hormone (TRH) (Norris, 1996). However, the role of TRH in amphibians is less clear. While studies indicate that TSH cells in the pituitary respond to TRH (Denver and Licht, 1988) and injection of TRH increases TH levels in adult frogs (Denver and Licht, 1989), TRH does not in fact alter the timing of tadpole metamorphosis (Denver, 1993). In larval amphibians, TRH can stimulate the pituitary to produce prolactin (PRL) (Castano *et al.*, 1992), a hormone considered to exert anti-metamorphic actions on target tissues and perhaps promote larval growth. Instead, the stress neurohormone, corticotropin-releasing factor (CRF), stimulates the thyroid axis in larval amphibians and other nonmammalian vertebrates (Denver, 1997a). While the primary function of CRF in vertebrates is to stimulate the stress axis via ACTH release from the pituitary (Norris, 1996), studies have demonstrated that CRF injections are effective in increasing whole body content of TH in several tadpole species (Denver, 1993, , 1997a). CRF has also been shown to directly stimulate the secretion of TSH protein from the turtle pituitary in tissue culture (Denver and Licht,

1991). CRF-stimulated release of TSH from the pituitary induces the thyroid gland to synthesize TH. CORT from the interrenals are suggested to enhance the capacity of TH to induce metamorphosis at later stages of development (Kikuyama *et al.*, 1993; Wright *et al.*, 1994). Levels of both TH and CORT are regulated through feedback mechanisms at the CNS throughout metamorphosis and both hormones stimulate metamorphic changes via receptor-mediated actions in target tissues.

The thyroid gland itself is completely developed by late premetamorphosis and early prometamorphosis (Dodd and Dodd, 1976). Several measures of thyroid activity (*e.g.*, radioiodine uptake, gland ultrastructure, plasma and tissue TH levels) have shown that thyroid activity increases significantly during prometamorphosis, peaks at metamorphic climax, and finally declines to a constant adult level (Dodd and Dodd, 1976; Regard *et al.*, 1978; Kikuyama *et al.*, 1993). There are two main forms of TH: thyroxine (T4) and the more bioactive form, 3,3', 5-triiodothyronine (T3). Pituitary TSH stimulates the production of thyroglobulin, the precursor for T4, and active iodine reacts with the tyrosine residues of the thyroglobulin to produce iodinated tyrosine residues (Norris, 1996). Coupling of these residues results in the formation of T4 or T3. Both T4 and to a lesser extent T3 are released from the thyroid gland (Leloup and Buscaglia, 1977); however, with greater than ten times the biological potency of T4, T3 is primarily responsible for TH-induced changes during metamorphosis (White and Nicholl, 1981).

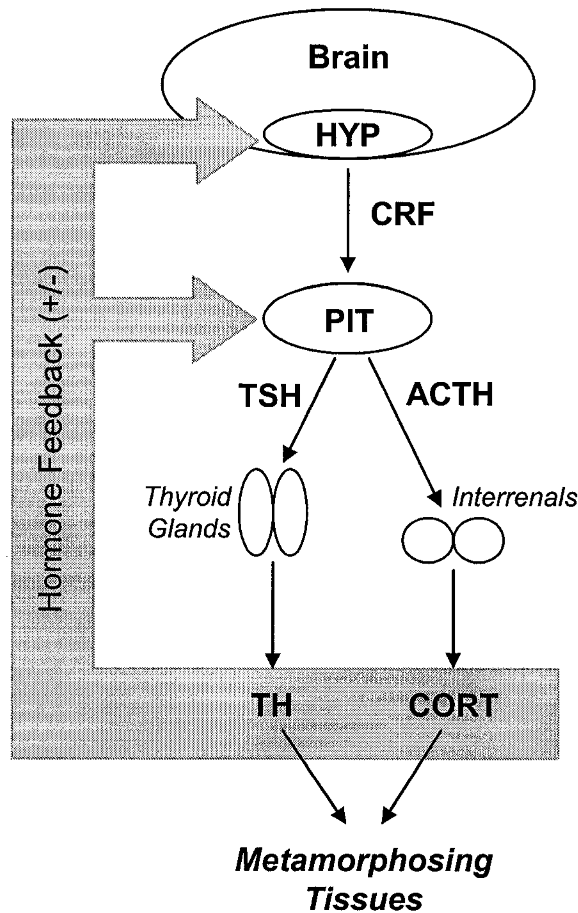

Figure 1.2 Endocrine control of amphibian metamorphosis by the brain-pituitary-thyroid axis. Hormone feedback effect can be either stimulatory (+) or inhibitory (-) depending on the developmental stage and target tissue. HYP, hypothalamus; CRF, corticotropin-releasing factor; PIT, pituitary; TSH, thyrotropin stimulating hormone; ACTH, adrenocorticotropin; TH, thyroid hormone; CORT, corticosteroids. Figure adapted from Denver *et al.* (2002).

1.2.2 Thyroid hormone action

During metamorphosis, larval tissues respond to TH in a stage- and tissue-specific manner. The ability of tissues to respond to TH is dependent on local hormone receptor activation and TH metabolic enzyme activity. Therefore, while the central thyroid axis regulates the synthesis and circulating levels of TH during development, the peripheral action of TH varies according to receptor and enzyme function in specific metamorphic tissues.

The genomic actions of TH are mediated by the nuclear thyroid receptor (TR), which is a member of the nuclear receptor superfamily (along with steroid, vitamin D and retinoic acid receptors) (Evans, 1988). The TR is encoded by two distinct genes (TRα and TRβ) (Bassett *et al.*, 2003). The binding of TH to the TR forms a TH–TR complex, which then bind to consensus sequences on the DNA (TH response element; TRE) as dimers. Homodimers of TRα or TRβ can form on most TREs, but the preferred configuration is likely a TR heterodimer with the retinoid-X receptor (RXR) (Puzianowska-Kuznicka *et al.*, 1997). TR-RXR heterodimers bind DNA and activate TRE-containing genes much more effectively than TR homodimers (Machuca *et al.*, 1995). The TR-RXR heterocomplex recruits cofactors that mediate the repressive or activational effects of TH (Sachs *et al.*, 2000; Shi, 2000; Buchholz *et al.*, 2006). Sachs *et al.* (2000) proposes that this dual function allows unliganded TRs to act as repressors of TH-inducible genes during early metamorphosis to ensure a proper period of tadpole growth, while the liganded form later acts as activators of TH-responsive genes to induce the metamorphic process.

During amphibian metamorphosis, TRα and TRβ transcripts are differentially expressed with respect to tissue and developmental stage (Kawahara *et al.*, 1991). TRα is generally detected early in development, shortly after embryogenesis and prior to TH production by the thyroid gland (Yaoita and Brown, 1990). TRα is constitutively expressed in many tadpole tissues (Veldhoen *et al.*, 2002). In contrast, TRβ transcript levels increase dramatically in response to rising TH levels during metamorphosis (Yaoita and Brown, 1990). TRβ mRNA levels are also induced in several tissues (*i.e.* brain, tail, hindlimb) with the administration of exogenous T3 (Krain and Denver, 2004). This autoinduction of TRβ is responsible for modulating the transcription of downstream TH-responsive genes whose products regulate diverse cellular changes in metamorphosing tissues (Tata *et al.*, 1993; Denver *et al.*, 1999).

The relative amounts of T4 and T3 are regulated within specific tissues through an enzymatic process known as deiodination, the removal of iodide atoms from rings of the iodothyronine structure (Norris, 1996). Becker *et al.* (1997) proposed that the activity of these iodothyronine deiodinases is responsible for the differential timing of metamorphosis in peripheral tissues. Two isoforms have been studied in anurans. One isoform, deiodinase type II (D2), catalyzes the conversion of T4 to T3, whereas the other isoform, type III (D3), selectively inactivates both T4 and T3, converting them into T2 or reverse T3, respectively. D2 mediates tissue sensitivity to circulating TH by regulating local T3 production. D2 activity and expression peak when a tissue (*e.g.*, hindlimb, tail) is undergoing metamorphic changes (Becker *et al.*, 1997). Conversely, D3 is the major inactivation enzyme for T4 and T3 and its activity is proposed to regulate tissue-specific TH availability when circulating TH levels are high (Kawahara *et al.*, 1999). Until very

recently it was thought that amphibians possessed only these two forms of deiodinase, while other vertebrates, with a type I deiodinase (D1) in the liver, had three forms (Sutija and Joss, 2006). However, a recent study by Kuiper *et al.* (2006) revealed that amphibians also possess D1, though its function in metamorphosis has not yet been determined.

1.2.3 Cross-regulation: a role for estrogen in metamorphosis?

Interaction between two endocrine pathways via receptor-mediated mechanisms, also termed hormone cross-regulation, plays an important role in directing vertebrate development; metamorphosis is no exception. There are considerable experimental data supporting the relationship between the stress and thyroid axes in amphibians. Some examples are a parallel increase in plasma corticosteroids and TH during metamorphosis (Krain and Denver, 2004), enhancement of metamorphic process by exogenous administration of corticosteroids (Wright *et al.*, 1994), and a demonstrated reduction in the effectiveness of TH in inducing metamorphosis by inhibiting corticosteroid synthesis in tadpoles (Kikuyama *et al.*, 1982). In comparison, the impact of sex steroids, specifically estrogen, on TH-dependent development has received little attention. This is surprising given the well-characterised relationships between thyroidal status and reproduction in several fish species (Cyr and Eales, 1996).

The majority of studies suggests that sex steroids inhibit amphibian larval development (Richards and Nace, 1978; Gray and Janssens, 1990; Hayes, 1997). For example, Gray and Janssens (1990) found that exposure to 17β-estradiol (E2) and testosterone (T) antagonised the metamorphic effects of exogenous T3 in tadpoles. In another study, E2 implants decreased both plasma T4 and T3 levels and reduced D2

activity (and consequently T3 production) in the kidneys of an adult ranid frog, *Rana ridibuna* (Vandorpe and Kuhn, 1989). Factors such as differences in E2 sensitivity (between species and developmental stages), exposure route and concentration make comparing results among the limited number of studies difficult. Hayes (1997) suggested that sex steroids act on central thyroid regulation, at the brain-pituitary-thyroid axis and that sex steroid-inhibition of metamorphosis could be a result of a down-regulation of TH levels or stimulation of TH-inhibitors, such as prolactin. However, sex-steroids may also affect peripheral TH sensitivity by modulating the expression of hormone receptors and enzymes that control TH actions.

For almost a century, the TH-dependent events of metamorphosis (morphological and functional) have fascinated the field of developmental biology as well as many other disciplines. With thyroid and reproductive axis cross-regulation mediating numerous physiological processes in vertebrates, anuran metamorphosis provides a unique model for examining estrogen-mediated effects on TH-driven development at several levels of biological organization. For the field of toxicology, this is also a timely focus, with increasing awareness of estrogenic compounds in the aquatic environment and evidence for their capacity to disrupt hormone action (Damstra *et al.*, 2002).

1.3 Endocrine disrupting compounds (EDCs)

Since the 1970s, there has been a global concern that chemicals in the environment, both natural and anthropogenic, possess the ability to disrupt normal functioning of the endocrine system. Exposure to these endocrine disrupting compounds (EDCs) has been linked to effects in wildlife and may also pose a risk to human health.

A recent comprehensive document released by the World Health Organisation entitled "Global Assessment of the State-of-the-Science of Endocrine Disruptors" listed 29 selected workshops/assessment reports on endocrine disruption between 1992 and 2002 (Damstra *et al.*, 2002). The same document defines an EDC as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations."

The mechanisms of action by which an EDC can alter hormone function are diverse. Receptor-mediated mechanisms have received the most attention but EDCs can also affect hormone synthesis, metabolism and transport. The timing of exposure also has a substantial influence on the nature of EDC effects. While exposure during mature life stages may be compensated by normal homeostatic response and, as such, may not result in permanent effects, developmental exposure could interfere with hormonal organisation of endocrine organs and result in permanent detrimental changes in function and subsequent response to hormone signals. These outcomes are referred to, respectively, as the activational and organisational effects of EDCs (Guillette *et al.*, 1995).

The scientific literature has been dominated by studies concerning EDCs that interfere with sex steroid hormone action, especially those that mimic the actions of endogenous estrogen (Propper, 2005). These estrogenic EDCs, also termed xenoestrogens, can have diverse effects on reproductive development and function in vertebrates and invertebrates (LeBlanc, 1999; Damstra *et al.*, 2002; Segner *et al.*, 2003). In fish, exposure to xenoestrogens has been shown to induce feminization of gonads and secondary sex characteristics (Kinnberg *et al.*, 2003; Parrott and Blunt, 2005), disrupt

hormone synthesis enzymes in the brain (Kishida *et al.*, 2001; Kazeto *et al.*, 2004), and lead to reproductive failure over multiple generations (Nash *et al.*, 2004). The best known estrogenic response is the mRNA and protein induction of vitellogenin, the female egg yolk precursor protein, in the liver of male fish (Wheeler *et al.*, 2005). Xenoestrogens enter water systems from numerous sources: sewage treatment effluent (Desbrow *et al.*, 1998), discharge from industrial manufacturing (Heberer, 2002) and agricultural runoff (Hanselman *et al.*, 2003) containing detergents, pesticides and pharmaceuticals.

With the widespread occurrence of xenoestrogens and their sometimes striking effects on reproductive morphology, the thyroid system has received comparatively little attention. The thyroid system is directly or indirectly involved in growth, development, metabolism and reproduction, making it susceptible to contaminant-induced disruption through multiple systems (Zoeller and Crofton, 2000; Boas *et al.*, 2006). A review by Brucker-Davis (1998) described the effects of over 40 pesticides and 45 industrial chemicals on the thyroid axis in numerous vertebrates. Some of the compounds identified as thyroid disruptors were also previously described as having estrogenic activity. This functional overlap is not surprising considering the extensive hormonal cross-regulation between endocrine axes, especially during development. With the focus of EDC research shifting towards examining the mechanisms behind the observed effects, it is important to expand our sometimes narrow concept of a target system for EDC actions. For example, compounds classified as estrogenic due to their effects on reproduction may also disrupt the immune, thyroid and stress response. In the same respect, using a well-characterised hormone response or system as a model for examining

these "non-target effects" will help elucidate the mechanisms behind the disrupted endocrine response. According to Guillette, "without this broader approach, research into endocrine disruption will become dominated by a narrow dogma, focusing on a few endpoints and mechanisms" (2006).

1.4 Amphibian development: a model for thyroid-mediated effects of EDCs

Tadpoles become competent to respond to exogenous TH at the time of hatching (Tata, 1968). Most morphological and physiological changes that occur during natural metamorphosis can be accelerated by treating tadpoles with TH, and can be slowed by blocking TH action (Dodd and Dodd, 1976; Kikuyama *et al.*, 1993). The obligatory TH-dependence of metamorphosis and the ability to manipulate this biological system are two major reasons for the recent initiatives to develop a metamorphosis assay to assess EDC effects on the thyroid system (OECD, 2004).

Morphological endpoints such as developmental stage and hindlimb differentiation as well as histological assessment of the thyroid gland are now being used as methods to detect contaminant-induced disruption of the thyroid axis (Tietge *et al.*, 2005; Opitz *et al.*, 2006). Although these endpoints are straightforward to assess and provide an overall measure of EDC effects, they are not specific to changes in thyroid axis function. In studies using mammals, plasma T3 and T4 concentrations are commonly used to assess the anti-thyroidal activities of EDCs (Boas *et al.*, 2006). However, the difficulty of obtaining blood from tadpoles limits the ability to detect treatment-related changes in circulating TH during metamorphosis.

Examination of TH-dependent gene expression provides an indirect method of evaluating thyroidal status since the expression of several genes (*i.e.*, TRβ, D3) is closely correlated with changes in circulating TH (Krain and Denver, 2004). Gene expression analysis is also conducive to the examination of tissue-specific TH-responsiveness, a condition not accurately measured with other endpoints, such as whole body TH levels. Therefore, a targeted gene-expression approach can provide valuable information regarding mechanisms involved in estrogenic disruption of normal TH action, especially if one examines both estrogen- and thyroid-regulated genes. While previous studies have demonstrated that estrogenic EDC can alter morphological endpoints such as the timing of metamorphosis, tail resorption, and tadpole body condition (Nishimura *et al.*, 1997; Bevan *et al.*, 2003; Goto *et al.*, 2006), none have assessed TH-dependent gene expression as an endpoint of estrogen-induced thyroid disruption.

The mechanisms directing metamorphosis and the genes involved in its regulation are well-conserved among anuran species (Denver *et al.*, 2002). Even so, there is significant variation in contaminant-induced developmental effects across species. The laboratory animal, *X. laevis* is the model species for the development of a comprehensive screening and testing strategy for thyroid-active EDCs (OECD, 2004). As a model species, *Xenopus sp.* have several advantages such as their rapid tadpole development and ease of rearing in the laboratory. In addition, genetic resources for Xenopus studies continue to rapidly develop, including complementary DNA (cDNA) sequencing, microarray production and transgenesis methods (Klein *et al.*, 2002). However, there are substantial differences in life-history strategies between *Xenopus* and other anuran species, such as Ranids, that influence their susceptibility to the effects of environmental

contaminants. Therefore, it is important that the research community also focus its efforts on characterising the developmental and molecular response of native or endemic amphibians to EDC exposure. Although the apparent global decline of amphibians cannot be attributed solely to environmental contaminants (Houlahan *et al.*, 2000; Kiesecker *et al.*, 2001), studying native species provides more appropriate information for assessing the risk of EDCs to the health of amphibian populations.

1.5 Thesis outline

The main objective of this thesis was to determine the effects of xenoestrogen exposure on amphibian metamorphosis. To accomplish this I used (1) toxicological and developmental endpoints to characterise species- and stage-specific responses to xenoestrogen exposure and (2) molecular endpoints to examine potential mechanisms of xenoestrogen-induced thyroid system disruption. The principle test animal used for this research was the Northern leopard frog (*Rana pipiens*). This species is native to a large proportion of North America and considered to be a sensitive test species for evaluating the developmental effects of environmental contaminants on metamorphosis (Degitz *et al.*, 2000; Crump *et al.*, 2002).

With the exception of the comparative toxicity study (Chapter 2), I used the synthetic estrogen, 17α-ethinylestradiol (EE2), for estrogenic exposures conducted for this thesis research. EE2 is structurally similar to its natural analogue, 17β-estradiol, but has a higher binding affinity to the estrogen receptor (Denny *et al.*, 2005). EE2 is used by millions of women worldwide as a main component of oral contraceptive formulations and hormone replacement therapies, resulting in its detection in surface waters in Canada,

United States and Europe (Ternes *et al.*, 1999; Metcalfe *et al.*, 2001; Yin *et al.*, 2002). Several full life-cycle experiments with various fish species have shown that, even at very low concentrations, exposure to EE2 has endocrine-disrupting effects on reproductive development and function. Some examples include stimulation of vitellogenin synthesis, formation of intersex gonads (ovotestes), and phenotypic sex reversal in male fish, as well as abnormal ovarian development and reduced egg production in female fish (Lange *et al.*, 2001; Nash *et al.*, 2004; Fenske *et al.*, 2005; Parrott and Blunt, 2005). Owing to its environmental occurrence and estrogenic properties, EE2 was selected as a model xenoestrogen to assess whether estrogenic exposure could alter growth, development and gene expression in the developing tadpole.

This thesis is organized into chapters based on 1) comparing the stage and species-specific toxicity of (xeno)estrogens, 2) establishing the critical periods of sensitivity to EE2 exposure through effects on sexual development and metamorphosis, 3) developing species-specific assays for analysis of target thyroid hormone- and estrogen-responsive genes and using these assays to characterise transcript levels over development and in response to T3, 4) examining the gene expression response in hormone-sensitive tissues (brain and liver) following EE2 exposure, and 5) determining whether EE2 exposure could alter the normal T3-induced endocrine response in metamorphosing tissues.

CHAPTER 2

(Xeno)estrogen exposure affects survival and growth of ranid tadpoles*

2.1 Introduction

Amphibians are a vital component of many ecosystems and their apparent global decline is of major concern. While habitat destruction and alteration are likely the main contributors to population declines (Houlahan *et al.*, 2000), other factors such as disease (Carey, 2000), global climate change and UV-B radiation (Kiesecker *et al.*, 2001), along with environmental contamination (Carey and Bryant, 1995; Davidson *et al.*, 2002) can influence amphibian health and survival. Amphibians are susceptible to the effects of contaminant exposure as the developmental part of their life-cycle is spent in the aquatic environment. During early larval development and throughout tadpole metamorphosis, contaminants can be absorbed through their highly permeable skin or ingested via their food. Metamorphosis, like all vertebrate development, is an intricately timed process that is highly sensitive to disruption by environmental stress. During the various stages of metamorphosis, contaminant exposure can result in paralysis, deformities, altered growth rate or death (Carey and Bryant, 1995).

Many life-history characteristics influence an individual's vulnerability to contaminant-induced effects. Several studies indicate that amphibian susceptibility to pesticide exposure appears to be highly dependent on the developmental stage at the time of exposure (Berrill *et al.*, 1994; Bridges, 2000; Edginton *et al.*, 2003; Greulich and

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* *Adapted from:* Hogan, N.S, Lean, D.R.S, and V.L. Trudeau. 2006. Exposure to estradiol, ethinylestradiol and octylphenol affect survival and growth of *Rana pipiens* and *Rana sylvatica* tadpoles. Journal of Toxicology and Environmental Health. 69: 1555-1569.

Pflugmacher, 2003). It has been suggested that juvenile fish and embryos might be more susceptible to toxic stress when they are exposed during "critical windows" in their development (Mantovani *et al.*, 1999). There is also significant variability in sensitivity to contaminants among amphibian species (Pauli *et al.*, 1999; Rosenshield *et al.*, 1999). However, developmental stage and species differences in sensitivity often strongly depend on the specific chemical considered (Berrill *et al.*, 1998). It is also suggested that differences in response to toxicant exposure between species and within populations of the same species is due to genetic variation (Bridges and Semlitsch, 2000, , 2001). Variability due to life-history characteristics support the use of more than one species and developmental stage of amphibian when assessing the toxicity of a particular contaminant.

There is growing concern about the presence of endocrine disrupting compounds (EDCs), especially in surface waters. These compounds are capable of interfering with hormone-dependent processes involved in vertebrate development and reproduction (Crisp *et al.*, 1998). EDC exposure during early life-stages can be particularly detrimental to an individual's future health and survival as effects can persist throughout development and into adulthood (Guillette *et al.*, 1995; Bigsby *et al.*, 1999). The natural and synthetic estrogens, also referred to as xenoestrogens, represent a diverse group of EDCs that exhibit estrogenic activity and are prevalent in the aquatic environment (Jobling *et al.*, 1995). Xenoestrogens enter water systems from several sources, including sewage treatment effluent (Desbrow *et al.*, 1998), industrial manufacturing of pharmaceuticals (Heberer, 2002) and agricultural runoff (Hanselman *et al.*, 2003). Release of these compounds into water systems has been linked to incidences of

reproductive impairment in fish, and laboratory studies have also demonstrated a wide range of effects in other non-target species (Metcalfe *et al.*, 2001).

4-*tert*-octylphenol (OP) is one of the two main degradation products of alkylphenol polyethoxylates (APEOs), non-ionic surfactants used as household and industrial detergents (Nimrod and Benson, 1996). APEOs are found in aquatic sediments, and surface and ground water. OP accounts for about 15% of the commercial alkylphenols released into the aquatic environment (Bennett and Metcalfe, 1998) and its concentrations are generally detected below 1 μg/L (~ 5 nM) in rivers and streams (Bennie *et al.*, 1997) with point source levels reaching 10-30 μg/L (~ 50-150 nM) (Rudel *et al.*, 1998).

17α-Ethinylestradiol (EE2) and 17β-estradiol (E2) are among the most commonly detected hormones in effluents from sewage treatment plants (STPs) and in surface waters (Desbrow *et al.*, 1998; Ternes *et al.*, 1999). EE2 is a synthetic estrogen used in oral contraceptive formulations and hormone replacement therapies (Maier and Herman, 2001). The presence of these estrogens in Canadian STPs has been documented with median concentrations of 9 ng/L (0.03 nM) for EE2 and 6 ng/L (0.02 nM) for E2 (Ternes *et al.*, 1999). A recent study of 139 US rivers reported concentrations of 830 ng/L (2.8 nM; maximum) and 73 ng/L (0.25 nM; median) for EE2, and 200 ng/L (0.73 nM; maximum) and 160 ng/L (0.59 nM; median) for E2 (Kolpin *et al.*, 2002).

Toxicity data on EDCs are available for many fish species as they are traditionally used as biomarker organisms for aquatic exposure (Rotchell and Ostrander, 2003). However, because amphibian development is a highly sensitive hormone-dependent process, there are also efforts to develop an amphibian model for the assessment of EDCs

(Kloas, 2002). Laboratory exposure to xenoestrogens can increase amphibian embryo mortality, induce both external and internal developmental abnormalities, and influence sexual development resulting in feminization and skewed sex ratios (Bevan *et al.*, 2003; Mackenzie *et al.*, 2003; Fort *et al.*, 2004; Levy *et al.*, 2004). However, basic toxicity data for specific EDCs are still required for many amphibian species and across various life-stages.

The main objectives of these studies were to assess the toxicity of three aquatic contaminants known to possess estrogenic properties using a 2-week LC_{50} assay and to compare the responses between two developmental stages and between two ranid species, *Rana pipiens* (Northern leopard frog) and *Rana sylvatica* (Wood frog), both native to North America. The variability in sensitivity among amphibian species and between developmental stages is an important factor to consider when assessing the toxicity of environmental contaminants. This type of toxicity data is useful in establishing water quality criteria and has important applications in ecological risk assessment. More specifically, this work has also enabled our laboratory to establish exposure concentrations for further studies assessing developmental effects of estrogenic EDCs at sub-lethal concentrations.

2.2 Methods

2.2.1 Animals and rearing conditions

Fertilized eggs (portions of 4 clutches) of *R. pipiens* and *R. sylvatica* were collected from an isolated, permanent pond near Old Chelsea, PQ, Canada (45°30'N, 75°47'W). The pond was in a rural area and not adjacent to any obvious sources of

contamination or agricultural runoff. Egg clutches were combined in the lab and maintained in 30 L-capacity glass aquaria containing aerated and dechlorinated City of Ottawa water (pH 6.5-7.0; dissolved oxygen >80%; 20-22 °C) under a 12:12 hour light:dark photoperiod. Both species were developmentally staged according to the Gosner staging table (Gosner, 1960). Tadpoles were fed a mixture of Nutrafin flakes (Rolf C. Hagen Inc, Montreal, PQ) and tadpole food (Carolina Biological Supply Co., Burlington, NC) daily *ad libitum*. The care and treatment of animals used in this study were in accordance with the guidelines of the Animal Care Committee, University of Ottawa and the Canadian Council on Animal Care.

2.2.2 Chemical preparation

Technical grade E2 (FW=272.4), EE2 (FW=296.4) and 4-*t*-OP (FW=206.3) (Aldrich Chemical Co., Milwaukee, WI) were dissolved at a concentration of 1 mg in 10 mL of 95 % ethanol (EtOH) and a series of stock solutions were made by serial dilution ranging from 100 mM to 2.5 mM. Eight nominal exposure concentrations (0.25-10 μM) were prepared by adding 10 μL of the appropriate stock solution for every 100 mL of water. Control dishes contained only water while vehicle control groups were treated with 0.01 % EtOH.

2.2.3 Exposure conditions and measurements

Stage-specific toxicity tests were conducted by exposing *R. pipiens* tadpoles to a toxicant for two weeks at two different stages of development: (1) stage 26 which corresponds to the beginning of exogenous feeding and (2) stage 36 which is when the tadpoles start developing hindlimbs. These stages were chosen because they represent

the beginning of two distinct stages during tadpole metamorphosis (pre- and prometamorphosis) and can be clearly identified by morphological examination. Ten tadpoles were exposed in four replicates at each concentration of E2, EE2 or OP. Stage 26 tadpoles, with an individual wet weight of approximately 20-25 mg, were held in a 250 mL-capacity culture dishes from Carolina Biological Supply (Burlington, NC), so that the average density at the beginning of the exposure period was 0.8-1g/L. For the stage 36 exposure, tadpoles were randomly assigned to 1.5 L culture dishes from Carolina Biological Supply. The individual wet weight of stage 36 tadpoles ranged between 1.0-1.2 g; therefore, tadpole density for the later exposure was approximately 6.6-8 g/L.

Species-specific differences in contaminant toxicity were evaluated in a second experiment by exposing both *R. pipiens* and *R. sylvatica* tadpoles for two weeks starting at stage 26. Exposures were conducted in triplicate 250 mL dishes for each concentration of E2, EE2 and OP. *R. pipiens* and *R. sylvatica* tadpoles have similar body weights at stage 26 so the rearing density for both species was approximately 0.8-1 g/L.

Exposure was static renewal for both experiments. New treatment solutions were set up daily in separate dishes and tadpoles were transferred to new solutions. Daily renewal prevented fouling of the water. Mortalities were recorded every 24 hr and water volume was then adjusted to maintain consistent rearing density. At the end of the 2-week exposure period, tadpoles were anesthetized in 1.5 g/L MS-222, blotted dry and individually weighed on a digital balance (to the nearest milligram).

2.2.4 Statistical analysis

The LC_{50} is the most common toxicological test used to determine the relative toxicity of a compound to a particular organism. It is the concentration to cause mortality

in 50% of the test subjects over a specific period of time. LC_{50} values and 95% confidence intervals for all exposures were calculated using an LC_{50} BAS calculation program written by Charles E. Stephan (US EPA, Duluth, MN, 1986). A trimmed Spearman-Karber analysis was used (Hamilton *et al.*, 1977). The data were not corrected for mortality in the vehicle control groups. 95% confidence intervals (CIs) were used to determine differences between species and stages; differences were considered significant if the CIs did not overlap.

Statistical analysis for weights was performed using the SPSS for Windows Version 11.0.0. Data were first assessed for normal distribution (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test). Data were log transformed when necessary to meet the assumptions of a parametric test. Nested analysis of variance (ANOVA) was used to test for differences between treatments and vehicle control in the body weight of tadpoles after a 2-week exposure to various concentrations of E_2, EE_2, and OP. The nested ANOVA determines this variability between subsamples (the dish replicates) and separates it from the variation between treatment groups and control (Sokal and Rohlf, 1981). When differences at the lower level (within treatments or controls) were significant, Tukey's multiple comparison post-hoc test was used to determine which treatments were different from the control. Significance was indicated if $p \leq 0.05$.

2.3 Results

Tadpole mortality in the water control and vehicle control groups (EtOH) ranged from 0-3% at the end of the two-week exposure period. There was no significant difference in body weight between water and vehicle control groups in any of the treatments (data not shown). Therefore, LC_{50} values and body weight measurements in the treatment groups were compared to the vehicle control group.

2.3.1 Acute toxicity

The LC_{50} results for the two different developmental stages of *R. pipiens* are presented in Table 2.1. Across both developmental stages OP was the most toxic whereas E2 was least. OP and EE2 were 4- and 2-times more toxic than E2 to stage 26 tadpoles, respectively. An LC_{50} value for stage 36 tadpoles exposed to E_2 could not be obtained because the highest concentration (10 μM) failed to produce ≥ 50% mortality. The main requirement for estimating an LC_{50} is that at least one mortality proportion must be ≤ 50% and at least one must ≥ 50%. Stage 26 tadpoles were significantly more sensitive to all three compounds than stage 36 tadpoles. There was a statistically significant difference in LC_{50} values among and between each developmental stage for EE2 and OP.

The LC_{50} results for exposure of both *R. sylvatica* and *R. pipiens* tadpoles (stage 26) are presented in Table 2.2. LC_{50} values for *R. sylvatica* are approximately two times lower than *R. pipiens* for all three compounds. Rank order of toxicity for both species is OP>EE2>E2 and the LC_{50} values are significantly different between species for each of the compounds.

2.3.2 *Developmental effects*

Results for body weight following exposure of *R. pipiens* at the two developmental stages are presented in Table 2.3. In the stage 26 exposure, tadpole body weight was significantly decreased in groups exposed to high concentrations of E2 (2.5, 5.0 and 7.5 μM). However, there was also a significant effect of dish ($p<0.05$) within the E2 treatment replicates. There was no body weight recorded at concentrations ≥ 2.5 μM of OP exposures because there was 100% mortality in these groups. At stage 36, body weight of tadpoles exposed to E2 and EE2 was significantly lower than control at concentrations ≥ 5 μM. In the lower range of concentrations (0.25-2.5 μM), exposure to OP markedly increased tadpole weight. Exposure to OP at 7.5 μM and 10 μM resulted in 100% mortality.

The results for exposures of both *R. pipiens* and *R. sylvatica* at stage 26 are presented in Table 2.4. There was no significant difference in body weight of exposed *R. pipiens* compared to control across all concentrations of E2, EE2 and OP except for a significant increase in body weight at the highest non-lethal concentration of EE2 (5 μM) and OP (2.5 μM). Higher concentrations of OP (≥ 5 μM), EE2 (≥ 7.5 μM) and E2 (10 μM) resulted in 100 % mortality in *R. pipiens* tadpoles. *R. sylvatica* tadpoles exposed to either E2 (0.5-5 μM) or EE2 (0.75-5 μM) had significantly higher body weight than control. Higher concentrations of E2 (≥ 7.5 μM), EE2 (≥ 7.5 μM) and OP (≥ 2.5 μM) resulted in 100% mortality of *R. sylvatica* tadpoles after a 2-week exposure.

Table 2.1 Two-week LC_{50} values and 95% confidence intervals (C.I.) for two developmental stages of *R. pipiens* exposed to three estrogenic EDCs (E2, 17β-estradiol; EE2, 17α-ethinylestradiol; OP, 4-*tert*-octylphenol). Initial number of tadpoles/treatment = 360.

	2-week LC_{50} (μM)			
	Stage 26		*Stage 36*	
Chemical	LC_{50}	95% C.I.	LC_{50}	95% C.I.
E2	5.57[1]	5.17-5.99	>10	n.o.
EE2	3.01[a,2]	2.71-3.33	4.17[b,1]	3.87-4.49
OP	1.36[a,3]	1.22-1.52	2.80[b,2]	2.74-3.44

Significant differences in LC_{50} values between stages are indicated by a different letter superscript (a,b) while differences between compounds within each stage are indicated by a different number superscript (1,2,3).

Table 2.2 Two-week LC_{50} values and 95% confidence intervals (C.I.) for *R. sylvatica* and *R. pipiens* post-hatch tadpoles (Stage 26) exposed to three estrogenic EDCs (E2, 17β-estradiol; EE2, 17α-ethinylestradiol; OP, 4-*t*-octylphenol). Initial number of tadpoles/treatment = 270.

	2-week LC_{50} (μM)			
	R. sylvatica		*R. pipiens*	
Chemical	LC_{50}	95% CI	LC_{50}	95% CI
E2	$2.50^{a,1}$	2.11-2.96	$4.56^{b,1}$	4.01-5.19
EE2	$1.89^{a,1}$	1.61-2.22	$2.75^{b,2}$	2.41-3.14
OP	$0.74^{a,2}$	0.66-0.83	$1.42^{b,3}$	1.18-1.82

Significant differences in LC_{50} values between species are indicated by a different letter superscript (a,b) while differences between compounds within each species are indicated by a different number superscript (1,2,3).

Table 2.3 Effect of 2-week exposure to E2, EE2, and OP at two life-stages (stage 26 and 36) on body weight of *R. pipiens* tadpoles.

E2	*n*[a]	#[b]	Mean[c]	SD	EE2	*n*	#	Mean	SD	OP	*n*	#	Mean	SD
Stage 26					**Stage 26**					**Stage 26**				
Control	4	40	0.036	0.008	Control	4	40	0.036	0.008	Control	4	40	0.036	0.003
0.25[d]	4	37	0.038	0.006	0.25	4	39	0.037	0.007	0.25	4	36	0.039	0.002
0.50	4	37	0.037	0.008	0.50	4	37	0.038	0.009	0.50	4	36	0.039	0.003
0.75	4	35	0.038	0.009	0.75	4	35	0.037	0.007	0.75	4	34	0.035	0.004
1.00	4	35	0.034	0.008	1.00	4	31	0.034	0.008	1.00	4	32	0.036	0.004
2.50	4	34	0.030	0.007	2.50	4	23	0.029	0.007	2.50	-	-	-	-
5.00	4	30	0.028*	0.005	5.00	3	16	0.030	0.007	5.00	-	-	-	-
7.50	4	13	0.025*	0.007	7.50	-	-	-	-	7.50	-	-	-	-
10.0	2	-	-	-	10.0	-	-	-	-	10.0	-	-	-	-
Stage 36					**Stage 36**					**Stage 36**				
Control	4	40	1.43	0.110	Control	4	40	1.43	0.110	Control	4	40	1.43	0.110
0.25	4	38	1.45	0.131	0.25	4	38	1.42	0.154	0.25	4	38	1.62*	0.158
0.50	4	37	1.42	0.153	0.50	4	38	1.43	0.142	0.50	4	35	1.70*	0.116
0.75	4	37	1.43	0.115	0.75	4	35	1.46	0.155	0.75	4	34	1.65*	0.193
1.00	4	37	1.37	0.174	1.00	4	33	1.44	0.161	1.00	4	34	1.77*	0.053
2.50	4	38	1.37	0.163	2.50	4	28	1.40	0.148	2.50	4	30	1.30*	0.116
5.00	4	35	0.88*	0.106	5.00	4	21	1.15*	0.177	5.00	3	14	1.32*	0.124
7.50	4	35	0.90*	0.146	7.50	3	18	1.09*	0.150	7.50	-	-	-	-
10.0	4	30	0.91*	0.135	10.0	-	-	-	-	10.0	-	-	-	-

There was 100% mortality in treatment concentration after 2-week exposure in spaces noted with (-).
[a] Number of replicates (dishes)
[b] Number of individual tadpoles measured
[c] Body weight in grams (g)
[d] Nominal concentration in μM
* Significant difference from control ($p<0.05$; ANOVA; Tukey post-hoc).

Table 2.4 Effect of 2-week exposure to E2, EE2, and OP on body weight of *R. pipens* and *R. sylvatica* tadpoles (Gosner stage 26).

E2	*n*[a]	#[b]	Mean[c]	SD	EE2	*n*	#	Mean	SD	OP	*n*	#	Mean	SD
R. pipiens					***R. pipiens***					***R. pipiens***				
Control	3	30	0.037	0.010	Control	3	30	0.037	0.010	Control	3	30	0.037	0.010
0.25[d]	3	28	0.040	0.009	0.25	3	29	0.037	0.013	0.25	3	26	0.038	0.008
0.50	3	28	0.039	0.008	0.50	3	28	0.037	0.010	0.50	3	23	0.036	0.009
0.75	3	27	0.041	0.007	0.75	3	24	0.038	0.011	0.75	3	25	0.039	0.009
1.00	3	25	0.038	0.008	1.00	3	24	0.038	0.009	1.00	3	20	0.040	0.010
2.50	3	23	0.035	0.007	2.50	3	18	0.043	0.007	2.50	2	9	0.054*	0.009
5.00	3	19	0.036	0.009	5.00	3	11	0.051*	0.010	5.00		-	-	-
7.50	3	12	0.032	0.010	7.50		-	-	-	7.50		-	-	-
10.0		-	-	-	10.0		-	-	-	10.0		-	-	-
R. sylvatica					***R. sylvatica***					***R. sylvatica***				
Control	3	29	0.029	0.010	Control	3	29	0.029	0.010	Control	3	29	0.030	0.010
0.25	3	27	0.034	0.014	0.25	3	27	0.031	0.014	0.25	3	28	0.033	0.016
0.50	3	22	0.045*	0.016	0.50	3	24	0.036	0.012	0.50	3	25	0.031	0.015
0.75	3	24	0.043*	0.013	0.75	3	23	0.041*	0.016	0.75	3	23	0.035	0.016
1.00	3	22	0.045*	0.021	1.00	3	18	0.041*	0.015	1.00	2	2	0.033	0.015
2.50	3	18	0.045*	0.012	2.50	3	14	0.043*	0.012	2.50		-	-	-
5.00	3	14	0.046*	0.014	5.00	3	11	0.041*	0.011	5.00		-	-	-
7.50		-	-	-	7.50		-	-	-	7.50		-	-	-
10.0		-	-	-	10.0		-	-	-	10.0		-	-	-

There was 100% mortality in treatment concentration after 2-week exposure in spaces noted with (-).
[a] Number of replicates (dishes)
[b] Number of individual tadpoles measured
[c] Body weight in grams (g)
[d] Nominal concentration in µM
* Significant difference from control ($p<0.05$; ANOVA; Tukey post-hoc).

2.4 Discussion

2.4.1 Acute toxicity

Two-week exposures to waterborne E2, EE2 and OP were toxic to *R. pipiens* tadpoles at both developmental stages tested. Limited data indicate that during various stages of larval development, amphibians show different degrees of sensitivity to pesticides and their metabolites (Berrill *et al.*, 1993; Berrill *et al.*, 1995). In this study, stage 26 tadpoles were approximately 2-fold more sensitive (as measured by a lower LC_{50} value) to all three compounds when compared to stage 36 tadpoles. This variation in response may be due to developmental differences in physiological, hormonal and metabolic pathways at specific life-stages. The ability of xenoestrogens to induce intestinal metabolic enzyme activity in carp (*Cyprinus carpino*) increases with age and is attributed to the differential toxicity of these compounds during fish development (Yokota *et al.*, 2002). Similarly, early stage tadpoles may not yet have the necessary detoxification enzymes present to breakdown the parent estrogenic compounds into their metabolites and therefore accumulate the toxic compounds to the point of lethality. Inherent differences in body size at the time of exposure can also influence the degree of toxicity. Stage 26 tadpoles are approximately 5-fold smaller and therefore have more body surface per body weight than stage 36 tadpoles. Consequently, they are likely to absorb more of the compound for their body weight.

R. sylvatica tadpoles were approximately 2-fold more sensitive than *R. pipiens* to the toxic effects of all three compounds. Results from previous experiments assessing effects of environmental contaminants on amphibian survival and development suggest that there are species differences in response, even between closely related *Rana* species

(Bridges and Semlitsch, 2000; Mackenzie *et al.*, 2003). Tadpole body weight between the two species used in the present study is similar during early larval stages; therefore, other developmental differences between species could contribute to the variation in acute toxicity. A faster rate of larval development in *R. sylvatica* compared to *R. pipiens* was suggested to contribute to species differences in effects of retinoic acid (Degitz *et al.*, 2000). Similarly, the 2-week exposure in the present study would also encompass a wider developmental period for *R. sylvatica* tadpoles and therefore, may explain the higher lethality in this species.

2.4.2 Developmental effects

Estrogen is known for its diverse growth promoting effects in vertebrates. In *Xenopus laevis* tadpoles, exposure to 1 μM E2 resulted in increased body weight and advanced developmental stage (Nishimura *et al.*, 1997). Similarly, exposure to E2 (0.1 nM and 10 nM) increased body weights of mummichog fry (*Fundulus heteroclitus*) (Urushitani *et al.*, 2002). In both studies, these effects on early growth were observed after a prolonged exposure to the hormone.

In the present study, only the highest concentrations of E2, EE2 and OP affected tadpole body weight after a two-week exposure; however, there did not seem to be a dose-dependent response. It did appear that exposure during the late stage of development had the greatest effect on body weight. It has been proposed that steroid hormones may influence growth of larval amphibians by modulating thyroid hormone activity, the major stimulatory hormone of metamorphosis (Hayes, 1997). Inhibition of larval growth in amphibians is considered one of the most sensitive indicators of developmental toxicity (Snawder and Chambers, 1990). Amphibians must reach a

minimum size in order to initiate the metamorphic process (Wilbur and Collins, 1973); therefore, decreased growth at any stage of tadpole development can compromise metamorphosis. In a study by Snodgrass *et al.* (2004) developmental exposure to coal combustion waste delayed tadpole growth of *R. sylvatica* and *R. clamitans*. Even at metamorphosis, treated animals were up to 40% smaller than their respective controls, demonstrating that adverse effects on tadpole growth during development may be permanent. Smaller metamorphs are less likely to survive predation, reach sexual maturity or effectively compete for resources (Werner, 1986). Therefore, environmental contaminants that influence tadpole size at critical periods during larval development may alter the timing of metamorphosis and long-term health.

Taking into consideration initial tadpole weights and rearing conditions in this study, the mean loading density during the stage 26 exposures (for *R. pipiens* and *R. sylvatica*) was approximately 1 g/L, whereas loading densities for stage 36 tadpoles were approximately 7 g/L. The latter density is above the recommended rearing density according to ASTM guidelines (ASTM, 2000). Although the length of exposures was only 2 weeks, density-induced stress may have affected the degree of toxicity at stage 36. Arthur and Dixon (Arthur and Dixon, 1994) determined that rearing density affected variability in fathead minnow wet weight over 28 days, with or without the addition of a toxicant. Density has also been shown to influence responses to contaminant exposure in amphibians (Cooke, 1979; Boone and Semlitsch, 2001). Therefore, it is not appropriate to use body weight as a parameter for comparing the relative toxicity of a contaminant between tadpole life-stages in the present study. However, if high density increases toxicity, then the compounds tested in this study would be less toxic to stage 36 tadpoles

if exposed at a lower density of 1 g/L. This would result in a larger difference in LC_{50} values between the two stages and would emphasize the importance of including multiple tadpole life-stages when assessing the toxicity of a contaminant to a particular amphibian species.

2.4.3 Environmental concentrations

It is unlikely that amphibian larvae ever experience E2 and EE2 concentrations in their natural habitats that approach the 2-week LC_{50} levels reported in this paper. However, our results provide the basic data necessary to design future studies examining the sublethal effects of these xenoestrogens in *R. pipiens* and *R. sylvatica*.

The most toxic compound examined in this study, irrespective of stage or species, was OP (stage 26 tadpole LC_{50} = 1.36-1.42 μM). As the two main breakdown products of alkylphenol polyethoxylates (APEOs), both OP and its counterpart nonylphenol (NP) are ubiquitous in the aquatic environment. NP constitutes 80% of the alkylphenols found in surface waters and sediments while 15% is OP. As a result, NP and its ethoxylates have been banned in many European countries; the Canadian Environmental Protection Act added NP to its Canadian Priority Substances List 2 in December 1995 (Maguire, 1999). Although OP is considered to be 10-20 fold more estrogenic than NP (White *et al.*, 1994), it has been often overlooked as an environmental threat because of its lower usage. In addition, OP, along with its major metabolite, octylcatchol, has been shown to bioconcentrate in various rainbow trout tissues over only a 4-day exposure (Ferreira-Leach and Hill, 2001). Blazquez *et al.* (1998a) found that 1 μM OP produced 50% mortality among immature male goldfish (*Carassius auratus*) 20 days after exposure.

In water, OP has been detected at concentrations of up to 13 μg/L (~63 nM) in an

estuary in the UK (Blackburn and Waldock, 1995); however, concentrations of approximately 1-10 μg/L (~5-50 nM) are more common (Bennie, 1999). As a persistent lipophilic compound, OP is found in sediments and sludge at higher concentrations than in surface water. Sediment samples taken from Great Lakes basin and St. Lawrence River area contained OP levels ranging from <0.01 – 1.8 μg/g dry weight (Bennie *et al.*, 1997) while in sediment and sludge from STPs, OP has been detected at concentrations of up to 12.1 μg/g dry weight (Lee and Peart, 1995). These sediment levels of OP, in the low ppm range, are higher than the LC_{50} concentrations for *R. pipiens* (1.36-2.80 μM; ~0.28-0.57 ppb) and *R. sylvatica* (0.74 μM; ~0.15 ppb). Therefore, species that over-winter as tadpoles under silt and on sediment may be exposed to toxic levels of OP for extended periods of time during sensitive larval stages.

CHAPTER 3

Critical periods of development: estrogenic exposure affects metamorphosis and alters sex ratios in *Rana pipiens*

3.1 Introduction

The plasticity of metamorphosis allows tadpoles to vary the timing of their development according to environmental conditions (Denver, 1997b). The brain translates external stimuli into neuro-hormonal signals which, in a developing tadpole, function to both organise and activate several hormone axes (*i.e.*, thyroid, reproductive, stress). During the transformation process from larval tadpole to juvenile frog, there are critical periods for the development and function of several endocrine systems and organs. While developmental plasticity allows the tadpole to respond to natural conditions so as to direct growth, metamorphosis, and reproduction, these processes are also susceptible to anthropogenic stressors, such as environmental contaminants. Therefore, in the context of critical periods, the developmental stage at which an amphibian is exposed to contaminants would affect its sensitivity to adverse effects.

The larval to juvenile period of metamorphosis can be divided into three distinct periods; premetamorphosis, prometamorphosis, and metamorphic climax (Etkin, 1964; Dodd and Dodd, 1976). Each period is characterised by unique morphological and biochemical transformations that are mediated by changes in the synthesis and action of endogenous hormones, especially thyroid hormone (TH). Most morphological and physiological changes that occur during natural metamorphosis can be accelerated by treating tadpoles with TH, and can be slowed by blocking TH action.

Gonadal differentiation is another hormone-directed process during amphibian development. Amphibians exhibit extremely diverse patterns of sexual differentiation that vary between and within species (Hayes, 1998). In several species (including Ranids) the critical period of sex differentiation occurs during the early stages of premetamorphosis (Richards and Nace, 1978; Ogielska and Kotusz, 2004). Exposure to exogenous sex steroid hormones during this window can dramatically change the phenotypic sex of tadpoles, resulting in complete sex reversal or abnormal gonadal intersex (where gonads contain both ovarian and testicular tissue) (Chang and Witschi, 1956; Richards and Nace, 1978).

Exposure to hormonally active contaminants, such as xenoestrogens, has the potential to influence several developmental processes in amphibians, particularly given the stage-specific endocrine control involved in amphibian growth, metamorphosis, and reproduction. The critical periods of sex differentiation and somatic growth occur during the aquatic life stages when, in the wild, tadpoles may be exposed diverse sources of estrogenic contaminants (*e.g.*, pharmaceuticals, pesticides, detergents). There is evidence that early exposure to high levels of estrogen increased mortality and the incidence of malformations, such as crooked vertebrae and swollen stomachs, in *X. laevis* embryos (Nishimura *et al.*, 1997; Bevan *et al.*, 2003). In addition, exposing ranid tadpoles to xenoestrogens throughout metamorphosis resulted in female-biased sex ratios and abnormal gonadal development (Mackenzie *et al.*, 2003). However, it is unknown whether short-term exposure during critical periods could also have lasting effects on metamorphic development and phenotypic sex.

The critical period or "window" is of particular significance when attempting to explain the developmental effects of xenoestrogen exposure. Experiments employing stage-specific exposures have been extremely effective in determining critical periods of sensitivity to xenoestrogens in other aquatic vertebrates, especially fish (Blazquez *et al.*, 1998b; van Aerle *et al.*, 2002; Anderson *et al.*, 2003). Establishing the sensitivity of different developmental stages not only provides valuable assessment information, but also provides important information concerning the modes of action of these developmental and reproductive toxicants. Targeted exposures during specific stages of tadpole development and follow-up assessment at metamorphosis would also provide information concerning the potential long-term consequences of xenoestrogen exposure.

The objective of this study was to evaluate the immediate and long-term effects of estrogenic exposure during critical periods of *R. pipiens* metamorphosis and gonadal differentiation. Tadpoles were exposed to 17α-ethinylestradiol (EE2) over three distinct developmental phases as well as for the entire length of metamorphosis. Metamorphic parameters were assessed following EE2 exposures and at metamorphic climax. In addition, histological analysis of the gonads was conducted for exposures encompassing the critical period of gonadal sex differentiation to determine whether a targeted exposure could affect gonadal development and sex ratios.

3.2 Methods

Details regarding the location from which eggs were obtained, laboratory rearing conditions and general maintenance are described in Chapter 2 (2.2 Methods; *2.2.1 Animals and rearing conditions*).

3.2.1 Developmental exposure regime

At the initiation of feeding (stage 27; approx. two weeks post hatch), healthy individuals were randomly assigned to tanks containing 12 L of water (1 tadpole/L) and the exposure regime began. Details of the experimental design and the developmental characteristics of the selected exposure periods are depicted in Figure 3.1A and 3.1B, respectively. In total, 27 tanks, each containing 12 tadpoles, were randomly assigned to one of 9 experimental groups to be treated with 17α-ethinylestradiol (EE2; Sigma-Aldrich, Oakville, ON) or vehicle (95% ethanol) for various periods. A stock solution of 100 μM EE2 was prepared in ethanol (95% EtOH) and 500 μL was added to the treatment water for a final nominal concentration of 5 nM EE2. A fresh stock solution of EE2 was made every two weeks over the duration of the experiment.

Throughout the exposure regime, the position of the tanks within the treatment room was altered weekly to minimize confounding effects of tank location on development. Tadpoles were fed a mixture of Nutrafin flakes (Rolf C. Hagen Inc, Montreal, PQ) and tadpole food (Carolina Biological Supply Co., Burlington, NC) daily *ad libitum*. A 75% water change was performed every 48 hr and any uneaten food and waste were removed from the bottom of the tanks at this time. Animals were monitored at each feeding for mortality and incidence of abnormalities and developmental staging was performed every 4-5 days.

For the Early and Mid exposure periods, surviving tadpoles in three of the EE2 exposure tanks (randomly selected) were sacrificed when 80% of the tadpoles in corresponding control tanks reached the designated Gosner stage (see Figure 3.1A). Tadpoles in the remaining three tanks continued to develop through to metamorphic

climax (stage 42) in the presence of the EtOH vehicle (final concentration 50 μL/L; 0.005%). Individuals were sacrificed upon reaching stage 42 (forelimb emergence). Water levels in each tank were adjusted accordingly to maintain a consistent loading density. The experiment was terminated at 154 days (22 wks) and remaining animals that had not yet reached metamorphic climax were counted.

3.2.2 Morphological measurements and gonadal histology

At the end of each exposure period, animals were anesthetized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, Sigma-Aldrich; 1 g/L in water). For tadpoles, body weight, total length and developmental stage were recorded. For metamorphs, body weight, snout-vent length (SVL) and days-to-metamorphosis (DTM) were recorded. Animals were fixed and decalcified in Cal-Ex II (Fisher Scientific, Ottawa, ON) for one week after which they were transferred into 70% ethanol for storage until histological processing.

Fixed specimens were removed from the ethanol and dissected. The abdominal cavity was opened, and the internal tissues were removed (except the gonads). Anatomical sex was initially determined by direct visual inspection using a binocular dissecting microscope (Zeiss 2000) (Figure 3.2). The animals were identified as male, female, or unknown anatomical sex. The kidneys were dissected out of the body cavity with the gonads attached (to minimize the risk of damage to the gonad) and placed in individual plastic tissue cassettes (Fisher Scientific). At this time, individuals were assigned a random number to facilitate blind analyses. Preserved tissues were dehydrated in a graded series of increasing ethanol concentration, cleared in xylene and embedded in paraffin using an automated Tissue-Tek VIP Vacuum Infiltration Processor (Sakura

E150/E300 Series). For a detailed tissue processing and embedding protocol, see Appendix 1.

Tissues were sectioned using a rotary microtome knife (Microm Heidelberg HM350). Serial 5 μm sagital sections were spread in a water bath (49°C) containing STA-On tissue section adhesive (Surgipath, Richmond, IL) and mounted on precleaned glass slides (Surgipath). Slides were dried at 57°C for 30 minutes and the sections were deparaffinized in xylene, rehydrated in ethanol and stained with hematoxylin and eosin. For a detailed staining protocol, see Appendix A. The stained sections were examined by an observer who was completely blind to the treatment and phenotypic sex as previously determined. Each sample was identified as female, male, or intersex (defined as the presence of male and female gonadal tissue within a single structure). Digital micrographs were captured using a Micro Publisher 3.3 Digital microscope camera (QImaging Corp. Burnaby, BC).

3.2.3 Statistical analysis

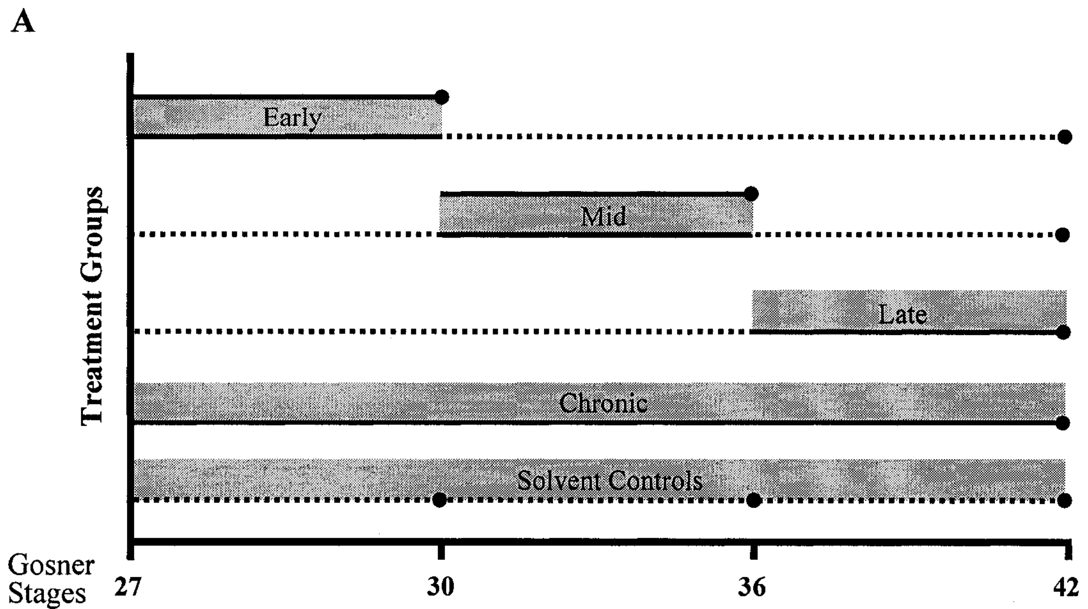
Differences in weight, length, and stage of tadpoles following the early and mid-metamorphosis exposures were analyzed using nested t-tests (individuals nested within tanks, replicate tanks nested within treatments). Since tadpole size could also be a function of developmental stage, weight and length were used as a covariate for stage within the statistical model; conversely, stage was used as a covariate for weight and length. For both exposure periods, there was no effect of tank or covariates in the analysis.

Differences in weight and SVL at metamorphosis were analyzed using nested ANOVAs followed by Dunnett's pair-wise comparison to control. There was no effect of

tank variation on response to EE2 treatment. DTM data did not conform to parametric assumptions so a Kruskal-Wallis ANOVA on ranks was used to test for an effect of tank within each treatment. Since there was no difference in DTM between tanks, data were pooled and differences between treatments were assessed using Kruskal-Wallis followed by Dunn's post-hoc test. A Spearman rank order correlation was performed to determine whether there was any relationship between the length of larval period and size. Developmental data were analyzed using SPSS 14.0 (SPSS Inc., Chicago, IL).

Chi-square analysis was used to compare mortality between the treatment and control groups. For statistical analyses of the sex ratios, individuals were classified as having either the male, female or intersex condition. The intersex condition was categorized as a sex distinct from normal males or normal females. Due to the low number of observations in either category, the Fisher's Exact test was used to determine differences in male:female (M:F) sex ratio between EE2-exposed and control groups. Statistical differences in the proportion of intersex between control and exposed groups were calculated using the Z-test. Proportion and sex ratios were analyzed using SigmaStat 3.1 (SPSS Inc., Chicago, IL). Statistical significance for all tests was set at $p \leq 0.05$.

A



B

Exposure	Stages[a]	Developmental Significance[b]
Early	27-30	Early premetamorphosis, limb bud development, no endogenous TH, gonadal differentiation
Mid	30-36	End of premetamorphosis, foot-paddle development, endogenous TH levels rising
Late	36-42	Prometamorphosis to climax, substantial hindlimb development and forelimb emergence, peak TH levels
Chronic	27-42	See above

[a] Based on Gosner (1960).
[b] For details refer to Etkin (1968), Dodd and Dodd (1976), Shi (2000).

Figure 3.1 Experimental design (A) and developmental characteristics (B) for exposures of *Rana pipiens* tadpoles to 17α-ethinylestradiol (EE2). There were three replicate tanks for each treatment group and corresponding solvent controls. Solid lines represent EE2 exposure (5 nM) and dotted lines represent exposure to solvent (EtOH; 0.005%). Animals were sampled and compared at the Gosner stages indicated (•).

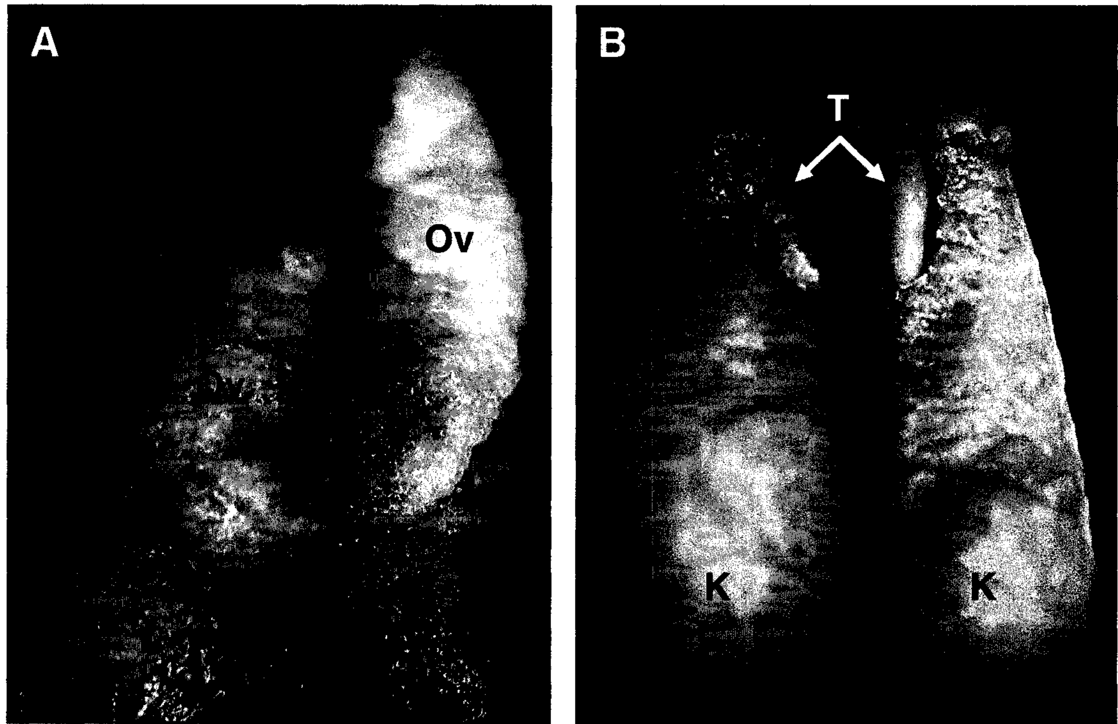


Figure 3.2 Photographs of female (A) and male (B) gonads at metamorphic climax (stage 42) before removal from body cavity. Bar represents 1 mm. Ov, ovary; T, testes; K, kidney.

3.3 Results

3.3.1 Morphology and development

Results for the growth and development of tadpoles exposed to EE2 and assessed during early and mid-metamorphosis are presented in Table 3.1. Tadpoles exposed during early metamorphosis had a significantly greater body weight ($p = 0.03$) and length ($p = 0.04$) than individuals from the control group (nested t-test). There was no effect of early exposure on developmental stage of the tadpoles ($p > 0.05$). In contrast, EE2 exposure during mid-metamorphosis did not affect tadpole body weight or length ($p > 0.05$). However, the EE2-treated tadpoles were developmentally delayed ($p = 0.006$).

Results for groups exposed during various periods of metamorphosis and assessed at metamorphic climax are presented in Table 3.2. Developmental exposure to EE2 did not affect body weight or SVL at metamorphic climax when compared to the control group ($p > 0.05$; ANOVA with Dunnet's post-hoc). However, the number of days it took for each group to reach metamorphic climax varied with the developmental period of EE2 exposure. Tadpoles exposed during early metamorphosis took the same number of days to reach metamorphic climax as the controls. However, tadpoles exposed during mid-metamorphosis or for their entire larval period (chronic) were delayed in reaching climax by a median of 16 and 18 days, respectively ($p \leq 0.05$; Kruskal-Wallis ANOVA with Dunn's post-hoc). Tadpoles exposed late in development also appeared to be delayed (by approx. 10 days) but this was not statistically significant. Spearman rank order correlations indicated that metamorph weight and length were not indicative of number of days to metamorphosis ($r = 0.004$ and $r = 0.03$, respectively; $p > 0.05$).

Mortality ranged between 14-31% over the course of the experiment, but was not significantly different between replicates or between control and treatment groups ($p > 0.05$; Chi-square). Although some individuals exhibited edema (distension of the body with fluid) when observed during daily monitoring, there was no difference between treated and control groups. Some tadpoles (1-4 individuals/treatment) had not reached metamorphic climax when the experiment was terminated at 154 days; however, there were no significant differences between control and treatment groups ($p > 0.05$; Chi-square). These developmentally delayed tadpoles displayed a range of developmental stages and were not included in the morphometric or sex ratio analyses.

3.3.2 Gonadal histology

The gonadal sex ratios for the control groups at stage 30, stage 36 and stage 42 are shown in Figure 3.3. Within the stage 30 control group, there were three animals identified with intersex gonads and described as having numerous immature oocytes within testicular tissue (Figure 3.4). The proportion of intersex (0.15) at stage 30 did not differ significantly from the other two control groups at later developmental stages ($p > 0.05$; z-test). At stage 36 (beginning of prometamorphosis) and at stage 42 (metamorphic climax), none of the gonads from control animals exhibited an intersex condition. A comparison of the male:female (M:F) sex ratios revealed that they did not significantly differ among the three developmental stages ($p > 0.05$; Fisher's Exact test).

At stage 30, the M:F sex ratio of the EE2 exposed group was significantly different from the control (3:12 *vs* 9:8 of control; $p \leq 0.05$; Fisher's Exact test; Figure 3.5). There were five intersex animals identified in the EE2 exposed group, but this

proportion (0.20) was not statistically different from the proportion of intersex (0.15) in the control ($p > 0.05$; z-test).

At metamorphic climax, the M:F sex ratio within the early-exposed group was skewed towards females ($p \leq 0.05$) as only two of 24 individuals had distinct testes (Figure 3.6). There was also a significantly greater proportion of intersex animals in this group (0.17) when compared to the control group in which there were no intersex ($p \leq 0.05$; z-test). Exposure to EE2 for the entire larval period (chronic) did not affect the M:F sex ratio at climax; however, this group had the largest proportion of intersex (0.30) when compared to the other two groups ($p \leq 0.01$ versus control; z-test).

Representative sections of intersex gonads from metamorphs in the EE2-exposure groups are shown in Figure 3.7. The number of oocytes within testicular tissue varied between 1-10 and usually occurred unilaterally on the left side and at the anterior end of the gonad. One individual (from the chronic exposure) was a bilateral intersex, with oocytes present in both the left and right gonads.

There were no observable differences in the morphology of the completely differentiated gonads (not intersex) from control and EE2 exposed frogs. At metamorphic climax, the ovaries were large structures completely filled with diplotene oocytes that contained a large round nucleus surrounded by a layer of darkly staining follicle cells (zona granulosa). In some cases, multiple nucleoli were visible in the nucleus of the primary oocytes. The oocytes were arranged in a gradient from the smallest in the most external part of the ovary to the largest in the center. Oogonia (immature oocytes) were observed along the periphery of the ovary in the germinal epithelium. Early signs of vitellogenesis were observed in some ovaries as indicated by

the appearance of cortical alveoli (vesicles) in the periphery of the follicular (granulosa) cells. Atretic or degenerating follicles were also observed in the ovaries of control and EE2-exposed groups. Testes were substantially smaller than ovaries (Figure 3.2). In the testes, primary spermatogonia were visible surrounded by Sertoli cells. However, sagital sectioning of the testes did not allow detailed assessment of the seminiferous tubules.

Table 3.1 Effect of 17α-ethinylestradiol (EE2; 5 nM) exposure during early and mid-metamorphosis on developmental endpoints in *R. pipiens* tadpoles[a]

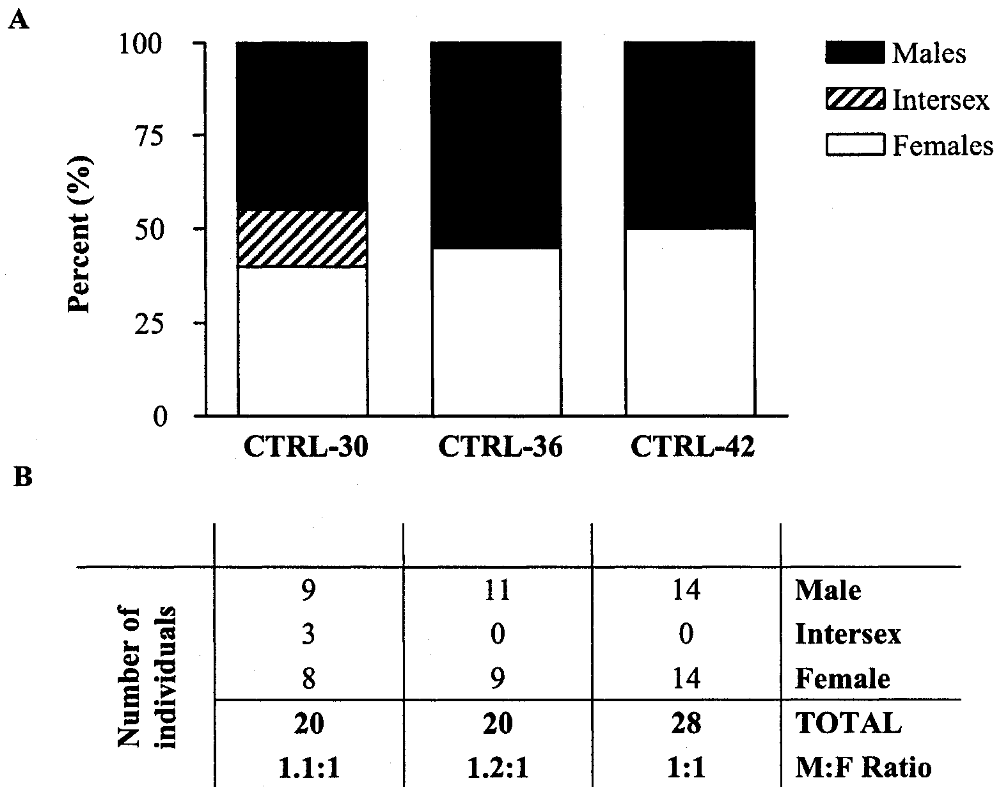
Exposure Period	Treatment	n[b]	Weight (g)	Length (mm)	Stage[c]	Mortality
Early	Control[d]	27	0.42 ± 0.09	33.5 ± 3.9	29.9 ± 1.4	9 (25%)
	EE2	25	0.48 ± 0.10*	37.6 ± 3.4*	30.1 ± 2.1	11 (31%)
Mid	Control	26	1.41 ± 0.15	69.1 ± 7.3	35.7 ± 0.8	10 (28%)
	EE2	27	1.43 ± 0.12	70.1 ± 5.9	34.6 ± 1.6*	9 (25%)

[a] Values are the mean ± SD.
[b] Initial sample size was 36 tadpoles (12 tadpoles per replicate, 3 replicates per treatment)
[c] Based on Gosner stages (Gosner 1960)
[d] Exposed to EtOH (0.005%)
* Significantly different from control ($p \leq 0.05$, t-test)

Table 3.2 Effect of 17α-ethinylestradiol (EE2; 5 nM) exposure during various periods of metamorphosis on developmental endpoints at metamorphic climax in *R. pipiens* [a]

Treatment	Exposure Period	n[b]	Weight (g)	SVL (mm)	DTM[c]	Mortality
Control[d]	--	28	1.38 ± 0.35	20.7 ± 2.1	105	7 (19%)
EE2	Early	24	1.26 ± 0.45	20.3 ± 2.4	102	9 (25%)
	Mid	29	1.41 ± 0.26	21.6 ± 0.9	121*	5 (14%)
	Late	28	1.28 ± 0.27	19.9 ± 1.2	113	7 (19%)
	Chronic	27	1.38 ± 0.26	20.7 ± 1.6	123*	5 (14%)

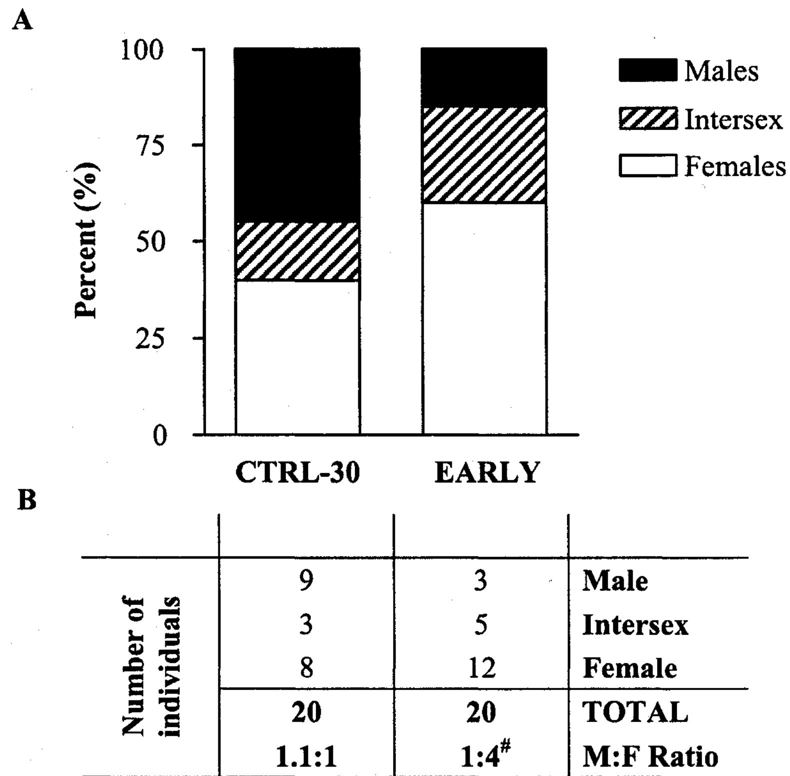
[a] Values are the mean ± SD.
[b] Initial sample size was 36 (12 tadpoles per replicate, 3 replicates per treatment)
[c] Median value
[d] Exposed to EtOH (0.005%)
* Significantly different from control ($p \leq 0.05$; Kruskal-Wallis ANOVA on Ranks)



Number of individuals	CTRL-30	CTRL-36	CTRL-42	
	9	11	14	**Male**
	3	0	0	**Intersex**
	8	9	14	**Female**
	20	**20**	**28**	**TOTAL**
	1.1:1	**1.2:1**	**1:1**	**M:F Ratio**

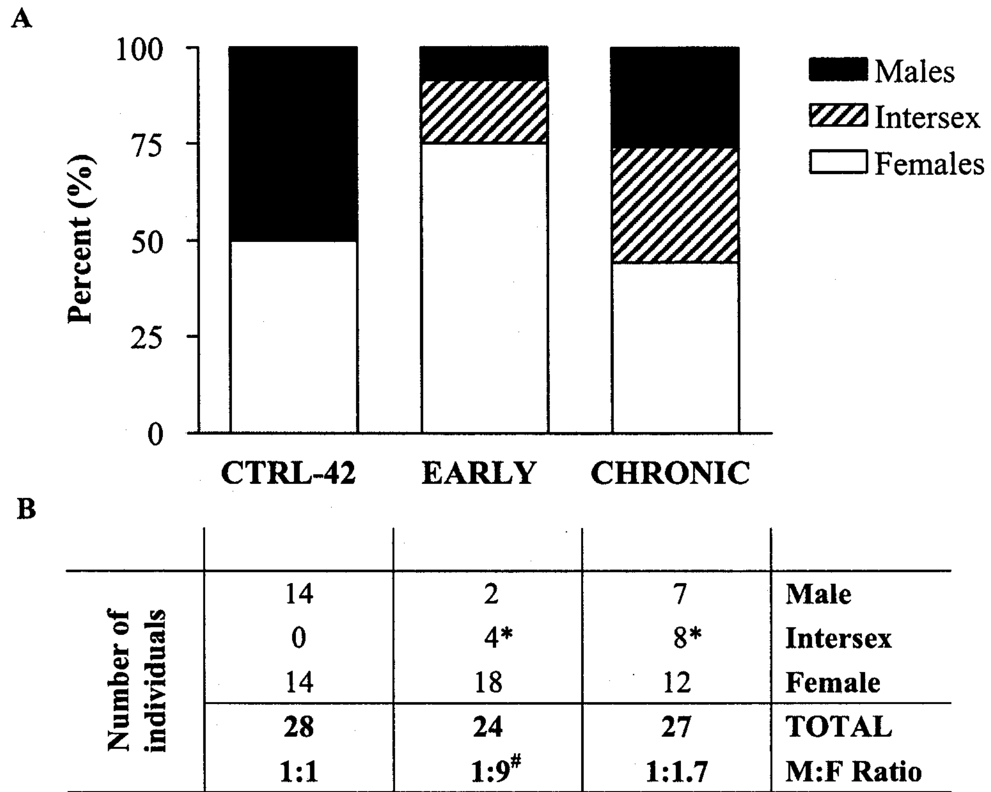
Figure 3.3 Sex ratios of *R. pipiens* at Gosner stages 30, 36 and 42 based on histological analysis of the gonads. All animals were exposed to the vehicle (EtOH; 0.005%). Graphical data are expressed as percent male, female, and intersex (A) while the numbers of individuals observed in each of the three conditions are indicated in the table (B).

Figure 3.4 Histological section of an intersex gonad from a stage 30 tadpole in the control group (CTRL-30). Bar represents 100 μm. OF, ovarian follicle; T, testicular tissue; K, kidney.



Number of individuals	CTRL-30	EARLY	
	9	3	**Male**
	3	5	**Intersex**
	8	12	**Female**
	20	**20**	**TOTAL**
	1.1:1	**1:4#**	**M:F Ratio**

Figure 3.5 Effect of EE2 exposure on gonadal sex ratios of tadpoles at stage 30 based on histological analysis. *R. pipiens* tadpoles were exposed to 5 nM EE2 at the beginning of larval development (stages 27-30; EARLY). Data for the control group (CTRL-30) were previously represented in Fig 3.3 and are presented here for comparison. Graphical data are expressed as percent male, female, and intersex (A) while the numbers of individuals observed in each of the three conditions are indicated in the table (B). Sex ratios for each group and incidence of intersex were compared to the control (CTRL-30). Symbol represent significant difference in sex ratio (#; Fisher's Exact test) compared to CTRL-30.



Number of individuals	CTRL-42	EARLY	CHRONIC	
	14	2	7	**Male**
	0	4*	8*	**Intersex**
	14	18	12	**Female**
	28	**24**	**27**	**TOTAL**
	1:1	**1:9#**	**1:1.7**	**M:F Ratio**

Figure 3.6 Effect of EE2 exposure on gonadal sex ratios at metamorphic climax (stage 42) based on histological analysis. *R. pipiens* tadpoles were exposed to 5 nM EE2 during either the beginning of larval development (stages 27-30; EARLY) or throughout the entire period of metamorphosis (stages 27-42; CHRONIC). Data for the control group (CTRL-42) were previously represented in Fig 3.3 and are presented here for comparison. Graphical data are expressed as percent male, female, and intersex (A) while the numbers of individuals observed in each of the three conditions are indicated in the table (B). Symbols represent significant differences in proportion of intersex (*; Z-test) and sex ratios (#; Fisher's Exact test) compared to the control (CTRL-42).

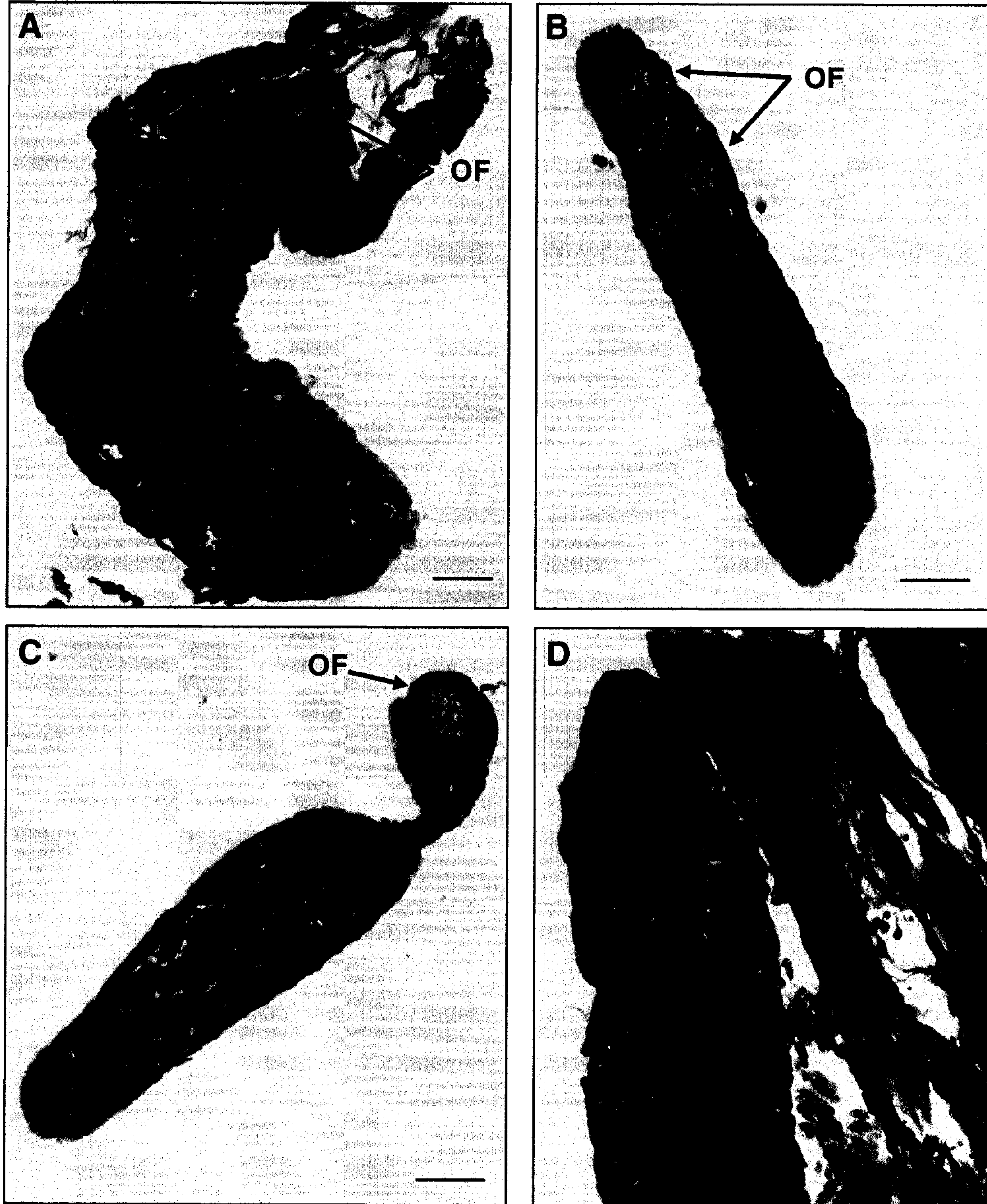


Figure 3.7 Histological sections of intersex gonads observed at metamorphic climax (stage 42). Individuals were exposed to 5nM EE2 at the beginning of larval development (EARLY; A) or throughout metamorphosis (CHRONIC; B-D). Bar represents 100 μm. OF, ovarian follicles; T, testicular tissue.

3.4 Discussion

3.4.1 Growth and metamorphosis

EE2 exposure during early larval development resulted in larger tadpoles at stage 30, as determined by greater body weight and SVL. Although the hormonal regulation of early tadpole growth is not well understood, premetamorphic development (from hatch to stage 34) is considered TH-independent due to the lack of a functional thyroid gland (Dodd and Dodd, 1976). Hormones such as prolactin (PRL) and growth hormone (GH) are considered to promote growth during the early stages of development; however, this hypothesis was generated from experiments where tadpoles were treated with mammalian hormone preparations (White and Nicholl, 1981; Kikuyama *et al.*, 1993). Mammalian PRL was reported to have a greater growth-promoting effect than mammalian GH in tadpoles, whereas mammalian GH was said to be more potent in juvenile frogs (Brown and Frye, 1969). Recent studies with transgenic *X. laevis* showed that while overexpression of xPRL or ovine PRL (oPRL) did not increase larval growth (Huang and Brown, 2000c), overexpression of GH resulted in larger tadpoles and metamorphs (Huang and Brown, 2000b). There is evidence of E2-induced GH production and release in goldfish (Trudeau *et al.*, 1992) in addition to E2-stimulation of GH and PRL mRNA in rainbow trout pituitary culture (Elango *et al.*, 2006). Our data suggest the hypothesis that EE2 may be stimulating early tadpole growth through estrogenic regulation of GH or PRL production.

Tadpoles exposed to EE2 during mid-metamorphosis (stage 30-36) were developmentally delayed immediately following exposure and in reaching metamorphic climax, as determined by staging and DTM, respectively. Interestingly, tadpoles exposed

during this period were also significantly delayed in reaching metamorphosis. This mid-metamorphosis period of EE2 exposure encompasses the end of premetamorphosis and beginning of prometamorphosis, the latter being characterised by the onset of thyroid gland function, rising TH levels, and TH-dependent morphogenesis. Gray and Janssens (1990) found that late premetamorphic *X. laevis* tadpoles exposed to E2 were resistant to T3-induced changes in development (body condition, gut length). E2 treatment has also been demonstrated to induce hypoactivity of the thyroid follicles in the eel (*Anguilla anguilla*) (Olivereau *et al.*, 1981). Although there is no direct physiological evidence in amphibians for estrogenic modulation of the thyroid system, it is possible that estrogenic suppression of TH action could delay tadpole development as observed here.

There was a stage-specific effect of EE2 exposure on the number of days required for tadpoles to reach metamorphic climax. Only the chronic and mid-metamorphic EE2 exposures delayed development, suggesting a heightened sensitivity to EE2 during the TH-responsive metamorphic stages. Late pre- and early prometamorphic stages have been recommended for assays testing thyroid antagonist and agonist actions (OECD, 2004). The thyroid gland and several other factors involved in TH regulation (*e.g.*, receptors and enzymes) are acquiring activity during this period of tadpole development. Therefore, this would likely be the most sensitive window for xenoestrogen-inhibition of the thyroid axis and associated developmental events during metamorphosis.

3.4.2 Sex ratios and gonadal differentiation

The phenotypic sex ratios of the control animals were examined at three stages of development. While there was a proportion of intersex tadpoles at stage 30, gonads appeared to be morphologically distinct (male and female) in all individuals by stage 36.

This supports a previous study by Humphrey *et al.* (1950) where, in *R. pipiens*, gonads were morphologically distinct by Taylor-Kollros (1946) stage VI which is approximately equivalent to Gosner stage 31. In amphibians, as with most vertebrates, the gonads go through a phase during early development where they are not yet completely differentiated into male and female organs (Witschi, 1967). However, many species, including *R. pipiens,* possess sexually distinct male and female gonads at metamorphic climax and are therefore considered sexually differentiated races (Gallien, 1974). The *R. pipiens* used in this study also completed their gonadal differentiation by late pre-metamorphosis as indicated by the absence of intersex at stage 36 and at metamorphic climax in the control animals.

There was a significant female-biased sex ratio immediately following the early EE2 exposure (stage 30), indicating that early larval development is a sensitive period for the feminizing effects of EE2. A previous study by Richards and Nace (1978) also showed that Taylor-Kollros stages I-VII (approximate Gosner stages 26-32) were the optimal developmental period for E2-induced feminization of *R. pipiens* tadpoles. Using known populations of genotypic male *X. laevis* tadpoles, early work by Chang and Witschi (1956) clearly demonstrated that E2 exposure during a specific period of development was able to hormonally sex reverse tadpoles producing 100 % phenotypic females. Therefore, the ability E2 to promote female gonadal development suggests that some of the EE2-exposed females and intersex individuals in the present study are sex-reversed males. However, while it is likely that the gonads of genotypic males are being feminized by EE2, this is speculative since the genotypic sex of the *R. pipiens* tadpoles in this study is unknown.

Tadpoles exposed to EE2 during the critical period of sex differentiation exhibited signs of feminization at metamorphic climax. Specifically, the tadpoles exposed early in development displayed a higher incidence of intersex and a strong female-biased sex ratio, while, in comparison, the control group contained no intersex individuals and exhibited a 1:1 M:F sex ratio. The chronic exposure also had the highest incidence of intersex animals at metamorphic climax. These results demonstrate that exposure to an estrogenic compound during a critical period of sex differentiation can result in altered gonadal development and sex ratios. Mackenzie *et al.* (2003) employed a continuous exposure regime to examine effects of estrogenic EDCs on gonadal development in Ranids and found that EE2 was effective in producing all female or female-biased sex ratios. While their exposure period encompassed stage 25 through stage 46 (complete tail resorption), the authors speculated that the specific stages of gonadal differentiation are likely the sensitive period for the developmental effects of xenoestrogens. In the current study, the feminizing effects of EE2 following early exposure, but still observed at metamorphic climax, confirm that estrogenic exposure targeted during the critical stages of gonadal differentiation can effectively disrupt amphibian sexual development.

Early exposure to EE2 altered sex ratios and increased the incidence of intersex at metamorphic climax, approximately 2-3 months following exposure, demonstrating that the effects of EE2 on gonadal differentiation are persistent. This agrees with previous data in *R. pipiens* where E2 exposures spanning the early stages of tadpole development produced near 100 % phenotypic female metamorphs (Richards and Nace, 1978). However, E2-treatment of another ranid species, *R. curtipes*, during its critical period of gonadal development did not affect sex ratios at metamorphic climax (Saidapur *et al.*,

2001). Although thereare few data on the potential reversibility of hormone-induced feminization in amphibians, studies in fish indicate that the persistence of xenoestrogen-induced sex reversal is highly variable and depends on species-specific modes of sex differentiation (Carlson *et al.*, 2000; Orn *et al.*, 2006). Whether there are species-specific characteristics that contribute to the persistence of EE2 effects in *R. pipiens* is an interesting question and remains to be explored.

The gonads of intersex animals displayed some interesting patterns in morphology. Only one individual (from the chronic exposure) exhibited bilateral intersex while the remaining affected individuals exhibited intersex in the left gonad only. Left-biased asymmetry in gonadal differentiation has been documented in vertebrates. In chickens, the gonads display an asymmetry in gonadal differentiation with only the left gonad becoming an ovary (Smith and Sinclair, 2004). In mammals, the right gonad grows faster than the left gonad (Mittwoch, 1996) and is functionally dominant as indicated by the fact that ovulation occurs more frequently from the right *vs* left ovary (Fukuda *et al.*, 2000). Asynchronous gonadal differentiation in amphibians may also contribute to the left-biased occurrence of intersex gonads. In most intersex individuals, the oocytes were localized in the anterior end of the gonad. Gonadal differentiation in amphibians proceeds from the anterior to the posterior end of the gonad (Hayes, 1998). Therefore, the location of oocytes in the intersex gonads may be attributed to the timing of EE2 exposure, which would have coincided with the differentiation of the anterior end.

No apparent abnormalities in oocyte condition (*e.g.*, size of oocytes, presence of atretic oocytes, etc.) or advancement in oocyte development were noted in the ovaries of EE2-exposed females of any group. Mackenzie *et al.* (2003) observed an increase in the

proportion (70-80%) of vitellogenic oocytes in *R. pipiens* following a chronic exposure to 1 and 10 μg/L EE2 (~ 3 and 30 nM). Variation in uptake and body burdens of EE2 related to specific water quality parameters, or inherent sensitivity of a different *R. pipiens* population may contribute to differences in effects between studies. Alternatively, measuring vitellogenin production or estrogen receptor expression in the metamorph liver may also be a sensitive endpoint for estrogenicity, as they have been shown to increase in fish following exposure to EE2 (Andersen *et al.*, 2003; Denny *et al.*, 2005).

3.4.3 Potential impacts at the population level

An important question remaining from this work is the biological significance of a two week delay in metamorphosis. While plasticity of metamorphosis is advantageous in that it allows amphibians to modulate their growth according to the optimal environmental conditions, a contaminant-induced delay in metamorphosis may leave tadpoles vulnerable to predation, habitat desiccation, and inadequate food supply. Late metamorphosis is also related to lower post-metamorphic growth rates and smaller mean adult size (Semlitsch *et al.*, 1988), traits that may contribute to reduced reproductive potential. Contaminants could influence the plasticity of metamorphosis by inducing a stress response or in the case of hormonally active compounds, interfere with the developmental action of hormones within individual tissues. Mechanistic studies will help determine whether estrogenic exposure is modulating the normal endocrine response to endogenous hormones (*e.g.*, TH, PRL, GH) that is necessary for proper metamorphosis.

The functional consequences of the intersex condition in amphibians is unknown within the context of this study. In fish, the degree of contaminant-induced intersex is inversely correlated with gamete quality and production (Jobling *et al.*, 2002). Furthermore, reduced reproductive success and abnormal behaviour in fish exposed to EE2 over multiple generations raises concerns for effects of xenoestrogen exposure at the population level (Nash *et al.*, 2004). Richards and Nace (1978) demonstrated that the treatment of *R. pipiens* tadpoles with hormones produced completely sex reversed animals and subsequent mating of these individuals with normal animals resulted in highly skewed sex ratios. Therefore, it is possible that breeding of xenoestrogen-induced intersex or sex reversed animals in wild populations would result in skewed sex ratios and eventual population decline. Reduced hatching success of eggs and the presence of juvenile intersex animals in an EE2-contaminated lake provides field-based evidence that xenoestrogens are capable of affecting amphibian fecundity and development (Park and Kidd, 2005). Experiments assessing the reproductive capacity of sex reversed amphibians would provide important information for considering the impact of xenoestrogens on native amphibian populations. However, with amphibians such as Ranids, rearing juveniles and breeding sexually mature adults in a laboratory setting is extremely difficult. Therefore, the aquatic and rapidly developing *Xenopus tropicalis* may be a suitable anuran model for examining transgenerational reproductive effects of early xenoestrogen exposure.

CHAPTER 4

Gene expression in the *Rana pipiens* tadpole brain: developmental profiles and thyroid hormone regulation*

4.1 Introduction

Amphibian metamorphosis is a dramatic larvae-to-adult transformation that is initiated and controlled by thyroid hormones (TH). The synthesis, release and action of TH are temporally regulated by both external (*i.e.* environmental) signals (Denver, 1998a) and internal hormone feedback acting on the neuroendocrine system (brain and pituitary) (Manzon and Denver, 2004). The central nervous system (CNS) regulation of the thyroid axis, together with tissue-specific TH metabolism, results in systematic morphological and physiological transformations in the developing tadpole (Shi, 2000). Individual tissues undergo their unique metamorphic changes at distinct developmental stages; their ability to respond to TH is in part related to local molecular events. This is most noticeably demonstrated by the stage-specific transformations of the hindlimb, tail and intestine (Shi *et al.*, 1996). Although not as visible, brain remodelling during metamorphosis is also extensive with the formation of many neuroendocrine structures being dependent on TH (Denver *et al.*, 1997; Denver, 1998b).

The genomic actions of TH are mediated by two members of the nuclear hormone receptor superfamily, the TH receptors: TRα and TRβ (Bassett *et al.*, 2003). During amphibian metamorphosis, TRα and TRβ are differentially expressed with respect to

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* *Adapted from:* Hogan, N.S, Crump, K.L., Duarte, P, Lean, D.R.S, and V.L. Trudeau. Hormone cross-regulation in the tadpole brain: developmental expression profiles and effect of T3 exposure on thyroid hormone- and estrogen-responsive genes in *Rana pipiens*. General and Comparative Endocrinology. *Submitted - in revision.*

tissue and developmental stage, with the head region exhibiting the highest amount of both transcripts (Kawahara *et al.*, 1991). TRα is generally detected early in development, shortly after embryogenesis and prior to TH production by the thyroid gland (Yaoita and Brown, 1990). Although TRα does not appear to be directly regulated by TH (Eliceiri and Brown, 1994), it is likely involved in the repression of early TH-responsive genes (Sachs *et al.*, 2000). In contrast, TRβ transcripts increase in response to rising TH levels during metamorphosis (Yaoita and Brown, 1990) and with the administration of exogenous T3 (Denver, 1997b; Krain and Denver, 2004). This autoregulation of TRβ is responsible for modulating the transcription of downstream TH-responsive genes that are involved in normal brain development and neuronal differentiation during metamorphosis (Tata *et al.*, 1993; Denver *et al.*, 1997; Sachs *et al.*, 2000).

While receptors mediate the genomic response to TH, deiodinase enzymes are responsible for co-ordinating the tissue-specific metabolism of TH during amphibian development (Galton, 1992). The activity of deiodinase type II (D2) catalyzes the conversion of thyroxine (T4) to the more bioactive triiodothyronine (T3), whereas type III (D3) inactivates both T3 and T4. D2 mediates tissue sensitivity to circulating TH by regulating local T3 production; D2 activity and expression peak when a tissue (*e.g.*, hindlimb, tail) is undergoing metamorphic changes (Becker *et al.*, 1997; Huang *et al.*, 2001). Conversely, D3 activity reduces local concentrations of TH and transgenic Xenopus laevis tadpoles that over-express D3 are resistant to T3-induced developmental changes associated with metamorphosis (Huang *et al.*, 1999). Developmental and T3-responsive changes in deiodinase expression in the pituitary (Manzon and Denver, 2004) and brain (Denver *et al.*, 1997) indicate that these enzymes regulate neuroendocrine TH

levels. However, these genes have yet to be examined simultaneously in the developing tadpole brain.

It is well established that TH is essential for normal brain development in vertebrates. A deficiency in TH during critical periods of development can result in extreme defects in brain maturation (Bernal *et al.*, 2003) and later impairment of neuropsychological function (Zoeller and Rovet, 2004). The cellular basis for these effects lies in the organisational role of TH in neuronal migration, synaptogenesis and differentiation of multiple cell types (Anderson, 2001; Howdeshell, 2002). However, the developing vertebrate brain is also a site for the synthesis and organisational effects of sex steroid hormones, especially estrogen (McEwen and Alves, 1999). Estrogen action is primarily mediated by two types of nuclear estrogen receptor (ER), namely estrogen receptor α (ERα) and ERβ, which like the TRs are members of the nuclear receptor family (Evans, 1988). Weiler *et al.* (1987) isolated the first ER, ERα, in an anuran, *X. laevis*, while ERβ has only recently been identified in *X. tropicalis* (Wu *et al.*, 2003). When activated by a ligand, the ER acts as a transcription factor, interacting with estrogen response elements (ERE) in the promoter region of the DNA to induce transcription of estrogen-responsive genes (Katzenellenbogen, 1996). While estrogen exerts its effects though the ER, estrogen synthesis in the vertebrate brain reflects the activity of aromatase, an enzyme that catalyzes the local conversion of testosterone to estrogen (Callard *et al.*, 1978b). Aromatase activity and estrogen synthesis plays an important role in the sex differentiation of the neuroendocrine system (MacLusky *et al.*, 1994; Resko and Roselli, 1997). Although aromatase activity has been detected in the adult anuran brain where estrogen synthesis is likely involved in the seasonal

reproductive cycle (Callard *et al.*, 1978a; Guerriero *et al.*, 2000), whether estrogen plays a role in the brain during anuran metamorphosis is unclear. Tadpoles have detectable whole body levels of estrogen (Bogi *et al.*, 2002) and there is indirect evidence supporting endogenous estrogen action in the tadpole brain (Trudeau *et al.*, 2005). However, there are currently no data on the expression of estrogen-responsive genes in the brain during metamorphosis.

Amphibian TH-dependent metamorphosis could provide a unique model for addressing the relationship between thyroid status and steroid hormone signalling at the level of the brain. Studies have shown that disruption of TH activity during tadpole development affects gonadal sex differentiation (Hayes, 1997; Goleman *et al.*, 2002) and formation of other sexually dimorphic structures (Robertson and Kelley, 1996), suggesting that TH are important for developmental and physiological events beyond those associated with metamorphosis. There is currently some evidence for hormone cross-regulation between the sex steroids and TH. For example, TH can enhance estrogen-induced ERα expression in the amphibian liver (Rabelo and Tata, 1993) and repress testosterone-induced aromatase activity in the mammalian ovary (Gregoraszczuk *et al.*, 1998). Characterising the developmental and hormonal regulation of thyroid hormone- and estrogen-sensitive genes in the metamorphic brain will provide a basis for exploring cross-regulation between the thyroid and reproductive axes in directing amphibian development.

The objective of this study was to assess the developmental expression and regulation of hormone receptors and enzymes involved in mediating TH and estrogen action in the developing tadpole brain. We cloned partial cDNAs for specific hormone

receptors (TRα, TRβ, and ERα) and enzymes (D2, D3, and aromatase) and developed both multiplex and simplex real-time PCR assays for sensitive detection of these target genes in *Rana pipiens*, the Northern leopard frog. Using this method, we (1) established developmental expression profiles for these genes in the brain from premetamorphosis to completion of metamorphic climax and (2) characterised their response in the tadpole brain following exposure to waterborne T3.

4.2 Methods

Details regarding the location from which eggs were obtained, laboratory rearing conditions and general maintenance are described in Chapter 2 (2.2 Methods; *2.2.1 Animals and rearing conditions*).

4.2.1 Tissue collection for developmental profiles

Brain tissue was collected from individuals at various stages of metamorphosis: (1) stage 30, foot-paddle development (premetamorphosis); (2) stage 36, hind-limb development (prometamorphosis); (3) stage 42, forelimb emergence and beginning of tail resorption (metamorphic climax); and (4) stage 46, tail completely resorbed (juvenile frog). These stages were chosen because they are distinct stages of metamorphosis initiated by dramatic hormonal changes within the thyroid system and they are clearly identifiable by morphological examination. Tadpoles were staged weekly and ten individuals were randomly collected at each of the four developmental stages. Animals were anesthetized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, Sigma-

Aldrich; 1 g/L in water) and sacrificed by decapitation. Whole brain was immediately dissected, frozen on dry ice and stored at -80 °C.

4.2.2 Exposure to T3

3,3′,5-Triiodo-L-thyronine (T3) and dimethyl sulfoxide (DMSO) were purchased from Sigma Canada Ltd (Oakville, ON). A concentrated stock of T3 (10 mM) was used to make three dilutions (0.01, 0.1, 1.0 μM) for addition to treatment water. Ten tadpoles (Gosner stages 33-34) were exposed to nominal concentrations of T3 (0.5, 5, 50 nM) or a DMSO solvent control (CTRL) for 48 hours. These stages of development represent a period where endogenous TH levels are beginning to rise to detectable levels (Regard *et al.*, 1978) and tadpoles are competent to respond to exogenous T3 (Shi *et al.*, 1996). In addition, the length of T3 exposure was based on the results of a preliminary time-course experiment where TRβ expression increased at 24 hrs and reached peak expression at 48 hrs (data not shown). The DMSO concentration was 0.005 % in all treatments. Each treatment contained ten tadpoles equally distributed between two 5 L glass tanks (loading density = 1 tadpole/litre). Tadpoles were not fed nor were chemicals renewed during the exposure period. Brain tissue was collected as described previously.

4.2.3 RNA isolation and cDNA synthesis

Total RNA for the developmental profile samples was obtained from whole brain using the QIAGEN RNeasy Mini Kit (including the RNase-free DNase set) as described by the manufacturer (Qiagen, Mississauga, ON). For T3 exposed samples, total RNA was isolated using TRIzol reagent as described by the manufacturer (Invitrogen Canada Inc., Burlington, ON). The RNA was then resuspended in RNase-free water and treated

with RQ1 RNase-Free DNase as described by the manufacturer (Promega, Madison, WI). DNase treated RNA was purified by organic extraction using phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform as described by Sambrook *et al.* (1989). The treated RNA was then precipitated with ethanol (99 %) and sodium acetate (0.3 M). The RNA isolated by both methods were resuspended in RNase-free water and stored at -80°C. Concentrations of RNA were determined spectrophotometrically using GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech, Piscataway, NJ).

Total cDNA was prepared from 2-3 μg total RNA and 200 ng random hexamer primers (Invitrogen) using Superscript II RNase H^- reverse transcriptase as described by the manufacturer (Invitrogen). The 20 μL reaction was diluted to a final concentration of 10 ng/μL before PCR amplification.

4.2.4 Cloning of ERα

A cDNA sequence for ERα was cloned from *R. pipiens* liver. Degenerate primers were designed against conserved regions of amphibian and fish sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/ index.html) and contructed using ClustalW (EMBL-EBI; http://www.ebi.ac.uk/clustalw/) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) programs. Primer sequences were: sense primer 5'-ACTATGGTGTCTGGTCTTGTG-3'; antisense primer 5'-TTCCCTTTCATCATTCCCACTTC-3'. The PCR mixture (50 μL final volume) contained 1.0x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM forward and reverse primers, 0.02 U Taq® DNA Polymerase, and 2 μL of cDNA template. All PCR reagents and primers were from Invitrogen. PCR amplification was performed on a Mastercycler® gradient thermalcycler (Eppendorf, Mississauga, ON) with the following

conditions: an initial 7 min denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min. After the last amplification cycle, the final extension step was completed at 72 °C for 7 min and samples were immediately cooled to 4 °C.

Amplification product was excised from a 1% agarose gel and purified using QIAQuick Gel Extraction Kit (Qiagen), ligated into the pCR 2.1 TOPO® cloning vector (TOPO TA cloning kit; Invitrogen), and transformed into *Escherichia coli* competent cells (One Shot TOPO 10 chemically competent cells; Invitrogen). Cells were then plated onto LB-agar plates containing ampicillin and X-gal. Positive colonies were selected, grown overnight in LB broth containing ampicillin, and plasmids were purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI). All procedures were carried out according to the manufacturer's instructions. Approximately 10 µL of purified plasmid was sent to the Canadian Molecular Research Services (CMRS; Ottawa, ON) for sequencing. The resulting partial sequence (175bp; accession no. DQ398027) was 86% similar to that of *X. laevis* ERα (Weiler *et al.*, 1987; accession no. L20735).

4.2.5 Real-time PCR

Coding sequences for *R. pipiens* TR-alpha (accession no. DQ398026), TR-beta (accession no. AY049025), D2 (Davey *et al.*, 1995; accession no. L42815), D3 (accession no. DQ398025), and L8 (accession no. AY452063) were obtained from GenBank. These sequences were used with Beacon Designer 2.1 (PREMIER Biosoft International, CA) to design primer and probe sets for multiplex assays with TRα, TRβ, and L8 in triplex and D2, D3 and L8 in triplex. Dual-labeled fluorescent probes for each

of the five genes listed above were purchased from Integrated DNA Technologies (IDT; Coralville, IA). Primers for simplex assays (SYBR Green I) of ERα and aromatase were designed using the Primer3 program using the previously described ERα partial cDNA sequence (accession no. DQ398027) and a *R. pipiens* partial cDNA sequence of aromatase (accession no. DQ449025)

The real-time PCR assays developed and used in this study were optimized according to the guidelines described in Applied Biosystems (Applied Biosystems, 1997). Probe and primer concentrations were optimized for minimum threshold cycle (Ct) and maximum change in fluorescence (dRn) in the two multiplex assays for each gene target, both in simplex and triplex. The optimal primer/probe concentrations and thermal cycle conditions are detailed in Table 4.1. Fluorescent probe- and SYBR Green I-based real-time PCRs (quantitative PCR; QPCRs) were assayed using an Mx4000 real-time polymerase chain reaction system (Stratagene, La Jolla, CA). For the probe-based multiplex assays, each 25 μL reaction contained 1.0x PCR buffer (Qiagen), 5 mM $MgCl_2$ (Qiagen), 800 μM dNTPs (Invitrogen), 300 nM passive reference dye (Stratagene), 1.25 U HotStarTaq™ (Qiagen), optimized concentrations of each forward/reverse primer set (Invitrogen) and associated probe (IDT), and 5 μL of diluted cDNA template. The thermocycle program included an initial enzyme activation step for 15 min (95 °C) followed by 40-45 cycles of denaturation for 15 sec (95 °C) and annealing/elongation for 1 min (60-62° C; Table 1). The primer sets used in SYBR Green I-based assays were optimized for concentration and annealing temperature to obtain a minimum Ct, maximum dRn and a single sequence-specific peak in the denaturation curve. For the SYBR Green I assays, each 25 uL reaction contained 1.0x PCR buffer (Qiagen), 2.5 mM

$MgCl_2$ (Qiagen), 200 μm dNTPs (Invitrogen), 100 nM passive reference dye (Stratagene), 1.25 U HotStarTaq (Stratagene), optimized concentrations of each forward/reverse primer set (Invitrogen), 0.25x SYBR Green I Dye (Molecular Probes, Eugene, OR), and 5 μL of diluted cDNA template. The thermocycle program for the SYBR Green I-based assays started with an enzyme activation step for 15 min (95° C), followed by 40-45 cycles consisting of: denaturation for 15 sec (95 °C), annealing for 5 sec (60-62° C; Table 2.1), elongation for 30 sec (72 °C), and 8 sec (80 °C). After this amplification phase, there was a denaturation step of 1 min (95 °C) followed by 40 cycles starting at 55 °C and increasing 1 °C/30 sec to generate a dissociation curve. Negative controls for the real-time PCR reactions included a no template control where RNase-free water was added to the reaction instead of the template (cDNA) and a no reverse transcriptase control where RNase-free water was added to the cDNA synthesis reaction (previously described) instead of the enzyme.

4.2.6 Data analysis

Before calculating the threshold cycle (Ct) for each QPCR reaction, the threshold for each target was corrected to ensure that it was within the linear range of the amplification curve. The relative standard curve method (Applied Biosystems, 1997) was used to interpolate relative mRNA abundance of target and reference genes within each sample. The standard curve was generated using a serial dilution of a cDNA mix of representative samples. Samples were run in duplicate and data for each target were averaged and normalized to L8 as the internal reference. Biological replicates were then averaged to achieve normalized gene expression ± SEM. Statistical analysis was performed using SigmaStat 2.03 software (SPSS Inc., Chicago, IL). Data were evaluated

for normality (Kolmogorov-Smirnov test) and equal variance (Levene's test). Data were log transformed when necessary and assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple comparisons (developmental profiles) or pair-wise comparisons between treatment and solvent control (T3 exposure experiment). Significance was set at $P<0.05$.

The ribosomal protein L8 is commonly used as an internal control for amphibian gene expression studies (Helbing *et al.*, 2003; Manzon and Denver, 2004) and its expression did not change with T3 treatment in this experiment. However, there was a decrease (~35%) in L8 expression in the metamorph and juvenile brain (Gosner stages 42 and 46) compared to the larval stages. This pattern was consistent in both the probe- and SYBR Green I-based reactions. To ensure that developmental expression patterns were not due to changes in L8, both L8-normalized and non-normalized data for all target genes were compared using the statistical analysis described above. Although the magnitude of change in the expression of the target genes between stages differed, it did not alter the overall developmental patterns of gene expression or the statistical results. Therefore, the L8-normalized data are reported.

Data for developmental profiles are presented as the mean percentage of maximum expression relative to the developmental stage with the highest expression. T3 dose-response data are presented as mean fold change in expression relative to the control group.

Table 4.1 Primer and probe sequence information and real-time PCR assay conditions for probe-based muliplex amplification of genes from the *Rana pipiens* brain.

Target	Element	Sequence (5'-3')	Amplicon Length (bp)	Probe (dye, quencher)	Primer/Probe (nM)	Annealing Temp (°C)
TRα	S primer	AGATGGCAGTGAAGCGAGAAC	150	Cy5, BQH2	900	60
	AS primer	GGTCTGAGGACATGAGCAGGA			300	
	S Probe	TGCAGCAGAGCCACTTCCGTGTCA			250	
TRβ	S primer	AAGGAACCAGTGCCAAGAATGT	86	FAM, BQH1	300	60
	AS primer	AACGCTTGCTGTCGTCCAAA			300	
	AS Probe	TTGCCATGCCAACAGCGATGCACT			150	
D2	S primer	TTTGGATCAGCCACCTGAC	78	Cy5, BQH2	600	62
	AS primer	TACAGCGGAGAACTCTTCCA			300	
	S Probe	ATAAGCCAGTTGCCAGCCTTCAGC			200	
D3	S primer	GGTACGGACAGAAGCTCGAC	80	FAM, BQH1	300	62
	AS primer	CTCCAGGGTCACCACCTC			600	
	S Probe	TTCTTCAAGTCGGCACACCTGGG			200	
L8	S primer	GTGTAGAAGAGAAGCCAGGTGAT	79	HEX, BQH1	56.25[a] / 80[b]	60[a] / 62[b]
	AS primer	GGATTGTGGGAGATGACGGTAG			56.25[a] / 80[b]	
	AS Probe	TGCCAGATGCACGAGCCAACTTGC			150	

TRα, thyroid receptor alpha; TRβ, thyroid receptor beta; D2, deiodinase type 2; D3, deiodinase type 3; L8, ribosomal protein L8. Primer and probe orientation indicated as S (sense) and AS (antisense).

[a] Conditions for TRα/TRβ/L8 multiplex
[b] Conditions for D2/D3/L8 multiplex

Table 4.2 Primer information and real-time PCR assay conditions for SYBR Green I-based amplification of genes from the *Rana pipiens* brain.

Target	Element	Sequence (5'-3')	Amplicon Length (bp)	Primer (nM)	Annealing Temp (°C)
ERα	S primer	GAACCCCTCTGTGAAAAGCA	220	100	60
	AS primer	CACAAAACCTGGCACTCGTT		100	
Aromatase	S primer	TCATTGTGGAAGGTGATTCG	97	300	60
	AS primer	ATCGTTCGGTGGACTTGAAA		300	
L8	S primer	GTGTAGAAGAGAAGCCAGGTGAT	79	100	62
	AS primer	GGATTGTGGGAGATGACGGTAG		100	

ERα, estrogen receptor alpha; L8, ribosomal protein L8.
Primer and probe orientation indicated as S (sense) and AS (antisense).

4.3 Results

4.3.1 Real-time PCR methods

For both the probe- and SYBR Green I-based assays, amplification efficiency, linearity and working range were determined by linear regression analysis of serial dilutions of cDNA. Standard curves were performed prior to and along with sample analysis. The linear range of detection was over three orders of magnitude. Reaction efficiencies (as determined by the standard curves) were close to 100% for all reactions.

4.3.2 Developmental expression profiles

Developmental profiles of thyroid hormone receptors (Figure 4.1), deiodinases (Figure 4.2), ERα and aromatase (Figure 4.3) were determined by measuring their mRNA expression in the whole brain at four stages of development: stage 30 (premetamorphosis), stage 36 (prometamorphosis), stage 42 (metamorphic climax), and stage 46 (juvenile).

TRα and TRβ mRNA were detected at all stages of development examined (Figure 4.1). Expression of TRα remained relatively constant in the brain during development (Figure 4.1A). In contrast, TRβ mRNA levels were low and unchanging during premetamorphosis and prometamorphosis (20% of maximum expression), significantly increased to a maximum at metamorphic climax ($P<0.001$), and then decreased to 80% of maximum in the juvenile frog ($P<0.001$; Figure 4.1C). D2 expression was detected at all stages of development examined and declined from tadpole stages through metamorphic climax with a significant decrease (~60% less than stage 30; $P<0.001$) in the juvenile brain (Figure 4.2A). In contrast, brain D3 expression was

extremely low at premetamorphosis and could not be consistently detected (Figure 2C). Levels increased to a detectable level at prometamorphosis and then were strongly upregulated at climax (99% above stage 36, $P<0.001$). This spike in D3 expression was followed by a significant decrease in the juvenile (50% less than climax; $P<0.001$). Expression of ERα was constant and relatively low during pre- and prometamorphosis then increased to a maximum at metamorphic climax (~90% above stage 36; $P<0.001$) and remained elevated in the juvenile (Figure 4.3A). Aromatase mRNA levels were also low and unchanging during pre- and prometamorphosis (20% of maximum), increased significantly at metamorphic climax ($P<0.001$) and reached maximum expression in the juvenile (4-5 fold above tadpole levels; $P<0.001$; Figure 4.3C).

4.3.3 Response to T3

To determine if the transcripts of interest were regulated in the brain by TH, Gosner stage 33-34 tadpoles were exposed to T3 for 48 hours. Exposure to T3 (0.5, 5, 50 nM) had no effect on survivorship; there was 100% survival in all groups.

TRα transcript levels did not change significantly upon T3 treatment (Figure 4.1B), whereas a significant 5-fold increase in TRβ expression was observed at 50 nM T3 ($P<0.001$; ANOVA; Figure 4.1D). Increases in D2 expression (1.7-fold) observed at 0.5 and 5 nM were not statistically significant; however, 50 nM T3 upregulated mRNA levels by 4-fold ($P<0.001$; ANOVA; Figure 4.2B). T3 treatment resulted in a dose-dependent increase in D3 mRNA levels with a significant induction at 50 nM (>7-fold; $P<0.001$; ANOVA; Figure 4.2D). A two-fold induction of ERα mRNA was observed in the brain after exposure to 50 nM T3 ($P<0.05$; ANOVA; Figure 4.3B). In contrast to ERα, aromatase expression exhibited an apparently dose-dependent decrease with increasing

T3 concentrations; compared to CTRL, 50 nM T3 exposure resulted in a 50% decrease in aromatase mRNA levels ($P<0.01$; ANOVA; Figure 4.3D).

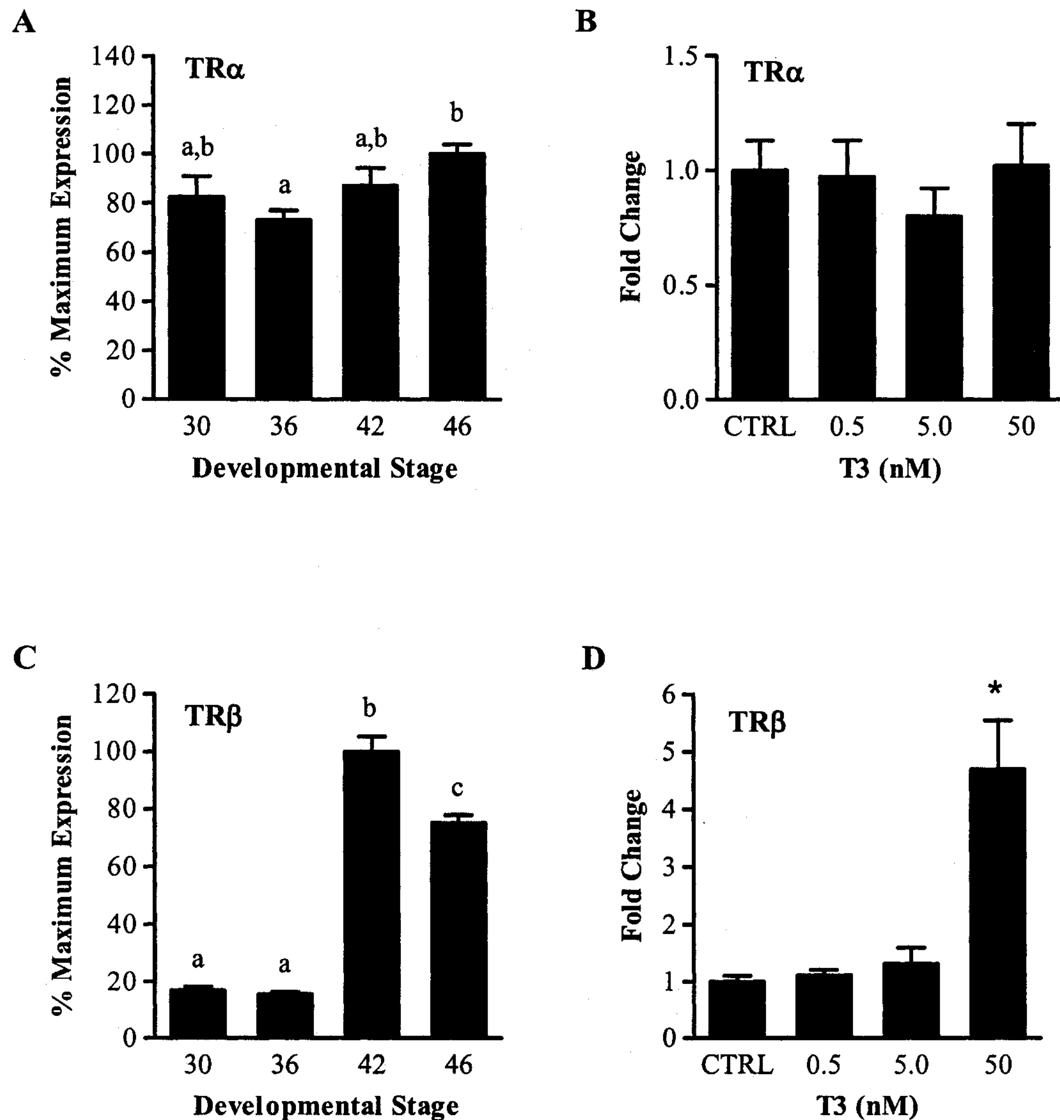


Figure 4.1 Expression of TRα (A,B) and TRβ (C,D) in the brain of *R. pipiens* tadpoles as determined by multiplex real-time PCR. Bars represent the mean + SEM (n=6-8 individuals). Data were analysed by one-way ANOVA followed by Bonferroni post-hoc comparisons. Significance is indicated if $p \leq 0.05$. Developmental profile data (A,C) are presented as the percent maximal expression relative to the developmental stage with the highest expression. Letters indicate significant differences in expression between stages. T3 exposure data (B,D) are presented as fold change relative to control (CTRL) animals. Asterisk (*) indicates significant differences between treatment and control. TRα, thyroid hormone receptor alpha; TRβ, thyroid hormone receptor beta.

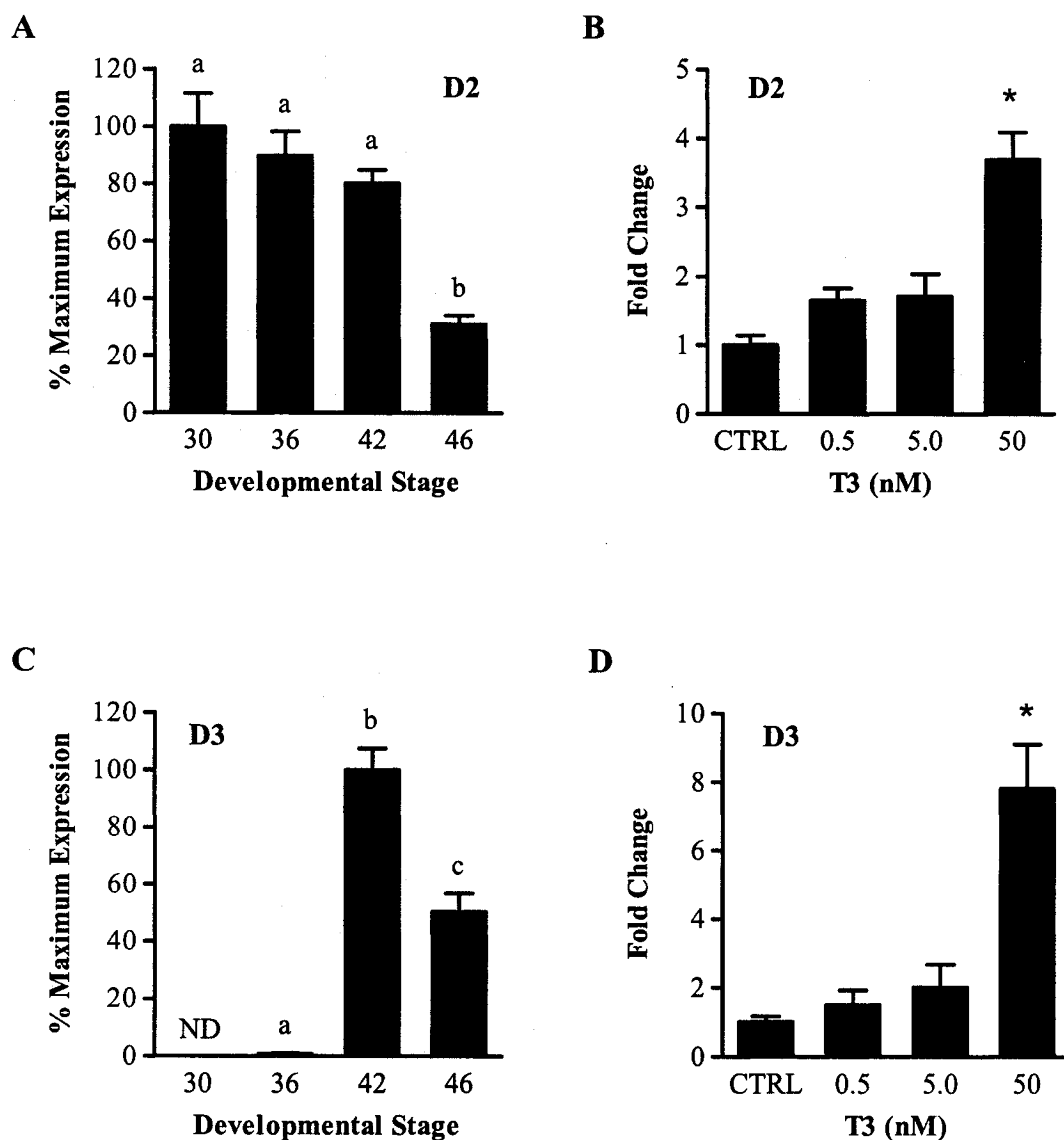


Figure 4.2 Expression of D2 (A,B) and D3 (C,D) in the brain of *R. pipiens* tadpoles as determined by multiplex real-time PCR. Bars represent the mean + SEM (n=6-8 individuals). Data were analysed by one-way ANOVA followed by Bonferroni post-hoc comparisons. Significance is indicated if $p \leq 0.05$. Developmental profile data (A,C) are presented as the percent maximal expression relative to the developmental stage with the highest expression. Letters indicate significant differences in expression between stages. T3 exposure data (B,D) are presented as fold change relative to control (CTRL) animals. Asterisk (*) indicates significant differences between treatment and control. ND, no detection. D2, deiodinase type II; D3, deiodinase type III.

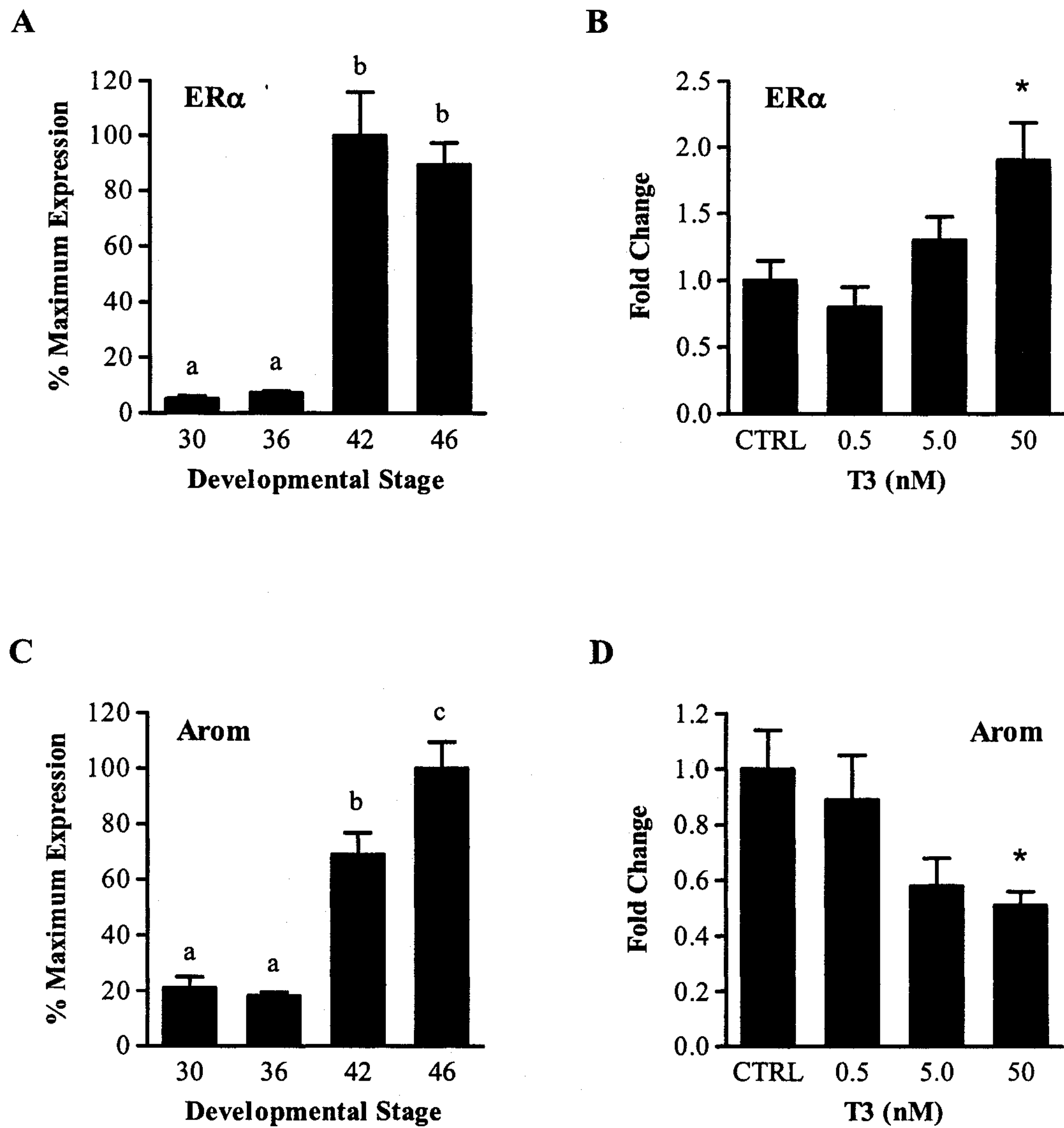


Figure 4.3 Expression of ERα (A,B) and Arom (C,D) in the brain of *R. pipiens* tadpoles as determined by SYBR Green real-time PCR. Bars represent the mean + SEM (n=6-8 individuals). Data were analysed by one-way ANOVA followed by Bonferroni post-hoc comparisons. Significance is indicated if $p \leq 0.05$. Developmental profile data (A,C) are presented as the percent maximal expression relative to the developmental stage with the highest expression. Letters indicate significant differences in expression between stages. T3 exposure data (B,D) are presented as fold change relative to control (CTRL) animals. Asterisk (*) indicates significant differences between treatment and control. ERα, estrogen receptor alpha; Arom, aromatase.

4.4 Discussion

The present study assessed the developmental and TH-regulated changes in gene expression in the *R. pipiens* brain during metamorphosis. Members of the nuclear receptor superfamily considered in this expression analysis were TRα, TRβ and ERα. Upon binding their associated ligands (TH and estrogen, respectively), these receptors can upregulate their own expression (autoregulation) and act as transcription factors to regulate the expression of several genes involved in growth, development, and reproduction. Also examined were mRNA levels of key enzymes involved in local hormone synthesis and metabolism; both forms of iodothyronine deiodinase (D2 and D3) are responsible for the specific tissue availability of THs while aromatase catalyses the conversion of androgen to estrogen.

Compared to conventional methods of assessing gene expression, real-time PCR allows detection of mRNA across a much greater dynamic range. In addition, probe-based chemistry allows the development of multiplex assays to detect two or more targets simultaneously; this type of assay is particularly appealing when considering multiple isoforms (*i.e.*, TRα and TRβ). This development and application of real-time PCR assays to selectively detect transcripts involved in amphibian metamorphosis has both confirmed previous data and provided novel information regarding their expression in the developing *R. pipiens* brain. This is also the first study to assess the developmental expression and TH-regulation of ERα and aromatase in the amphibian brain.

4.4.1 Thyroid hormone receptor and deiodinase expression

Expression of TRβ in the brain was upregulated at metamorphic climax and was induced by exogenous T3. Similar results were recently obtained by Northern blot analysis of TRβ in the *X. laevis* brain where expression was also correlated with increasing whole body levels of T3 and T4 (Krain and Denver, 2004). In contrast to TRβ, TRα expression remained relatively constant in the brain during metamorphosis and did not respond to T3 exposure. An earlier study measuring receptor expression in the whole "head" of *X. laevis* tadpoles also demonstrated that TRα, although present at higher levels than TRβ, did not change during development (Kawahara *et al.*, 1991). The constitutive expression of TRα throughout metamorphosis and elevation of TRβ at climax observed in this study supports the dual-function of TRs proposed by Sachs *et al.* (2000). This model states that TRs function as repressors or activators of TH-inducible genes in a temporal and spatial manner to ensure proper timing of metamorphic events. These regulatory actions are tissue-specific depending on where the TRs are expressed. In premetamorphic tadpole, there is no circulating T3 and TRα levels are high in relation to TRβ in the brain (Shi, 2000). According to Buchholz *et al.* (2006), the role of unliganded TRα may be to repress certain early-response genes which, in the present study, may be necessary to direct normal brain development during the transition from tadpole to a juvenile frog. In contrast, neuroendocrine regions of the brain that form during later stages would rely on rising T3 levels to autoinduce TRβ expression and in turn activate other TH-responsive genes.

This is the first comparative analysis of D2 and D3 expression in the tadpole brain and supports the hypothesis that coordinated deiodinase expression in the developing

amphibian brain works to control local TH levels. As with other tissues during development (Becker *et al.*, 1997), stage-specific regulation of the deiodinases in the brain would be important for TH-dependent timing of tissue remodelling during metamorphosis. However, difficulty in obtaining enough tissue and low transcript levels have previously limited deiodinase gene expression analysis in the amphibian brain (Kawahara *et al.*, 1999). The probe-based multiplex provides a sensitive method for assessing both D2 and D3 mRNA levels in developing tadpole tissues.

D2 gene expression was high in the tadpole brain and decreased through metamorphic climax and juvenile stages. Using *in situ* hybridisation and immunostaining, Cai and Brown (2004) previously showed that high D2 expression and ventricular cell proliferation are colocalised in the *X. laevis* brain during premetamorphosis. When this cell replication was complete at metamorphic climax, the D2 signal also decreased. The ventricular cells of the mammalian brain also express high levels of D2 during the TH-sensitive neonatal period of development (Guadano-Ferraz *et al.*, 1997; Guadano-Ferraz *et al.*, 1999). Becker *et al.* (1997) concluded that high D2 levels correspond with the time a particular tissue undergoes TH-induced changes; however, detailed expression analysis in the brain during development by slot blot analysis was difficult due to low mRNA levels. Higher D2 during early development, as observed here, may allow the brain to respond to very low levels of circulating T4 by locally converting it into the physiologically active T3 for TH-induced tissue transformation.

Treatment with T3 induced D2 expression in the brain of early prometamorphic tadpoles. However, if the negative feedback response is functional in the brain, as has

been demonstrated in the pituitary (Manzon and Denver, 2004), T3 treatment should result in a down-regulation of D2 in an effort to maintain local TH concentrations. Incubation of *X. laevis* pituitaries with T4 or T3 downregulated thyroid stimulating hormone (TSHβ), which is responsible for activating the release of THs from the thyroid gland (Manzon and Denver, 2004). If T3 exposure lowered circulating levels of T4 via this mechanism, then the observed increase in D2 expression after T3 exposure may represent a compensatory response by the brain to increase TH-sensitivity as cell proliferation is dependent on thyroid status.

Of all the genes assessed in this study, D3 displayed the greatest change in expression over the developmental stages examined and in response to T3. Transcript levels were non-detectable during premetamorphosis, were elevated at prometamorphosis and exhibited a nearly 100-fold increase at metamorphic climax. Exposure to T3 also induced D3 expression in the brain as seen with *X. laevis* (Denver, 1997b). However, results from previous expression analyses of D3 in the brain have been variable. Northern blot analysis also determined that levels at premetamorphosis were undetectable but did not detect any increase at climax (Kawahara *et al.*, 1999) whereas D3 expression in *R. catesbeiana* brain could not be assessed by slot blot analysis due to a weak hybridization signal from the cDNA probe (Becker *et al.*, 1997). The dramatic developmental upregulation of D3 seen here using real-time PCR is likely a response to the rise in endogenous TH characteristic of late prometamorphosis (Leloup and Buscaglia, 1977; Regard *et al.*, 1978; Krain and Denver, 2004) and would serve to protect the brain from high circulating TH levels through the inactivation of local T4 and T3.

4.4.2 Estrogen receptor and aromatase expression

The developmental expression profile of ERα in the brain was similar to that of TRβ and D3; mRNA levels were constant during the tadpole stages and increased significantly at metamorphic climax. Exogenous T3 also induced ERα mRNA in the brain. There is both direct and indirect evidence that THs facilitate E2-activation of ERα and other estrogen-responsive genes in the liver of *Xenopus laevis* (May and Knowland, 1981; Kawahara *et al.*, 1989). Rising TH levels during *X. laevis* metamorphosis, as well as administration of TH to immature hepatocytes, induces the capacity for E2-stimulated gene transcription (Rabelo and Tata, 1993; Rabelo *et al.*, 1994). While these studies examined ER/TH interactions in the liver, the brain is also a target for both thyroid hormone and estrogen feedback and our results suggest that THs may be involved in estrogen action and regulation of ERα in the tadpole brain. However, the mechanism by which T3 modulates ERα expression is uncertain within the context of this study. As in the liver, rising levels of TH and associated transcripts (*i.e.,* TRβ) in the brain during metamorphosis could facilitate estrogen activation and autoinduction of ERα. In this context, exposure to T3 at later developmental stages (when the brain is more sensitive to TH) should also produce a greater inductive response. We propose that, in the developing tadpole, interactions between the ER and THs mediate stage- and tissue-specific hormone sensitivity in the neuroendocrine brain.

Aromatase expression in the *R. pipiens* brain at metamorphic climax and in the juvenile frog was higher compared to the tadpole stages. While it has been previously reported that aromatase expression increases during metamorphosis in the *X. laevis* ovary (Miyashita *et al.*, 2000), this is the first description of aromatase expression in the anuran

brain during metamorphosis. Aromatase is encoded by the *cyp19* gene in vertebrates (Simpson *et al.*, 1994). An increase in the expression of aromatase in the teleost fish brain during larval development (Sawyer *et al.*, 2006) and its up-regulation by exogenous E2 (Kishida *et al.*, 2001) is attributed to the presence of an estrogen response element (ERE) in the promoter of a brain-specific aromatase gene (*cyp19b*) (Tchoudakova *et al.*, 2001; Chang *et al.*, 2005). An ERE half site has been identified in the promoter region of the single aromatase gene found in *X. laevis* (Akatsuka *et al.*, 2005) and there are data indicating that a short-term waterborne exposure (96 hr) to the synthetic estrogen, ethinylestradiol (EE2) increases aromatase mRNA in the brain early prometamorphic *R. pipiens* tadpoles (Duarte *et al.*, 2006; also see Chapter 5). Although the cDNA amplified in our expression analysis is a partial sequence and the full length gene from *R. pipiens* has yet to be cloned, we propose that aromatase in the *R. pipiens* brain is also estrogen-responsive and that increasing expression during metamorphosis is a reflection of increased estrogen production in the brain. However, the function of estrogen-responsive genes, such as ERα and aromatase, and local estrogen production in the brain during metamorphosis (*e.g.*, neuronal proliferation, apoptosis, sex differentiation) remains to be explored.

Exposure to T3 decreased aromatase expression in the brain in a dose-dependent manner. Others have demonstrated that reducing endogenous TH levels (by thyroidectomy) results in enhanced circulating estrogen in gonadotropin-treated rats (Tamura *et al.*, 1998). In addition, T3-inhibition of estrogen production and aromatase expression in ovarian granulosa cells, which provides a mechanistic explanation for the higher aromatase mRNA levels and increased estrogen synthesis in hypothyroid

mammals (Hatsuta *et al.*, 2004). However, our data indicate that the thyroid-responsive brain of a developing amphibian is also a site for TH-inhibition of aromatase. Competition between receptors may be involved in this inhibition response. The consensus sequence of the ERE half-site is identical to that of a TRE and there is *in vitro* evidence for competitive binding between liganded TR and ER to the same ERE half-site (Zhu *et al.*, 1996). An increase in T3-bound TR could therefore interfere with ER-induced expression of estrogen responsive genes, such as aromatase, via the ERE. We hypothesize that the developmental induction of TR by TH in the brain during metamorphosis enhances competitive estrogen-thyroid receptor interactions at the ERE; this would regulate aromatase expression and influence estrogen synthesis in the developing brain. The functional importance of this hormone interaction has been demonstrated through the thyroid hormone and estrogen cross-regulation of other genes involved in reproductive behaviour, specifically lordosis, in the rodent (Vasudevan *et al.*, 2001; Zhu *et al.*, 2001). In the developing tadpole, TH may influence sex steroid regulation of genes involved in cellular development, and perhaps sexual differentiation, of the brain during metamorphosis.

The expression of many genes assessed in this study is consistent with the data on their respective receptor levels and enzyme activities. For example, Eliceiri and Brown (1994) found that *X. laevis* TRβ protein, but not TRα, exhibited similar developmental and TH-induced patterns compared to its mRNA levels. Deiodinase activity also corresponds to expression across various *R. catesbeiana* tissues (Becker *et al.*, 1997). High aromatase activity is also indicative of an increased number of aromatase-containing cells and mRNA abundance throughout the various brain regions in teleost

fish (Forlano *et al.*, 2001). However, whether the observed developmental and TH-induced changes in estrogen-sensitive gene expression are functionally significant within the metamorphosing *R. pipiens* brain is an important question and warrants further consideration. Localization of aromatase and ERα transcripts in the brain would also provide some insight concerning their role in brain development.

Through the development and use of real-time PCR methods to measure mRNA in the ranid frog, *R. pipiens*, this work has further established that the developing amphibian brain is an active site for the regulation of TH-responsive genes. In addition, it appears that reproductive endocrine genes in the brain are also developmentally regulated by TH. The cross-regulation between these two major endocrine axes and their roles in directing brain development is a novel and worthwhile focus for further comparative neuroendocrine studies. Finally, this work has provided the tools and the basis for examining how other environmental factors (*e.g.*, stress, endocrine disruptors) affect TH- and estrogen-regulated gene expression during amphibian development.

CHAPTER 5

Tissue-specific effects ethinylestradiol (EE2) on gene expression in brain and liver of the *Rana pipiens* tadpole

5.1 Introduction

Estrogens are involved in many aspects of development, growth, sexual differentiation, and reproductive behaviour in vertebrates. During development, estrogen is particularly important for early organisation and subsequent function of endocrine tissues (McEwen and Alves, 1999). Estrogen action is primarily mediated by the nuclear estrogen receptor (ER), a ligand activated transcription factor that is capable of binding to estrogen response elements (ERE) in the promoter region of the DNA and activating transcription of estrogen-responsive genes (Katzenellenbogen, 1996). One such example is the estrogen stimulation of hepatic ER to induce transcription and subsequent production of vitellogenin proteins which are important for oocyte maturation and egg production in oviparous vertebrates such as fish and amphibians (Pakdel *et al.*, 1991; Tata *et al.*, 1993).

Endocrine disrupting compounds (EDCs) in the aquatic environment that can mimic estrogen action are of significant concern because of their potential effects on wildlife health. These xenoestrogens have demonstrated diverse effects on reproductive development and function in vertebrates and invertebrates (LeBlanc, 1999; Damstra *et al.*, 2002; Segner *et al.*, 2003). In fish, exposure to xenoestrogens has been shown to induce feminization of gonads and secondary sex characteristics (Kinnberg *et al.*, 2003; Parrott and Blunt, 2005), disrupt hormone synthesis enzymes in brain (Kishida *et al.*, 2001; Kazeto *et al.*, 2004), and lead to reproductive failure over multiple generations

(Nash *et al.*, 2004). A well-characterised estrogenic response and biomarker of xenoestrogen exposure is the induction of vitellogenin mRNA and protein in the liver and presence of vitellogenin in the plasma of male fish (*e.g.*, Wheeler *et al.*, 2005).

Although the main focus has been on fish, there is also evidence of xenoestrogen-induced endocrine disruption in amphibians. Laboratory studies have shown that exposure to 17β-estradiol (E2) or xenoestrogens during tadpole development can produce 100% females or female-biased sex ratios and induce abnormal gonadal phenotypes (i.e., intersex) (Mackenzie *et al.*, 2003; Levy *et al.*, 2004). However, not all actions of xenoestrogens are confined to the reproductive system. E2 is an endogenous regulator of fish thyroid function at several levels in the thyroid cascade (Cyr and Eales, 1996) and the potential therefore exists for xenoestrogens to influence thyroidal status (Brown *et al.*, 2004). There are several reports that xenoestrogen exposure can alter the timing of metamorphosis, tail resorption, and tadpole body condition (Nishimura *et al.*, 1997; Bevan *et al.*, 2003; Christensen *et al.*, 2005; Goto *et al.*, 2006), which suggests that thyroid function in developing amphibians may also be a sensitive target for estrogenic endocrine disruption.

Xenoestrogen exposure can alter endocrine function in various tissues by affecting different factors that mediate hormone action (*e.g.*, receptors, enzymes, binding proteins). Thyroid hormones (TH) control amphibian metamorphosis through activation of two distinct thyroid hormone receptors, alpha and beta (TRα and TRβ), which in turn regulate gene transcription (Shi, 2000; Buchholz *et al.*, 2006). Within specific tissues, deiodinase activity mediates the availability of TH; deiodinase type II (D2) is responsible for the conversion of thyroxine (T4) to the more biologically active TH, triiodothyronine

(T3), while deiodinase type III (D3) degrades T4 and T3 (Becker *et al.*, 1997). The brain controls both the thyroid and reproductive axes through both endocrine feedback mechanisms and local hormone production/metabolism. Previous gene expression analyses revealed that these receptors and enzymes are developmentally regulated by T3 during metamorphosis in the *R. pipiens* tadpole brain (Chapter 4). The same study measured aromatase and ERα in the brain and it was determined that their expression increased over metamorphosis. In the vertebrate brain, aromatase is responsible for the enzymatic conversion of testosterone to 17β-estradiol (E2) (Callard *et al.*, 1978b; Simpson *et al.*, 1994). However, it has not yet been determined if these transcripts in the tadpole brain are estrogen-responsive. The liver is also an important endocrine organ; in addition to being a site for the ER-mediated induction of vitellogenin and other reproductive genes, hepatic deiodinase activity is involved in the regulation of circulating thyroid hormone levels in fish (Cyr and Eales, 1996) and may have a similar function in tadpoles (Galton and Hiebert, 1987).

The synthetic estrogen, 17α-ethinylestradiol (EE2), is an active component of oral contraceptives and hormone replacement therapy. This potent endocrine modulator is detected in the aquatic environment at low ng/L (pM-nM) concentrations (Ternes *et al.*, 1999). At high concentrations (>1 μM) waterborne EE2 exposure causes mortality in ranid tadpoles (Hogan *et al.*, 2006) while developmental exposure to low nM concentrations delays metamorphosis and alters gonadal sex differentiation (Mackenzie *et al.*, 2003; also Chapter 3). Investigating changes in the expression of hormone receptors and enzymes in target tissues, such as the brain and liver, can provide insight into potential mechanisms behind EE2-induced endocrine disruption in tadpoles.

The aim of the present study was to examine the effects of EE2 exposure on hormone receptors and enzymes in brain and liver of the premetamorphic *R. pipiens* tadpoles. Using previously developed multiplex and simplex real-time PCR methods (Chapter 4), I assessed changes in thyroid hormone receptor (TRα and TRβ) and estrogen receptor (ERα) mRNA following a 96 hr waterborne exposure to a range of nominal EE2 concentrations. In addition, I examined the expression of iodothyronine deiodinases and aromatase, key enzymes responsible for local synthesis and availability of thyroid hormones and estrogen, respectively. The results indicate that EE2 1) alters TH metabolism in the liver and 2) stimulates estrogen action and synthesis in the brain and 3) differentially regulates ERα in the brain and liver.

5.2 Methods

Details regarding the location from which eggs were obtained, laboratory rearing conditions and general maintenance are described in Chapter 2 (2.2 Methods; *2.2.1 Animals and rearing conditions*).

5.2.1 EE2 Exposure

A stock solution of 10 mM EE2 was prepared in ethanol (EtOH). This stock was serially diluted to make three dilutions (2, 20, 200 μM) for addition to treatment water. Ten late premetamorphic tadpoles (stages 32-35) were exposed to nominal concentrations of EE2 (0.1, 1.0 and 10 nM) or an EtOH solvent control (CTRL) for 96 hrs. Exposures were carried out in 10L of aerated water (loading density ~ 1 tadpole/L). The final EtOH concentration was 0.005% (50 μL/L). Tadpoles were fed 2 hours prior to a complete water change and renewal of EE2 at 48 hr. At the end of the exposure period, tadpoles

were anesthetized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, Sigma-Aldrich; 1 g/L in water) and sacrificed by decapitation. Brain and liver were dissected immediately and frozen on dry ice until storage at -80 °C.

5.2.2 RNA isolation, cDNA synthesis and real-time PCR

Total RNA was obtained from tissues using the QIAGEN RNeasy Mini Kit (including the RNase-free DNase set) as described by the manufacturer (Qiagen, Mississauga, ON). RNA was resuspended in RNase-free water and concentrations were determined spectrophotometrically using GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech, Piscataway, NJ) prior to cDNA synthesis. Total cDNA was prepared from 2-3 μg total RNA and 200 ng random hexamer primers (Invitrogen) using Superscript II RNase H- reverse transcriptase as described by the manufacturer (Invitrogen). The 20 μL reaction was diluted to a final concentration of 5-10 ng/uL before PCR amplification.

Expression of individual gene targets was assessed by fluorescent probe- and SYBR Green I-based real-time RT-PCR (QPCR) assays using an Mx4000 real-time quantitative polymerase chain reaction system (Stratagene, La Jolla, CA) as described previously in Chapter 4. Briefly, dual-labelled fluorescent probes were designed with primer sets for multiplex assays of TRα, TRβ, and L8 in triplex and D2, D3 and L8 in triplex while ERα primers were used in a SYBR Green I-based assay. For each individual target, relative quantity was determined using standard plots that were generated using serial dilutions of a representative pool of sample cDNA. Triplicate data for the amplification of each target cDNA were averaged and normalized to ribosomal L8.

5.2.3 Data Analysis

Gene expression data were assessed for normality (Kolmogorov-Smirnov) and homogeneity of variance (Levene's test) and were transformed if necessary to meet parametric assumptions. Data were then analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni pair-wise comparisons. Significance was indicated if $p \leq 0.05$. Data are presented as the mean (+SEM) fold change in expression relative to the control group. Analyses were performed using SigmaStat 3.1 software (SPSS Inc., Chicago, IL).

5.3 Results

Differences in the expression of TH-responsive genes following EE2 exposure, expressed as fold-change relative to (vehicle) control animals, are shown in Table 5.1. In the brain, both TRα and TRβ expression appeared to increase with EE2 concentration but the difference was not statistically significant. There was no effect of EE2 on D2 mRNA in the brain. There was no detectable amplification of D3 in the brain; however, this is likely due to the developmental stage of the tadpoles in used in this exposure (late premetamorphosis; stages 32-35). Previous analysis of D3 expression during development (presented in Chapter 4) demonstrated that D3 mRNA approaches detectable limits in the brain between stages 30 and 36 when measured using real-time PCR. In the liver, there was no significant effect of EE2 on TRα and TRβ expression. There was also no amplification of D2 in the liver which is consistent with the absence of D2 activity and expression in this tissue in amphibians at any developmental stage (Galton, 1988; Becker *et al.*, 1997). There was a significant effect of EE2 on D3

expression in the liver. Whereas 0.1 nM EE2 had no statistically significant effect, both 1 nM and 10 nM EE2 decreased transcript levels by ~ 50 % when compared to the control ($p=0.002$ and $p=0.003$, respectively).

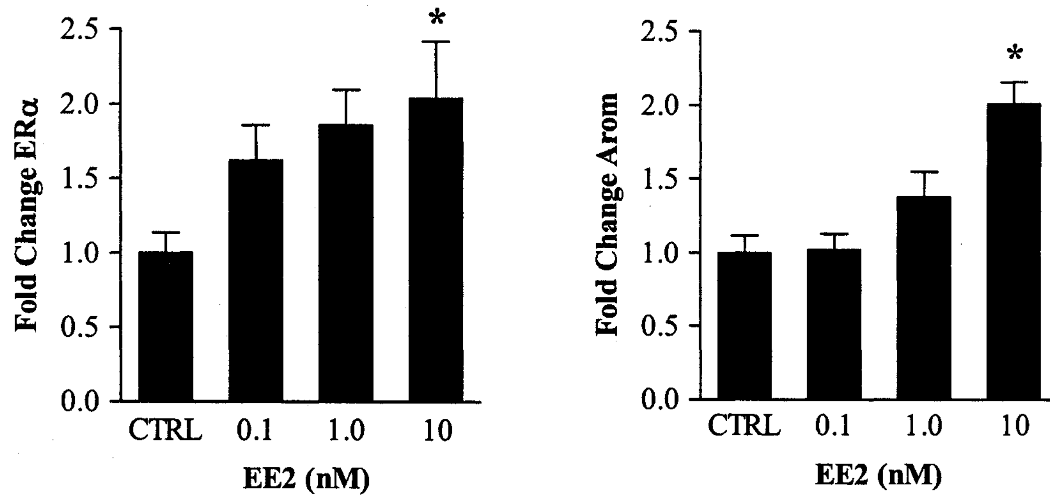
EE2-induced changes in the expression of ERα and aromatase, are shown in Figure 5.1. There were significant, although opposite, effects on the expression of ERα in the brain compared to liver following EE2 exposure. In the brain (Fig 5.1A), ERα mRNA levels were upregulated with increasing EE2 concentrations with a significant 2-fold induction at 10 nM ($p=0.02$). An increase in aromatase mRNA levels with increasing EE2 concentration was also observed in the brain. This induction was significant at 10 nM EE2 (~ 2-fold; $p<0.001$). However, in the liver (Fig 5.1B), exposure to EE2 decreased ERα expression by ~ 50% at the 1 and 10 nM concentrations ($p=0.008$ and $p=0.03$, respectively). Aromatase transcripts were not detected in the tadpole liver.

Table 5.1 Changes in thyroid hormone receptor (TRα and TRβ) and deiodinase type II and III (D2 and D3) mRNA expression in the brain and liver of *R. pipiens* tadpoles following a 96 hr EE2 exposure as determined by multiplex real-time PCR[a]

			Thyroid hormone receptors		Deiodinase type II and III	
Tissue	**Treatment**	**nM**	**TRα**	**TRβ**	**D2**	**D3**
Brain	Control	--	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.11	ND[b]
	EE2	0.1	1.18 ± 0.09	1.06 ± 0.12	0.84 ± 0.07	
		1.0	1.45 ± 0.05	1.33 ± 0.12	1.05 ± 0.13	
		10	1.51 ± 0.09	1.46 ± 0.19	0.99 ± 0.10	
Liver	Control	--	1.00 ± 0.11	1.00 ± 0.14	ND	1.00 ±0.14
	EE2	0.1	1.56 ± 0.13	1.33 ± 0.19		0.85 ± 0.07
		1.0	1.15 ± 0.05	0.99 ± 0.14		0.51 ± 0.14 *
		10	0.86 ± 0.12	0.72 ± 0.08		0.48 ± 0.05 *

[a] Mean ± SEM fold-change in expression relative to control (EtOH vehicle; 0.005%); n = 8 individuals.
[b] ND = no detection
* Significantly different from control ($p \leq 0.05$; ANOVA with Bonferroni post-hoc)

A) BRAIN



B) LIVER

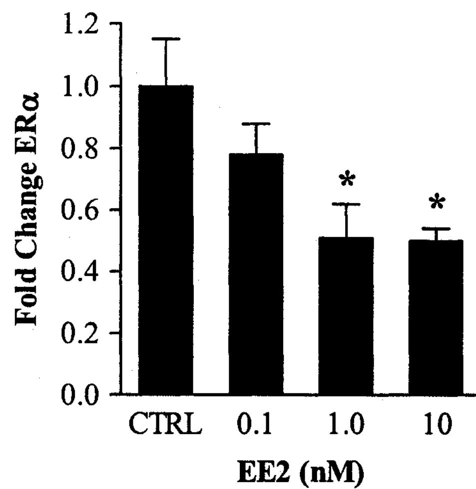


Figure 5.1 Changes in ERα and aromatase expression in the brain (A) and liver (B) of *R. pipiens* tadpoles following 96 hr EE2 exposure as determined by SYBR Green I real-time PCR. No aromatase mRNA was detected in the liver. Data were normalized to L8 and expressed as fold change relative EtOH vehicle control (CTRL). Values represent mean + SEM (n = 8 tadpoles/treatment). Data were assessed by one-way ANOVA followed by Bonferroni pair-wise comparisons. An asterisk (*) indicates significant difference from control ($p \leq 0.05$). ERα; estrogen receptor alpha.

5.4 Discussion

In the environment, EE2 concentrations in surface water can range from below the limit of detection up to 50 ng/L (0.2 nM) (Aherne and Briggs, 1989; Desbrow *et al.*, 1998; Yin *et al.*, 2002). Although levels of EE2 in the aquatic environment are relatively low compared to other contaminants, the *in vivo* potency of EE2 in fish is 10- to 50-fold higher than endogenous E2 (Segner *et al.*, 2003; Thorpe *et al.*, 2003). In the current study, the nominal EE2 concentrations used for exposure of *R. pipiens* tadpoles ranged from 0.1 nM to 10 nM. In a recent study by Mackenzie *et al.* (2003), *R. pipiens* tadpoles were exposed to 3 nM and 30 nM EE2 in a similar static renewal system. Through water chemistry analysis, they determined that tadpoles were actually exposed to an average concentration of EE2 that was 41% of the nominal concentration over a 48-h period. In the current study, the actual concentrations of EE2 were likely also less than the nominal concentrations and are, therefore, reflective of environmental levels. In addition, 10 nM EE2 has been previously shown to affect metamorphosis and gonadal sex differentiation in *R. pipiens* (Mackenzie *et al.*, 2003; Chapter 3).

Deiodinase activity in specific tissues mediates the availability of TH; deiodinase type II (D2) is responsible for the conversion of thyroxine (T4) to the more biologically active TH, triiodothyronine (T3). Failure to amplify D2 transcripts in the liver supports previous reports that hepatic D2 activity and mRNA levels are very low or non-detectable during the tadpole life stages (Galton, 1988; Galton and Hiebert, 1988). In other tadpole tissues (*e.g.*, tail and hindlimb), high levels of D2 activity and expression correlate with the developmental period during which a tissue undergoes TH-induced changes (Becker *et al.*, 1997). However, in the absence of hepatic D2 activity, the circulating plasma

provides the T3 necessary to drive TH-dependent changes in the liver (Galton, 1992; Becker *et al.*, 1997).

In contrast to D2, D3 mRNA is detectable in the tadpole liver. The results show that EE2 decreased D3 expression in the liver of *R. pipiens* tadpoles by 50% at both the 1 nM and 10 nM concentrations. In a study by Vandorpe and Kuhn (1989), E2 implants elevated endogenous E2 levels and decreased plasma TH levels in an adult ranid frog, *R. ridibunda.* The authors proposed that the reduction of circulating TH was due to decreased D2 activity in the kidney; however, they did not measure deiodinase activity in the liver. In immature rainbow trout, E2 injected intraperitoneally also depressed plasma T3 levels; this response was attributed to a decrease in the amount of functional hepatic D2 activity (Cyr *et al.*, 1988). This E2-suppression of TH levels raises the possibility that EE2 exposure could be having similar effect on TH levels in the tadpole. The observed reduction in hepatic D3 mRNA following exposure to EE2 may be a metabolic response in order to maintain normal circulating TH levels necessary for metamorphosis. A similar compensatory response was observed in the tilapia (*Oreochromis niloticus*) where an induced hypothyroid condition resulted in a downregulation of D3 activity in the liver (Mol *et al.*, 1999). It also seems plausible that D3 mRNA would decrease in the brain during periods of reduced TH levels to maintain local T3 homeostasis, as reported in the rat brain (Tu *et al.*, 1999). However, due to the early developmental stage of the tadpoles used in this study, D3 expression was not measurable. Although hepatic D3 mRNA appears to be sensitive to EE2 in the tadpole, examination of whole body TH levels, enzyme activity and D3 expression in other tissues (*e.g.*, kidney) would help elucidate the TH metabolic response to this xenoestrogen.

Exposure to EE2 stimulated ERα mRNA in the *R. pipiens* tadpole brain. Previous studies have demonstrated that E2 induces whole body levels of ERα mRNA in *X. laevis* tadpoles (Nishimura *et al.*, 1997) and juveniles (Bogi *et al.*, 2002) but this is the first report of a brain-specific induction of ERα in an amphibian. Denny *et al.* (2005) recently reported that EE2 binds with high affinity to the rainbow trout and fathead minnow ER. Following estrogen binding, an ER interacts with an ERE to induce its own transcription or that of other estrogen-responsive genes. Using an *in vivo* somatic gene transfer technique, Trudeau *et al.* (2005) showed that EE2 could activate an ERE-thymidine kinase-luciferase (ERE-TK-LUC) construct in the *X. laevis* tadpole brain as measured by a 2-fold increase in brain luciferase activity. As this technique is based on the transcriptional mechanism of action of estrogen, a positive effect indicates EE2-activation of the ER in the brain.

The estrogenic action of EE2 in the tadpole brain is further demonstrated with the induction of aromatase mRNA. It is hypothesized that E2 directly controls aromatase gene expression in the *X. laevis* gonad via an ERE in the gene promoter (Akatsuka *et al.*, 2005). Upregulation of aromatase mRNA and activity by E2 in the teleost fish brain (Kishida and Callard, 2001; Sawyer *et al.*, 2006) is attributed to the presence of a single or multiple EREs in a brain-specific aromatase isoform responsible for synthesis of neuroestrogen (Tchoudakova *et al.*, 2001; Chang *et al.*, 2005). In the current study, there was a parallel induction of ERα and aromatase in the *R. pipiens* brain. Similarly, in a recent study by Lyssimachou *et al.* (2006), a three-day exposure to EE2 (50 ng/L) induced brain aromatase mRNA and ERα in juvenile Atlantic salmon (*Salmo salar*). This pattern suggests that EE2-stimulation of ERα results in increased receptor-mediated ERE-

activation of the aromatase gene to stimulate aromatase expression. Given that basal and EE2-induced aromatase mRNA corresponds to levels of enzyme activity in the fish brain (Forlano *et al.*, 2001; Lyssimachou *et al.*, 2006), exposure to EE2 could also result in increased aromatase activity and estrogen production in the tadpole brain.

Estrogens produced from increased aromatase activity has a number of physiological functions, especially during development when aromatase activity and estrogen synthesis are involved in sex differentiation of the brain (Callard *et al.*, 1978b). Inappropriate estrogen production and transcription of estrogen-responsive genes caused by xenoestrogen exposure could significantly disrupt the early organisation of neural pathways and affect the responsiveness of the brain to subsequent hormone signals. Estradiol feminizes gonads of *X. laevis* tadpoles when exposure occurs during the period of sex differentiation (Witschi, 1967; Miyata and Kubo, 2000) and studies have demonstrated a correlation between xenoestrogen-induced aromatase expression and enzyme activity, and sex differentiation in fish (Fenske and Segner, 2004; Kuhl *et al.*, 2005). However, whether aromatase is involved in xenoestrogen-induced gonadal feminization in amphibians has not been examined. With evidence that EE2 exposure has similar immediate and persistent feminizing effects in *R. pipiens* tadpoles (Chapter 3), further work investigating the relationship between EE2, aromatase expression in the brain, and sexual differentiation is warranted.

Interestingly, EE2 down-regulated hepatic ERα mRNA in a dose-dependent manner. This response is in contrast to the classic estrogenic upregulation of ERα mRNA observed in several species of adult fish (Pakdel *et al.*, 1991; Sabo-Attwood *et al.*, 2004) and *X. laevis* (Tata *et al.*, 1993; Lee *et al.*, 1995). However, in the tadpole liver, there is

evidence that TH plays an important role in facilitating the stimulation of estrogen-responsive genes by E2. For example, during *X. laevis* metamorphosis, the capacity for E2 to induce hepatic ERα and vitellogenin mRNA in tadpoles is dependent on rising TH levels (Rabelo *et al.*, 1994). Moreover, *in vitro* administration of T3 to immature hepatocytes potentiates E2-induced ERα and vitellogenin (Rabelo and Tata, 1993). Therefore, if EE2 exposure decreases circulating TH or local metabolism of TH in the liver as previously suggested, this can modulate the ability of EE2 to induce the production of ERα mRNA. Another interpretation of the differential response in the liver is that EE2 has a suppressive effect on circulating E2 levels in the tadpole. A recent study by Martyniuk *et al.* (2006) found that EE2 exposure (0.1 and 1 nM) decreased serum E2 levels in the adult male goldfish (*Carassius auratus*). Aromatase mRNA was also induced in the goldfish brain, indicating that EE2 could stimulate neuroestrogen synthesis pathways in spite of low plasma E2 levels. As with a normal estrogenic response, tissue-specific sensitivity to xenoestrogen-induced changes in circulating hormone levels may be attributed to several factors, such as the receptor complement (different subtypes and isoforms) and presence of cofactors (coactivators and corepressors) (Diel, 2002). This is similar to the hormonal control of metamorphosis; the ability of circulating TH to induce tissue-specific transformations is exceedingly dependent on local hormone receptor levels and enzyme activity.

In summary, waterborne exposure to EE2 modulates gene expression in the brain and liver of *R. pipiens* tadpoles. The genes targeted in this study play a significant role in vertebrate development; nuclear hormone receptors mediate TH and estrogen action and enzymes regulate their metabolism and availability. The induction of ERα and aromatase

in the brain suggests that EE2 can disrupt normal estrogen signalling. Suppressed hepatic deiodinase and ERα expression indicate differential effects of EE2 in the liver that could be related to altered TH status. These results indicate that levels of EE2 present in the environment could induce a similar abnormal endocrine response in developing tadpoles and as a result, may negatively affect hormone-dependent processes such as metamorphosis and sex differentiation. However, much of the data regarding xenoestrogen effects on molecular and biochemical endpoints related to hormone function is drawn from studies with fish. Critically missing is similar research examining contaminant induced-disruption in developing amphibians. Such research would provide insight into the role of estrogen in metamorphosis, which has implications for understanding how vertebrate development may be disrupted by estrogenic pollutants.

CHAPTER 6

Estrogenic modulation of T3-induced gene expression in the tadpole

6.1 Introduction

Thyroid hormones (TH) orchestrate the structural and functional changes of larval tissues during amphibian metamorphosis (Dodd and Dodd, 1976; Kikuyama *et al.*, 1993). Regulation of thyroid function and TH action involves central feedback mechanisms (Manzon and Denver, 2004) as well as peripheral availability of TH to the thyroid hormone receptors (TR) (Galton, 1992). Metamorphosing tissues exhibit distinct developmental (Krain and Denver, 2004) and spatial (Becker *et al.*, 1997) patterns in the expression of TH-regulated genes, resulting in differential tissue sensitivity to circulating TH that drives coordinated transformations characteristic of metamorphosis (Shi *et al.*, 1996). Therefore, the ability of TH to induced thyroid-dependent gene expression is essential for normal tadpole development. In this same context, environmental contaminants that interfere with the competence of a tissue (or cell) to respond to TH, directly or indirectly, would be capable of disrupting amphibian metamorphosis.

Endocrine disruption by environmental contaminants is a current concern for wildlife and human health (Damstra *et al.*, 2002). To date, the occurrence of estrogenic endocrine disrupting compounds (EDCs) and their disruption of the reproductive system have received the most attention (Daston *et al.*, 1997; Kime, 1999; Propper, 2005). However, not all effects of xenoestrogens are confined to sexual development and reproductive function. There are many reasons to suspect that a TH-dependent process, such as metamorphosis, could be susceptible to disruption by xenoestrogens. Both

endocrine axes are centrally controlled by the brain–pituitary neuroendocrine axis and there is extensive hormonal cross-regulation between the reproductive and thyroid endocrine pathways in vertebrates (Cyr and Eales, 1996; Vasudevan *et al.*, 2002). However, the relationship between estrogen and thyroid status during amphibian development has not been widely studied. Gray and Janssens (1990) found that estrogen antagonised TH-induced metamorphosis in tadpoles. In addition, the estrogenic EDC, nonylphenol, had an inhibitory effect on rate of metamorphosis and tail resorption in bullfrog tadpoles (*R. catesbeiana*) (Christensen *et al.*, 2005). Whether xenoestrogens alter the progression of anuran metamorphosis through TH-dependent mechanisms has not yet been examined.

Metamorphosis provides a unique model for examining the thyroid-disrupting ability of EDCs. Morphological endpoints used to measure thyroid axis disruption include developmental staging, limb growth and tail resorption. In a recent report, the Organization for Economic Cooperation and Development (OECD) recommended the development of a metamorphosis assay for evaluating thyroid disruption by EDCs (OECD, 2004). The report emphasizes that molecular approaches are especially useful for identifying potential mechanisms by which EDCs affect TH-dependent metamorphosis. Treatment of tadpoles with the active form of TH, triiodothyronine (T3) precociously induces the expression of several genes that regulate central and peripheral T3 action (Helbing *et al.*, 2003). This provides an ideal system for examining how EDC exposure modulates the normal endocrine response to T3. From a developmental perspective, the ability of xenoestrogens to alter normal T3-responsiveness could be detrimental to the progression of amphibian metamorphosis.

The objective of this study was to determine whether exposure to a xenoestrogen could alter the normal endocrine response to TH in a developing tadpole. In a short-term *in vivo* assay, tadpoles were issued a T3 challenge in the presence or absence of EE2 exposure and the T3-induced gene expression patterns were compared between groups. Relative transcript levels were assessed in the both the brain and tail as they represent both a central and peripheral target of T3 action and exhibit distinct expression patterns during metamorphosis (Berry *et al.*, 1998; Krain and Denver, 2004).

6.2 Methods

Details regarding the location from which eggs were obtained, laboratory rearing conditions and general maintenance are described in the Methods section of Chapter 2 (2.2 Methods; *2.2.1 Animals and rearing conditions*).

6.2.1 EE2-T3 Challenge

17α-ethinylestradiol (EE2), 3,3′,5-triiodo-L-thyronine (T3) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). EE2 was dissolved in ethanol (EtOH) to give a 100 μM stock solution and this was added to treatment water for a final nominal concentration of 5 nM (~ 1 μg/L). A 10 mM T3 stock solution was prepared in DMSO and used to make two dilutions (0.1 and 1 μM) for addition to treatment water.

A schematic diagram of the exposure regime is shown in Figure 6.1. The exposure was divided into two 48-hour phases. In the first phase, tadpoles were pre-exposed to 5 nM EE2 or the EtOH vehicle. At the end of this first phase (48 hr), tadpoles

were fed a mixture of Nutrafin flakes (Rolf C. Hagen Inc, Montreal, PQ) and tadpole food (Carolina Biological Supply Co., Burlington, NC) *ad libitum.* The second phase was initiated 2 hr after feeding. Following a complete water change and renewal of EE2 or EtOH, tadpoles were exposed to one of three nominal waterborne concentrations of T3 (0, 5, 50 nM in DMSO). A waterborne concentration of 5 nM (~ 3.4 μg/L) T3 has been used by others to produce a total plasma T3 concentration similar to the 8 nM concentration observed in tadpoles at metamorphic climax (Shi and Brown, 1993; Denver *et al.*, 1997). The 50 nM (~ 33.7 μg/L) dose is considered supraphysiological in that it would be expected to induce the maximal physiological response. Exposures were carried out in tanks containing 8-10 tadpoles in 10 L of aerated water (loading density ~ 1 tadpole/L). All treatments (six in total) contained both EtOH and DMSO during the EE2-T3 48 hr exposure period at a combined concentration of 0.01% (100 μL/L). At the end of the exposure, tadpoles were anesthetized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, Sigma-Aldrich; 1 g/L in water) and sacrificed by decapitation. Tissues (brain and tail) were dissected and frozen on dry ice for storage at -80°C.

6.2.2 RNA isolation, cDNA synthesis and real-time PCR

Total RNA was obtained from brain and tail tissues using the commercially available TRIzol reagent as described by the manufacturer (Invitrogen Canada Inc, Burlington, ON). The RNA was resuspended in RNase-free water and treated with RQ1 RNase-Free DNase as described by the manufacturer (Promega, Madison, WI) and subsequently purified using a phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by chloroform as described by Sambrook *et al.* (1989). The treated RNA was then precipitated with ethanol and sodium acetate. RNA was resuspended in RNase-free

water and concentrations were determined spectrophotometrically using GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech, Piscataway, NJ) prior to cDNA synthesis.

Total cDNA was prepared from 2-5 μg total RNA and 200 ng random hexamer primers (Invitrogen) using Superscript II RNase H- reverse transcriptase as described by the manufacturer (Invitrogen). The 20 μL reaction was diluted to a final concentration of 5-10 ng/uL before PCR amplification.

Expression of individual gene targets were assessed by fluorescent probe- and SYBR Green I-based real-time PCRs (QPCRs) assays using an Mx4000 real-time quantitative polymerase chain reaction system (Stratagene, La Jolla, CA) as described previously in Chapter 4. Briefly, dual-labelled fluorescent probes were designed with primer sets for multiplex assays of TRα, TRβ, and L8 in triplex and D2, D3 and L8 in triplex while ERα primers were used in a SYBR Green I-based assay. Relative quantity was determined using standard plots that were generated for each target using serial dilutions of a representative pool of sample cDNA. Triplicate data for the amplification of each target cDNA were averaged and normalized to ribosomal L8.

6.2.3 Data Analysis

Gene expression data were assessed for normality (Kolmogorov-Smirnov) and homogeneity of variance (Levene's test) and were transformed if necessary to meet parametric assumptions. Changes in gene expression were assessed using two-way analysis of variance (ANOVA) with T3 dose and EE2 exposure as main factors. When main effects were significant, pairwise multiple comparisons were performed using the Holm-Sidak method. Additionally, when a statistically significant interaction between

T3 dose (0, 5, and 50 nM) and EE2 exposure (0 and 5 nM) was detected, post-hoc comparisons were made using Holm-Sidak. Data are presented graphically as the mean (+SEM) fold change in expression relative to the vehicle-treated animals (- EE2; 0 nM T3). All analysis was performed using SigmaStat 3.1 software (SPSS Inc., Chicago, IL). Values were considered to be significantly different when $p \leq 0.05$.

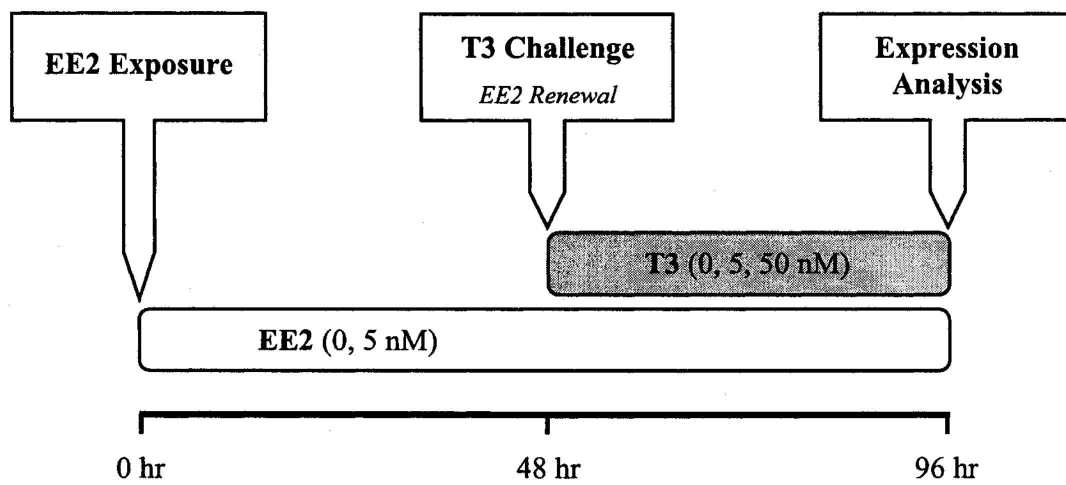


Figure 6.1 Schematic diagram of the EE2/T3 challenge exposure. Tadpoles were treated with or without EE2 for 48 hr then administered a T3 challenge (0, 5 or 50 nM). EE2 was also renewed at 48 hr. Solvent controls were EtOH and DMSO (each 0.005% of total volume) for EE2 and T3, respectively. There were six treatment groups in total, each containing ten tadpoles/treatment. EE2, 17α-ethinylestradiol; T3, 3,3′,5-triiodo-L-thyronine; EtOH, ethanol; DMSO, dimethyl sulfoxide.

6.3 Results

TRα expression in the tadpole brain (Figure 6.2A) was not affected by T3 ($F=0.76$; $p=0.48$). Although tadpoles exposed to EE2 had lower TRα transcript levels ($F=11.6$; $p=0.002$), this effect was independent of the T3 dose (T3 x EE2; $F=1.66$; $p=0.20$). TRα expression in the tail (Figure 6.2B) was upregulated by T3 ($F=6.49$; $p=0.004$). However, this induction was only significant at the 5 nM dose ($p=0.003$) and not the 50 nM dose when compared to controls (0 nM T3). There was no effect of EE2 exposure ($F=0.09$; $p=0.77$) on TRα expression and there was no interaction between factors (T3 x EE2; $F=0.01$, $p=0.99$).

In the brain, T3 induced TRβ expression (Figure 6.3A) in a dose-dependent manner ($F= 169.29$; $p<0.001$) while exposure to EE2 decreased overall TRβ levels ($F=6.41$; $P=0.02$). A significant interaction (T3 x EE2; $F=4.65$; $p=0.02$) indicated that EE2 exposure altered the dose-response induction of TRβ by T3. For example, in the absence of EE2, both doses of T3 upregulated TRβ expression 2.5-fold and 6.5-fold when compared to controls ($p\leq0.001$). However, in the EE2-exposed animals, only the 50 nM T3 dose was capable of inducing TRβ expression (5-fold; $p<0.001$) and there was no inductive effect at the 5 nM dose; the response was significantly different (lower) when compared to the groups that did not receive EE2 (5 nM, $p=0.01$; 50 nM, $p=0.002$). TRβ expression in the tail (Figure 6.3B) was also upregulated by T3 ($F=97.94$, $p<0.001$). Both doses of T3 increased TRβ levels approximately 9-fold when compared to controls ($p<0.001$). Unlike in the brain, exposure to EE2 had no effect on TRβ expression in the tail ($F=0.61$; $p=0.44$) and there was no interaction (T3 x EE2; $F=0.002$, $p=0.99$).

In the brain, D2 expression (Figure 6.4A) was induced by T3 in a dose-dependent

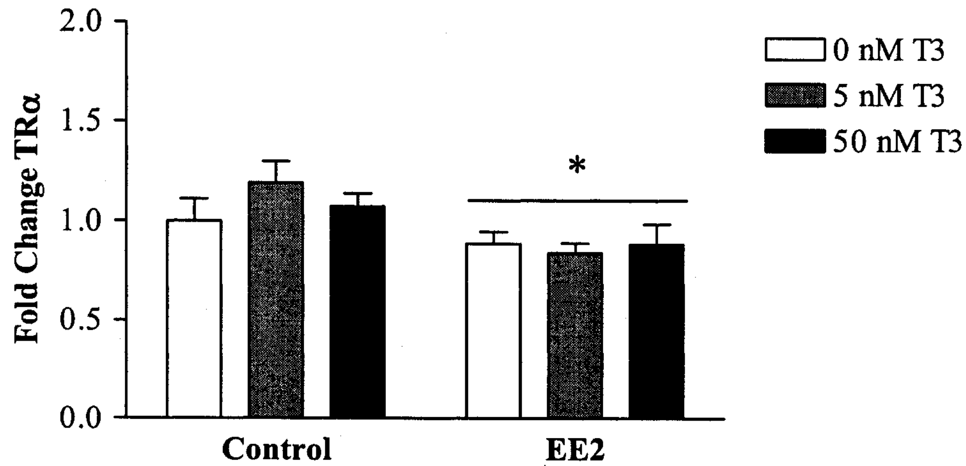
manner ($F=46.23$; $p<0.001$). D2 expression was also lower in animals exposed to EE2 ($F=5.14$; $p=0.03$) but this effect was independent of T3 dose (T3 x EE2; $F=0.99$, $p=0.38$). D2 expression was also induced by T3 ($F=33.03$; $p<0.001$) in the tail (Figure 6.4B). However, the presence of an interaction (T3 x EE2; $F=5.39$; $p=0.008$) indicated that the inductive effect of T3 was altered by exposure to EE2. Pair-wise comparisons revealed that although 50 nM T3 increased D2 levels in both EE2 exposure groups, T3 had a greater inductive effect in tadpoles that were exposed to EE2 (4.6-fold) when compared to non-exposed tadpoles (2.3-fold) ($p=0.001$).

D3 transcripts in the brain (Figure 6.5A) also responded to T3 ($F=26.70$ $p<0.001$) but only the 50 nM T3 dose upregulated D3 levels compared to controls ($p<0.001$). D3 expression in the brain was higher in animals exposed to EE2 ($F=4.79$; $p=0.03$) but this was independent of T3 effects (T3 x EE2; $F=0.36$; $p=0.70$). In the tail, T3 induced D3 expression (Figure 6.5B) in a dose-dependent manner ($F=104.09$; $p<0.001$) and although exposure to EE2 decreased D3 levels ($F=17.59$; $p<0.001$), this effect was independent of the inductive effects of T3 (T3 x EE2; $F=2.24$, $p=0.12$).

In the tadpole brain, EE2 exposure altered the inductive effect of T3 on ERα expression (Figure 6.6A), as indicated by a significant interaction between factors (T3 x EE2; $F=4.65$; $p=0.02$). Overall, tadpoles exposed to EE2 had lower ERα expression ($F=20.19$; $p<0.001$). In the absence of EE2, both doses of T3 induced ERα expression in the tadpole brain approximately 3-fold compared to the controls ($p<0.05$). However, there was no inductive effect of T3 on ERα expression among the EE2-exposed animals. Pair-wise comparisons at 5 nM and 50 nM T3 confirmed that EE2 exposed tadpoles had significantly lower ERα levels compared to non-exposed animals ($p<0.001$ and $p=0.02$,

respectively). Conversely, ERα expression in the tail (Figure 6.6B) was not affected by T3 dose ($F=3.18$; $p=0.052$) or EE2 exposure ($F=0.07$; $p=0.78$) and there was no interaction between these two main factors (T3 x EE2; $F=0.56$; $p=0.58$)

A) BRAIN



B) TAIL

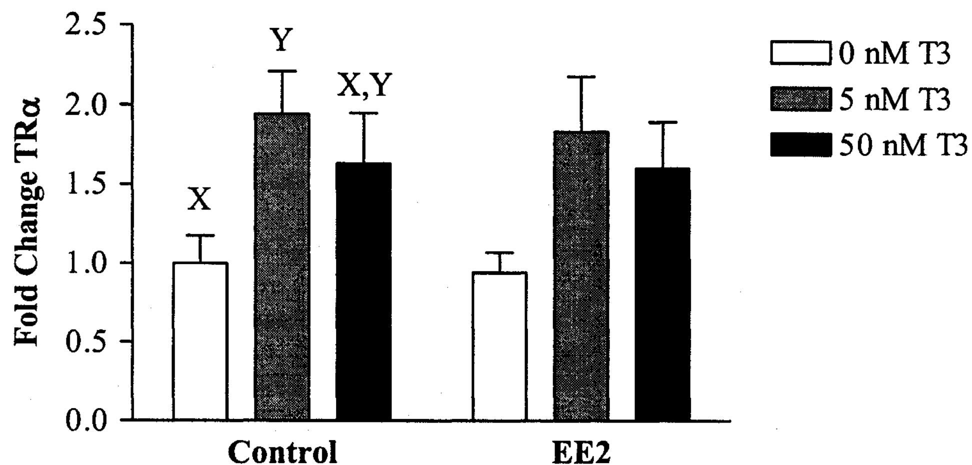
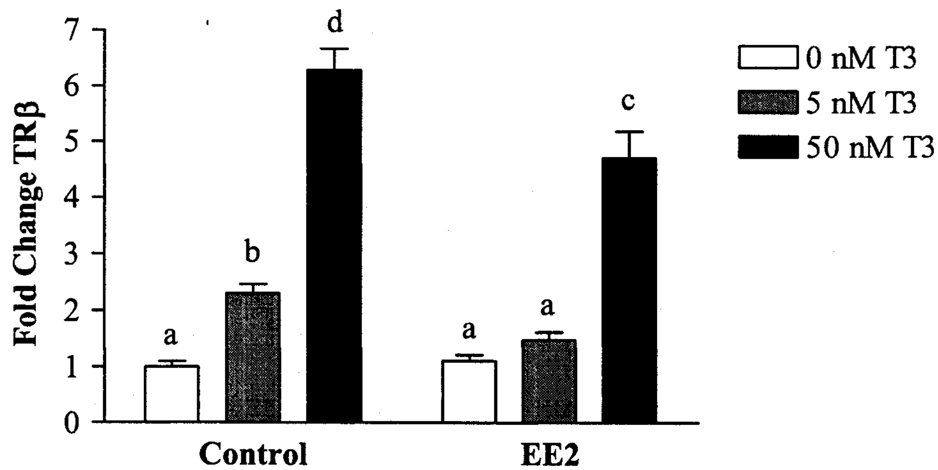


Figure 6.2 Effect of EE2 exposure (5 nM) on T3-induced TRα expression in brain **(A)** and tail **(B)** of *R. pipiens* tadpoles. Data were normalized to L8 and expressed as fold change relative to the 0 nM T3-treated control. Values represent mean + SEM (n = 6-8 tadpoles/treatment). Data were assessed by two-way ANOVA followed by Holm-Sidak pair-wise multiple comparisons (significance at $p \leq 0.05$). In the brain **(A)**, there was a main effect of EE2 exposure (*). In the tail **(B)**, there was a main effect of T3 dose (upper case letters; X-Y). TRα, thyroid hormone receptor alpha.

A) BRAIN



B) TAIL

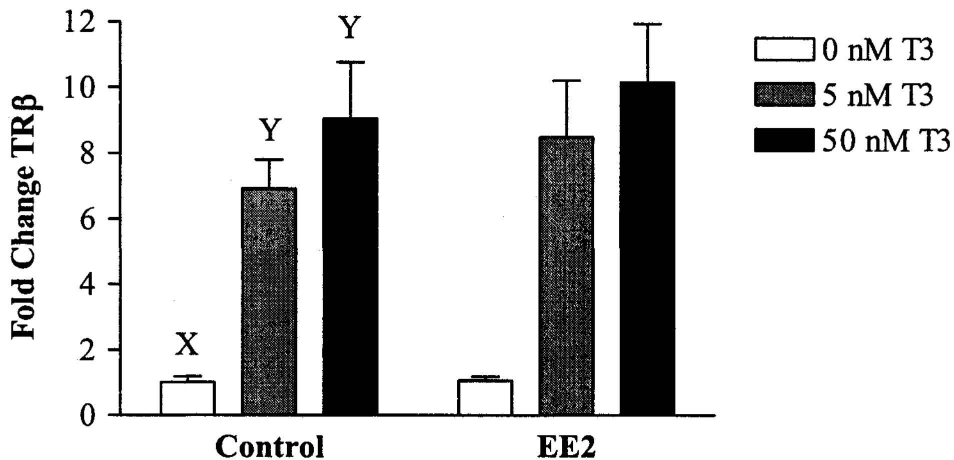
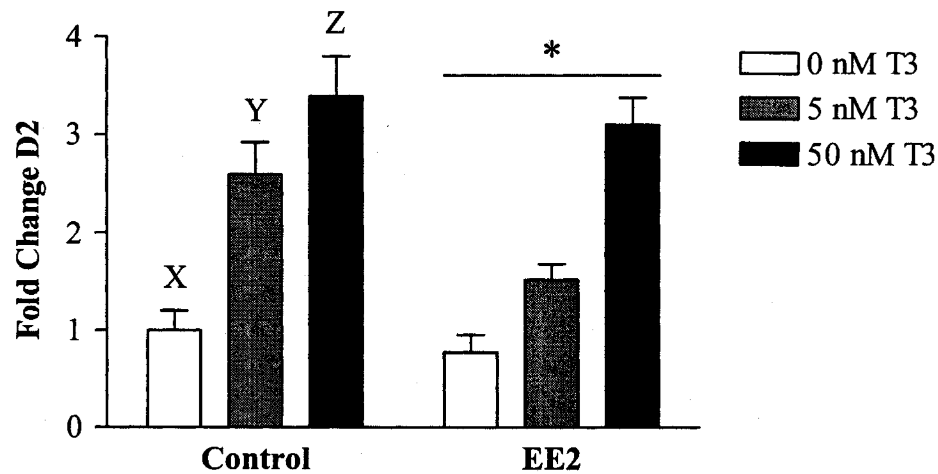


Figure 6.3 Effects of EE2 exposure (5 nM) on T3-induced TRβ expression in the brain **(A)** and tail **(B)** of *R. pipiens* tadpoles. Data were normalized to L8 and expressed as fold change relative to the 0 nM T3-treated control. Values represent mean + SEM (n = 6-8 tadpoles/treatment). Data were assessed by two-way ANOVA followed by Holm-Sidak pair-wise multiple comparisons (significance at $p \leq 0.05$). In the brain **(A)**, there was an interaction (T3 x EE2) and subsequent post-hoc comparisons identified significant differences within and between factors (lower case letters; a-d). In the tail, **(B)**, there was a main effect of T3 dose (upper case letters; X-Y). TRβ, thyroid hormone receptor beta.

A) BRAIN



B) TAIL

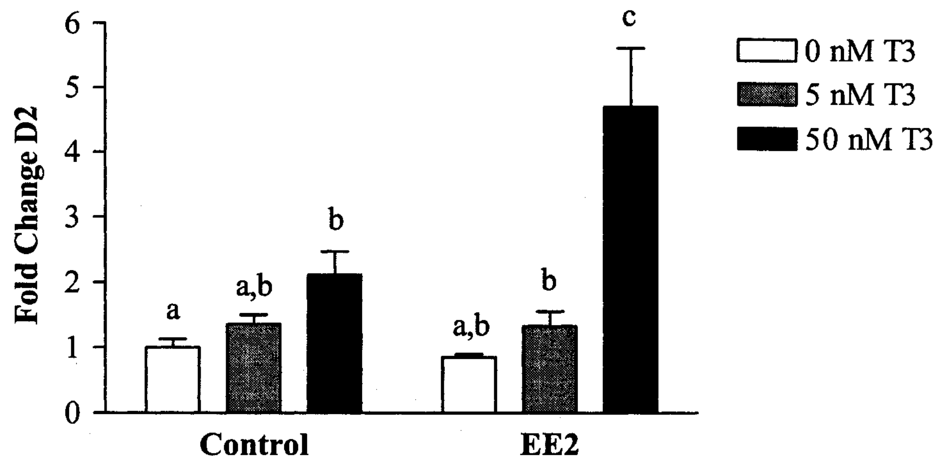
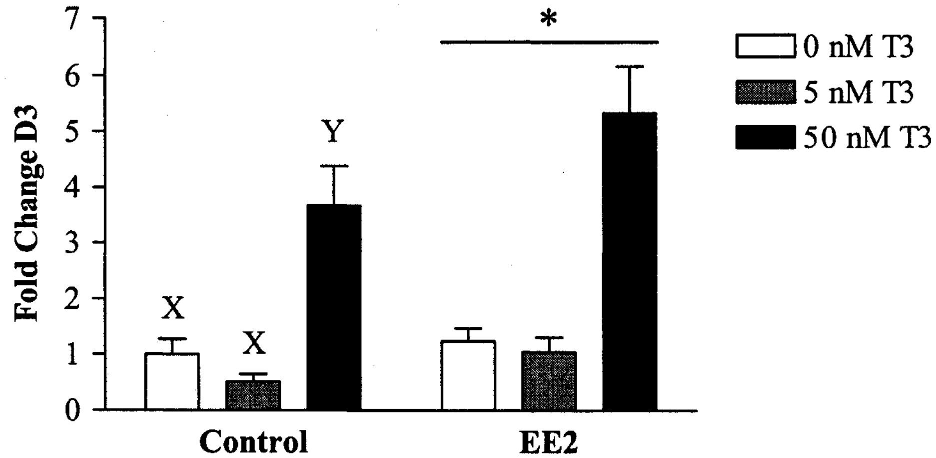


Figure 6.4 Effects of EE2 exposure (5 nM) on T3-induced D2 expression in the brain **(A)** and tail **(B)** of *R. pipiens* tadpoles. Data were normalized to L8 and expressed as fold change relative to the 0 nM T3-treated control. Values represent mean + SEM (n = 6-8 tadpoles/treatment). Data were assessed by two-way ANOVA followed by Holm-Sidak pair-wise multiple comparisons (significance at $p \leq 0.05$). In the brain **(A)**, there were main effects of T3 dose (upper case letters; X-Z) and EE2 exposure (*). In the tail **(B)**, there was an interaction (T3 x EE2) and subsequent post-hoc comparisons identified significant differences within and between factors (lower case letters; a-c). D2, deiodinase type II.

A) BRAIN



B) TAIL

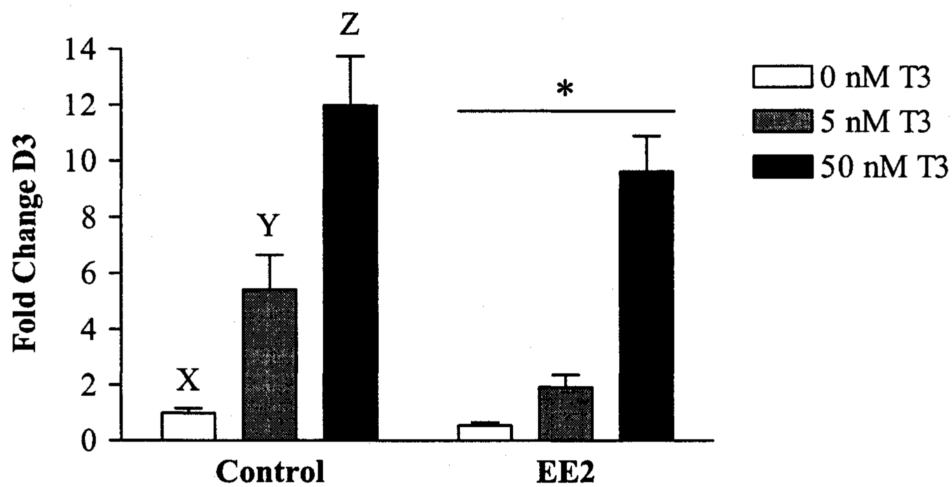
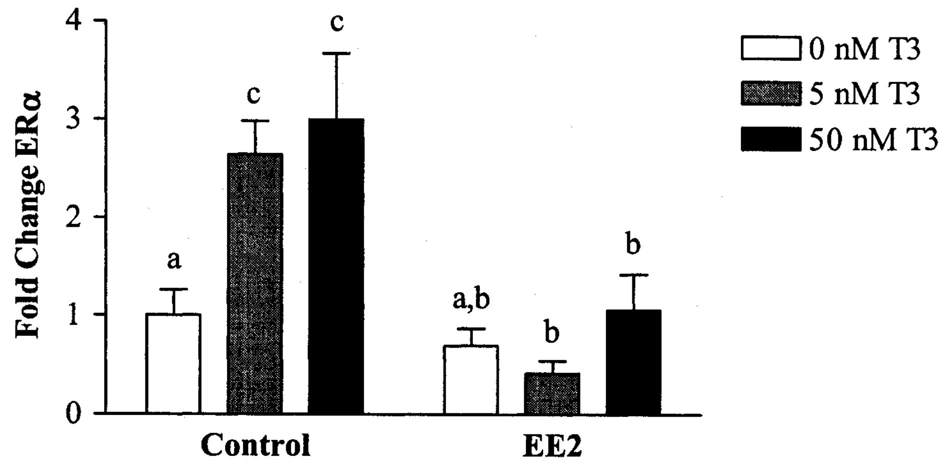


Figure 6.5 Effects of EE2 exposure (5 nM) on T3-induced D3 expression in the brain **(A)** and tail **(B)** of *R. pipiens* tadpoles. Data were normalized to L8 and expressed as fold change relative to the 0 nM T3-treated control. Values represent mean + SEM (n = 6-8 tadpoles/treatment). Data were assessed by two-way ANOVA followed by Holm-Sidak pair-wise multiple comparisons (significance at $p \leq 0.05$). In the brain **(A)** and the tail **(B)**, there were main effects of T3 dose (upper case letters; X-Z) and EE2 exposure (*). D3, deiodinase type II.

A) BRAIN



B) TAIL

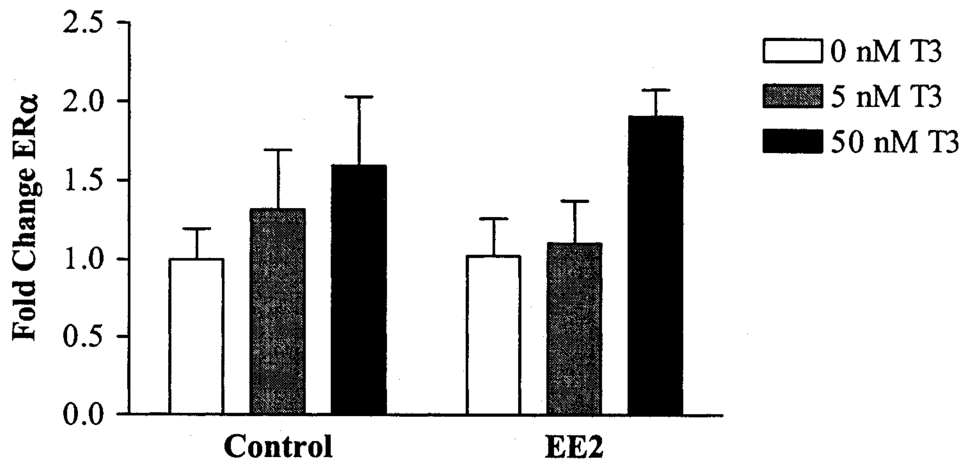


Figure 6.6 Effects of EE2 exposure (5 nM) on T3-induced ERα expression in the brain **(A)** and tail **(B)** of *R. pipiens* tadpoles. Data were normalized to L8 and expressed as fold change relative to the vehicle-treated control (No EE2; 0 nM T3). Values represent mean + SEM (n = 6-8 tadpoles/treatment). Data were assessed by two-way ANOVA followed by Holm-Sidak pair-wise multiple comparisons (significance at $p \leq 0.05$). In the brain **(A)**, there was an interaction (T3 x EE2) and subsequent post-hoc comparisons identified significant differences within and between factors (lower case letters; a-c). In the tail **(B)**, there were no effects of either factor. ERα, estrogen receptor alpha.

6.4 Discussion

The aim of this study was to examine whether EE2, a synthetic estrogen and potent EDC, could affect tissue-specific responsiveness to T3 in a tadpole. Metamorphosis is dependent on the ability of T3 to regulate tissue-specific changes in gene expression. Therefore, disruption of the normal endocrine response to T3 could represent a mechanism by which estrogen affects thyroid-dependent developmental events. The physiology of a tadpole at early prometamorphosis is well-suited for short-term assays as several TH-regulated genes are strongly and rapidly induced by exogenous T3. The ability of tissues to respond to T3 is dependent on the tissue-specific expression of thyroid hormone receptors and deiodinases. This tissue specific-sensitivity to T3 is therefore considered when discussing potential mechanisms by which EE2 alters the normal endocrine response in the tadpole.

Induction of TRα and TRβ expression by T3 was greater in the tadpole tail compared to the brain. This tissue-specific sensitivity in gene expression was also observed by Zhang *et al.* (2006), who measured gene expression by real-time PCR. They found that T3 exposure resulted in the greater change in TRα and TRβ expression in the *X. laevis* tail compared to brain and hindlimb and concluded that TRβ was the more sensitive transcript to T3 effects. The developmental functions of TRα and TRβ are complex and involve the repression and activation of T3-responsive genes (Buchholz *et al.*, 2006). During metamorphosis, T3 exerts its action by binding to TRβ which then dimerizes with other receptors, primarily the retinoic X receptor (RXR). This complex binds to thyroid response elements (TRE) to induce transcription of TRβ itself (autoinduction) and that of other TH-responsive genes. There is evidence that TR-RXR

heterodimers differentially regulate TRβ in the presence or absence of T3, and that developmental expression patterns of RXR are comparable to those of TR within individual tissues (Wong and Shi, 1995). Tissue differences in the levels of TRs or other elements (*i.e.*, RXR, co-activators and co-repressors) necessary for TRE activation can contribute to the differences in T3 sensitivity between tissues.

Exposure to EE2 inhibited or reduced the stimulatory effect of T3 on TRβ expression in the tadpole brain, demonstrating that a xenoestrogen is capable of modulating the responsiveness of the brain to T3. The ability of T3 to stimulate TRβ is crucial for the regulation of other TH-inducible genes. For example, basic transcription element binding protein (BTEB) has a TRE in its promoter and has been identified as a T3-responsive gene in the amphibian brain (Denver *et al.*, 1997). Developmental regulation of BTEB by T3 is important for brain development, specifically the growth of neurons and astrocytes (Denver *et al.*, 1999). Another T3-responsive gene, basic leucine zipper protein (bZIP), plays an important role in transcriptional regulation during cell differentiation in the tadpole brain (Denver *et al.*, 1997) and tail (Brown *et al.*, 1996). Therefore, it is possible that inhibition of TRβ induction and subsequent modulation of these target genes would have detrimental effects on T3-dependent developmental events in the brain during metamorphosis.

Local deiodinase activity is an important mediator of tissue-specific responses to T3 through control of TH metabolism. D2 activity converts the relatively inactive T4 to the biologically active T3. The results show that D2 expression was induced to a greater extent by T3 in the brain than was observed in the tail. This response is likely a reflection of differing temporal requirements for T3 in the brain and tail. I have previously

demonstrated that D2 mRNA is higher in the brain during mid-metamorphosis (Chapter 4) and others have shown that this increase corresponds to a period of development when cells in the brain are undergoing their T3-dependent proliferation (Cai and Brown, 2004). Conversely, D2 expression in the tail peaks at metamorphic climax, which is when this tissue requires T3 to stimulate apoptosis and tissue resorption (Becker *et al.*, 1997; also see Appendix B, Figure 1.C). Therefore, it appears that the ability of T3 to induce D2 expression is dependent on the tissue-specific requirements for T3 at a particular developmental stage.

Exposure to EE2 generally suppressed D2 expression in the brain. A decrease in D2 expression, and therefore less conversion of T4 to T3, may be indicative of reduced local availability of T3. Modulation of D2 could have considerable effects on brain development. Cai and Brown (2004) demonstrated that blocking D2 activity with the deiodinase inhibitor, iopanic acid (IOP), prevented T4-stimulated cell replication in the *X. laevis* tadpole brain. The fact that EE2 exposure also decreased TRβ expression in the brain further supports the idea the EE2 is reducing local T3 production by directly or indirectly suppressing D2. EE2 exposure had no effect on T3-stimulated D2 expression in the tail except for a potentiating effect at the highest dose of T3. Presently, the physiological relevance for this dose-dependent regulation of D2 expression by T3 in the tadpole tail is uncertain.

Whereas D2 drives the production of T3, the activity of D3 removes iodine from both T3 and T4, resulting in inactive metabolites. Both concentrations of T3 upregulated D3 mRNA in the tail but T3 had a substantially lesser response in the brain. Others have also shown that exogenous T3 has a greater inductive effect on D3 in the tadpole tail

compared to the brain as indicated by both enzyme activity (Becker *et al.*, 1995) and mRNA level (Shintani *et al.*, 2002). It has been suggested that the major role of D3 is to prevent exposure of the tissues to excessive levels of TH (Galton, 1992). Given that the brain is actively using T3 during the tadpole stages for T3-dependent development, inactivation of TH by D3 should be minimal when compared to tissues, such as the tail, that are not yet undergoing their T3-induced metamorphic changes. However, the high dose of T3 is likely supraphysiological, and thus the brain is responding by increased D3 which I predict would inactivate exogenous T3 to some degree.

The results demonstrate that EE2 had a stimulatory effect on overall D3 expression in the brain while having a suppressive effect in the tail. Differential modulation of D3 expression by EE2 may reflect the tissue-specific involvement of prolactin (PRL) in the regulation of T3-responsive genes. Shintani *et al.* (2002) demonstrated that PRL induced D3 mRNA expression in *X. laevis tail* cultures and in whole animal but had no effect on D3 in the brain. They and others also provide data supporting the antagonistic action of PRL on T3-dependent upregulation of TRβ and metamorphic function, such as tail regression (Tata *et al.*, 1991; Baker and Tata, 1992; Shintani *et al.*, 2002). In mammals, estradiol (E2) is an important physiological regulator of PRL production by the pituitary (Fink, 1988). Early studies have demonstrated that E2 treatment increases PRL levels in the rat (Maurer and Gorski, 1977) and directly stimulates PRL release from the pituitary *in vitro* (Lieberman, 1982). This estrogenic induction of PRL is mediated by increased levels of PRL mRNA (Maurer, 1982). In addition, injections of E2 elevated the synthesis and release of PRL in the female catfish pituitary (Singh and Singh, 1981) and E2 stimulated PRL expression in primary pituitary

cell cultures of female masu salmon depending on the reproductive stage (Onuma *et al.*, 2005). The evidence of estrogenic stimulation of PRL in other vertebrates suggests that EE2 could have similar effects on PRL levels or gene expression in tadpoles. Therefore, the observed decrease in D3 expression could be a response to the anti-metamorphic effects of EE2-stimulated PRL. This is further supported by the fact that overexpression of PRL in *X. laevis* specifically inhibits tail resorption, resulting in "tailed" frogs (Huang and Brown, 2000a).

The upregulation of ERα mRNA in the brain following T3 exposure agrees with the results of a previous T3-dose response experiment (Chapter 4). Several studies have demonstrated the potentiating effect of T3 on E2-stimulated ERα and vitellogenin expression in the *X. laevis* liver (Rabelo and Tata, 1993; Rabelo *et al.*, 1994). This evidence, together with the observed increase in brain ERα during metamorphosis, led to the proposal that T3 treatment also stimulates the capacity for endogenous estrogen to autoinduce ERα expression in the brain. In this study, however, the detection of ERα mRNA in the tadpole tail was somewhat unexpected. Preliminary analysis of ERα expression in the tail during metamorphosis showed that mRNA levels are upregulated 16-fold at metamorphic climax compared to levels in the tadpole (see Appendix B, Figure 1.E). However, from the present data, it appears that ERα in the tadpole tail is not directly regulated by T3. Thus, the role of ERα in the tail, as well as its cellular localization and hormonal regulation, remains to be investigated.

Based on the idea that T3 facilitates endogenous E2 activation and autoinduction of ERα, I hypothesized that EE2 would potentiate T3-induced ERα expression. However, exposure to EE2 completely prevented the upregulation of ERα expression by T3 in the

tadpole brain. This effect is observed at both the physiological and supraphysiological concentrations of T3, suggesting a true inhibitory effect of EE2 on T3 action. One possible mechanism for this is that EE2 exposure increased levels of ligand bound ERα, either through stimulation of aromatase or by EE2 activation of the ER. An increase in ligand-bound ER could interfere with the ability of T3-TR complex to regulate gene expression. The cross-regulation between E2 and T3 on gene expression in the mammalian brain is attributed to competitive binding of hormone receptors to identical ERE and TRE half-site sequences (Zhu *et al.*, 1996). Similarly, an increase in the amount of liganded ERα would compete with TR at the ERE or TRE to inhibit T3-TR actions. A more indirect mechanism may involve EE2 acting centrally to decrease T3 levels, as discussed in Chapter 5. Although the mechanisms proposed here are speculative, this inhibition response indicates that EE2 exposure could have significant effects on the developmental regulation of estrogen responsive genes during metamorphosis.

In conclusion, the results of the present study demonstrate that EE2 exposure at low concentrations for relatively short periods can alter the molecular response of *R. pipiens* tadpoles to T3. Although the mechanisms of EE2 modulation of gene expression are as yet unclear, interaction between T3 and EE2 at the receptor level is possible. Although a functional link between estrogenic modulation of T3-induced gene expression programs and disrupted metamorphosis remains to be demonstrated, this "challenge" assay together with real-time PCR could provide a sensitive and relatively rapid system for examining the potential thyroid-disrupting effects of other EDCs.

CHAPTER 7

General discussion

7.1 Thesis summary

The first objective of this thesis research was to assess the toxicological and developmental effects of xenoestrogen exposure during amphibian larval life-stages. Baseline toxicity data for acute xenoestrogen exposure were established for two ranid species, *R. pipiens* (Northern leopard frog) and *R. sylvatica* (Wood frog) and at two stages of tadpole development (Chapter 2). Although the results indicated that *R. sylvatica* tadpoles were more sensitive to the lethal effects of xenoestrogens, the morphological characteristics and developmental rate of *R. pipiens* made it the optimal species for subsequent work examining the sublethal effects of xenoestrogens. A developmental exposure was conducted to determine estrogen-sensitivity of *R. pipiens* tadpoles over distinct periods of metamorphosis (Chapter 3). Histological analysis revealed that a short-term exposure to EE2 during the hormone-sensitive period of gonadal differentiation resulted in female-biased sex ratios. Moreover, female-biased sex ratios and abnormal gonadal morphology were also apparent at metamorphic climax. This indicates that the dramatic effects of early exposure to EE2 can persist for at least several months after EE2 exposure ceased. Tadpoles that were also exposed to EE2 during mid-metamorphosis, a period of intense TH activity, exhibited delayed development and took longer to reach metamorphic climax. Collectively, these results suggest that a stage-dependent sensitivity to xenoestrogens is related to the critical period of hormone action for sex differentiation and metamorphosis.

The second objective of this thesis was to identify potential mechanisms by which xenoestrogens could alter TH-dependent metamorphosis. This required cloning and sequencing partial cDNAs for target genes in *R. pipiens*. Multiplex and simplex real-time PCR assays were developed for sensitive detection of transcripts in specific tissues. To better understand hormone action and potential cross-regulation between thyroid hormone and estrogen, we examined changes in hormone receptors (TRs and ERα) and enzymes (deiodinases, aromatase) in the brain throughout metamorphosis. Developmental changes in the expression of these genes and their regulation by exogenous T3 further established that temporal regulation of receptors and enzymes mediate hormone action in the tadpole brain (Chapter 4). Further, reproductive endocrine genes in the brain were also shown for the first time to be developmentally regulated by TH. These results suggest that cross-regulation between thyroid hormone and estrogen is involved in hormone signalling during metamorphosis.

The real-time PCR technique was used to assess the effects of EE2 on 1) gene expression in specific endocrine tissues in the tadpole (Chapter 5) and 2) the ability of T3 to induce the normal endocrine response in metamorphosing tissues (Chapter 6). The observation that EE2 induced both ERα and aromatase mRNA levels indicates that EE2 can disrupt normal estrogen signalling in the brain. However, the suppression of hepatic deiodinase and ERα expression demonstrates the differential effects of EE2 in the liver compared to brain, which may be related to altered TH status. These results indicate that levels of EE2 present in the environment could induce a similar abnormal endocrine response in developing tadpoles and as a result, may adversely affect hormone-dependent process such as sex differentiation and metamorphosis. To determine whether

xenoestrogen exposure alters tissue-specific thyroid hormone responsiveness, I compared the ability of T3 to regulate the gene expression in tadpoles that were either unexposed or exposed to EE2. Results from this short-term "challenge" assay demonstrated that xenoestrogen exposure alters the ability of T3 to induce the hormone receptors and enzymes that mediate the metamorphic response. This evidence for cross-regulation raises broader questions concerning the role of TH-estrogen interactions in development. Such interactions could also have implications for our understanding of how endocrine disruptors can affect both sexual development and metamorphosis.

7.2 Developmental sensitivity and persistent effects

Hormone signalling is important for the organisation of endocrine tissues during early vertebrate development and as such, disruption of this signalling during critical periods can have significant morphological and functional effects. It is clear from my results that xenoestrogen exposure during metamorphosis can have very different consequences depending on the period of development at which exposure occurs. Pulsed exposure of aquatic vertebrates to EDCs is the most likely "environmental" scenario. Snowmelt, heavy rain events and seasonal application of pesticides will all deliver EDCs to the receiving water system. If exposure corresponds to a critical period of development, a substantial proportion of individuals could have delayed metamorphosis and/or undergo abnormal sexual development. Both outcomes have the potential to compromise the fitness and survival of the entire population.

Although the developmental delay observed following EE2 exposure at mid-metamorphosis suggested an effect on the thyroid hormone axis, these results alone do

not specifically indicate that the effects were the direct result of disruption of thyroid axis activity. This was my motivation to develop a targeted gene expression approach to assess the expression of nuclear hormone receptors that mediate in hormone action and enzymes that regulate hormone synthesis and metabolism. The period in early development at which TH/E2 interactions become apparent remains to be determined. The T3-challenge assay could be applied to this question Moreover, future stage-specific exposures could employ other TH-dependent developmental endpoints as measures of endocrine disruption, such as hindlimb length, total body length *vs* tail length, head width and rate of tail resorption.

The developmental exposure experiment demonstrated for the first time the persistent effects of brief, early estrogen exposure on gonadal differentiation and metamorphosis. Follow-up gonadal assessment in terrestrial juveniles and young adults would provide a better indication of the permanence of the intersex condition and the reproductive potential of the oocytes within the intersex gonads. A role for aromatase in the ovarian differentiation of *R. rugosa* has also been recently demonstrated by Kato *et al.* (2004). They showed that aromatase expression was high during ovarian differentiation; however, when the process of ovarian differentiation was sex-reversed by testosterone, aromatase mRNA decreased in the gonads. It remains uncertain whether xenoestrogen-induced gonadal feminization of genotypic males is affecting the expression of the same genes that are involved in normal ovarian differentiation. *In situ* hybridization or immunostaining for aromatase, nuclear hormone receptors and other proteins in the developing ovaries from an EE2-exposed female population could reveal

differences in the developmental expression programs that would be indicative of genotypic females and sex-reverse males.

7.3 Hormone cross-regulation in development

Amphibian metamorphosis provides a unique opportunity to study hormonal interplay in the brain during TH-dependent development. Cross-regulation between thyroid hormone- and estrogen-responsive genes has been previously reported in the amphibian liver (May and Knowland, 1981; Rabelo and Tata, 1993; Rabelo *et al.*, 1994) and results presented in this thesis indicate that there are similar endocrine interactions during metamorphosis in the tadpole brain. Of particular interest is the developmental expression and TH-regulation of ERα and aromatase, especially with the question of TH involvement in gonadal development and reproduction in amphibians (Hayes, 1998). The positive regulation of ERα by T3 and the negative regulation of aromatase suggest a complex relationship between thyroid status and estrogen-sensitive genes in the brain during metamorphosis.

The importance of sex steroids in influencing the sexual differentiation of the mammalian brain during specific periods of development is well established (MacLusky *et al.*, 1994). In *R. pipiens*, while it is apparent that the gonads are morphologically distinct testes or ovaries by prometamorphosis, it is unknown whether the brain is sexually differentiated at metamorphic climax. Although aromatase exhibits sexually dimorphic patterns in the vertebrate brain, there was relatively little variation in aromatase mRNA among individual tadpoles in this study either at climax or following T3 exposure. While this suggests that basal and T3-regulated aromatase expression do

not differ between sexes, critically missing are studies localizing aromatase to specific brain structures (*i.e.*, hypothalamus, preoptic area, and telencephalon). Comparing the distribution of aromatase and assessing differential responses to T3 between male and females using *in situ* hybridization is a priority for future research.

Both TH and estrogen play important organisational roles in the developing neuroendocrine system of vertebrates (Toran-Allerand *et al.*, 1999; Bernal *et al.*, 2003) and there is considerable evidence of hormone cross-regulation in the brain at the many levels of biological organisation (*e.g.*, molecular, physiological, behavioural, *etc.*; (Vasudevan *et al.*, 2002; Farach-Carson and Davis, 2003). The interminable controversy regarding the endocrine-disrupting effects of the pesticide, atrazine, and its potential impact on amphibian populations, appears to be rooted, in at least in part, by an incomplete understanding of amphibian sex differentiation (Hayes, 2005; Jooste *et al.*, 2005). Therefore, in this context of endocrine disruption, the interplay between these two major endocrine axes and their roles in directing sex differentiation of the developing brain and gonads is a novel focus for further study.

7.4 Mechanisms of thyroid hormone-estrogen interactions: the potential of amphibian models

Model systems with unique characteristics provide researchers with the opportunity to "dissect" and ultimately understand complex processes. Amphibians, especially anurans, are powerful and amenable models because of the dependence of metamorphosis on TH, and the stage-specific activation of both the thyroid and reproductive axes. While I have shown that several genes may be cross-regulated, given

the major effects observed on metamorphosis and sexual development, there are potentially many other players in this interaction.

One possible site of action of EE2 or other EDCs to delay metamorphosis is production and release of thyroid stimulating hormone (TSH). An *in vitro* method has been developed and used by Denver (1988) to study the effects of hypothalamic hormones on TSH secretion in other adult anuran species and could be used to test whether EE2 acts directly on the pituitary. Manzon and Denver (2004) recently demonstrated that TH down-regulated TSHβ mRNA in the *X. laevis* tadpole pituitary; therefore, TSHβ gene expression may also be a sensitive measure of altered TH levels *in vivo*.

While it is clear that there is TH-estrogen cross-regulation in *R. pipiens* brain, it remains to be determined if the effects on gene expression are direct or indirect. Inhibition of aromatase during periods of high TH activity could be used to study the effects of TH independent of estrogen. Importantly, TH synthesis in tadpoles can be effectively inhibited by several methods (*e.g.*, treatment with perchlorate), allowing the investigator to study estrogen action independent of TH. To this end, we have used somatic gene transfer to show that EE2 increases expression of a minimal ERE-driven reporter gene *in vivo* in the perchlorate-treated *X. laevis* tadpole brain (Trudeau *et al.*, 2005). Moreover, Turque *et al.* (2005) have established stable lines of transgenic *X. laevis* expressing TRE-driven reporter genes that can be used to screen potential thyroid-active pollutants. These approaches will help to determine which genes are directly responsive to these hormones or hormonally-active contaminants.

7.5 Concluding remarks

Amphibian metamorphosis has long intrigued ecologists and developmental biologists. More recently however, metamorphosis has become a tool for toxicologists in their effort to understand the impacts of environmental contaminants on vertebrate development. My thesis contributes to this research area by examining the toxicological and developmental effects of xenoestrogens during metamorphosis and by determining changes in the expression of target genes involved in hormone-dependent developmental processes. This multi-parameter approach has provided valuable information regarding both the normal endocrine function and disrupted endocrine response in a native amphibian, *Rana pipiens*, the Northern leopard frog. Through this work, we have strengthened our knowledge of *R. pipiens* biology and gained insight into how hormone cross-regulation may be involved in development and endocrine disruption in the anuran tadpole.

APPENDIX A: Histological methods

Schedule for Tissue Processing and Embedding in Paraffin

The Tissue-Tek® VIP™ Vacuum Infiltration Processor (Sakura E150/E300 Series) was used for dehydration, clearing and infiltration of tissues in paraffin:

Dehydration of tissues (45 minutes for each step)

1. 70% Ethanol at 40°C
2. 80% Ethanol at 40°C
3. 90% Ethanol at 40°C
4. 95% Ethanol at 40°C
5. 100% Ethanol at 40°C
6. 100% Ethanol at 40°C

Clearing tissues of ethanol

7. Xylene (three 45 min. cycles)

Infiltration of tissues in paraffin

8. Paraffin at 58°C (four 45 min. cycles)

Embedding of tissues in paraffin

9. Processed tissues were embedded in paraffin blocks with the ventral side facing down for longitudinal ventral to dorsal sectioning

Harris' Hematoxylin and Eosin Y Staining Procedure for Gonad Paraffin Sections

Deparaffinize and hydrate sections

1. Xylene (three 3 min. cycles)
2. 100% Ethanol (three 1 min. cycles)
3. 95% Ethanol (1 minute)
4. Tap water (30 seconds)
5. Rinse sections in tap water

Stain sections with Hematoxylin and Eosin-Y

6. Hematoxylin (Richard Allan Scientific, Kalamazoo, MI) (2 minutes 30 seconds)
7. Tap water (30 seconds)
8. Clarifier II (Richard Allan Scientific) (30 seconds)
9. Tap water (30 seconds, agitate)
10. Bluing agent (1 minute)
11. Tap water (1 minute)

12. 95% Ethanol (30 seconds)
13. Eosin-Y (Richard Allan Scientific) (1 minute 30 seconds)

Dehydrate and clear sections

14. 100% Ethanol (three 1 min. cycles)
15. Xylene (three 1 min. cycles)
16. Mount slides with Permount (Fisher)

Blue (Hematoxylin basic dye binds to basophilic tissues): nucleus
Pink (Eosin Y acidic dye binds to eosinophilic tissues): cytoplasm

APPENDIX B: Developmental gene expression profiles in the tadpole tail

This is a preliminary analysis of developmental changes in thyroid hormone receptors (TRα and TRβ), deiodinases (D2 and D3), and estrogen receptor (ERα) gene expression in the *R. pipiens* tadpole tail during metamorphosis.

Methods Vehicle-treated animals from the developmental exposure detailed in Chapter 3 were used for this analysis. Tails were dissected at stages 27, 30, 36 and 42 and immediately placed on dry ice before storage at -80°C. Isolation of total RNA and synthesis of cDNA was carried out using methods described in Chapter 6. Expression of thyroid hormone receptor (TRα and TRβ), deiodinase enzymes (D2 and D3) and L8 ribosomal protein cDNA targets were assessed by a fluorescent probe-based real-time PCR and ERα transcripts were detected using SYBR Green I as described in Chapter 4 with all details regarding reaction mixtures and conditions reported therein. The reactions were performed in triplicate, and the data obtained were averaged and normalized to the ribosomal L8 control. Data were then analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni pair-wise comparisons. Significance was indicated if $p \leq 0.05$. Data are presented as the mean (+SEM) fold change in expression relative to stage 27. Analyses was performed using SigmaStat 3.1 software (SPSS Inc., Chicago, IL).

Results TRα mRNA levels were the same at stages 27 and 30 (premetamorphosis), followed by a significant increase of 1.5-fold at stage 36 ($p < 0.01$) and reaching maximum expression at metamorphic climax (2-fold increase over stage 27 levels: $p < 0.001$). TRβ was expressed at a constant level at the tadpole stages and increased 30-fold at metamorphic climax ($p < 0.001$). D2 and D3 levels were also constant in tadpoles and the expression of both transcripts increased at climax 18-fold and 10-fold, respectively ($p < 0.001$). ERα mRNA was detected in the tadpole tail at all three tadpole stages and increased 18-fold at metamorphic climax ($p < 0.001$).

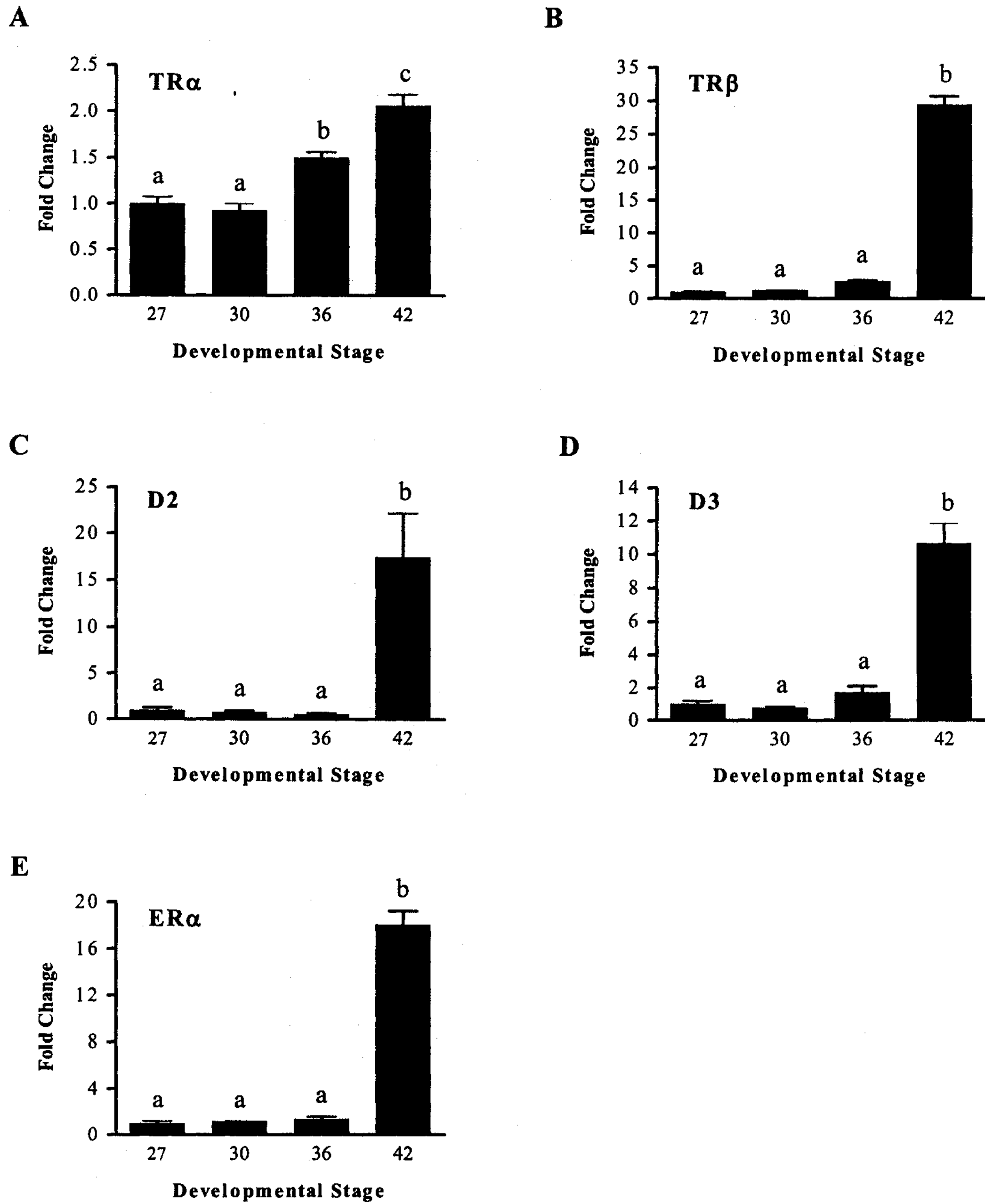


Figure 1. Developmental expression profiles of TRα **(A)**, TRβ **(B)**, D2 **(C)**, D3 **(D)**, and ERα **(E)** in the tail of *R. pipiens* during metamorphosis. Bars represent the mean + SEM (n = 8 individuals). Data were analysed by one-way ANOVA followed by Bonferroni post-hoc comparisons. Significance is indicated if $p \leq 0.05$. Data are presented fold change over stage 27. Letters indicate significant differences in expression between stages.

APPENDIX C: List of manuscripts not included in thesis

Croteau, MC, **Hogan, NS**, Gibson, J, Lean, DRS, and VL Trudeau. 2005. Toxicological threats to herpetofauna in urban and suburban environments. In *Herpetological Conservation*. Editors: R. Jung-Brown and J. Mitchell. (In press).

Martyniuk, CJ, Crawford, AB, **Hogan, NS**, and VL Trudeau. 2005. GABAergic modulation of the expression of genes involved in GABA synaptic transmission and stress in the hypothalamus and telencephalon of the female goldfish (*Carassius auratus*). Journal of Neuroendocrinology 17: 269-275.

Martyniuk, CJ. **Gallant, NS**, Marlatt, V, Wiens, S, Woodhouse, A, and VL Trudeau. 2006. Recent perspectives on estrogen and estrogen receptors in teleost fishes. In *Fish Endocrinology (Vol 2)*. Editors: M. Reinecke, G. Zaccone and B.G. Kapoor. Science Publishers, USA.

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