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**Chronic Exposures to the Herbicide Atrazine and the Pharmaceutical Finasteride Disrupt Sex Steroid
and Thyroid Hormone Signalling and Gonadal Development in Frogs**

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disrupt sex steroid and thyroid hormone signalling and
gonadal development in frogs**

Valérie S. Langlois

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valérie

RÉSUMÉ

Les perturbateurs endocriniens tels que les pesticides et les produits pharmaceutiques peuvent altérer le développement des amphibiens et contribuer à leur déclin mondial. Le premier objectif de cette thèse de doctorat était d'investiguer si l'atrazine (ATZ), un herbicide couramment utilisé sur les cultures de maïs et de soya, perturbe le développement de *Lithobates (Rana) pipiens* (grenouille léopard) à des concentrations détectées dans les écosystèmes canadiens. Des expositions à long terme de 0,1 et 1,8 µg/L ATZ ont été effectuées sur des têtards de *L. pipiens* en mésocosmes semi-contrôlés. L'atrazine a réduit le succès de la métamorphose, induit un ratio sexuel favorisant les femelles, altéré l'activité de la 5β-réductase (srd5beta; une enzyme impliquée dans la conversion de la testostérone en 5β-dihydrotestostérone), et finalement, affecté l'expression génique du récepteur des estrogènes α dans le cerveau et de la déiodinase de type 3 dans la queue des têtards. Le second objectif était de caractériser les fonctions des enzymes 5α-réductase (srd5alpha; de type 1, 2 et 3) et srd5beta chez les grenouilles. Ces enzymes convergent dans l'évolution puisqu'elles possèdent des fonctions biologiques similaires (ex.: la réduction de la testostérone, la biosynthèse de l'acide biliaire et l'érythropoïèse), par contre, les srd5alpha et srd5beta ne partagent pas d'ancêtre commun. L'utilisation du RT-PCR en temps réel a permis de détecter l'expression génique de ces enzymes au début du développement de *Silurana (Xenopus) tropicalis* (xénope tropical). De plus, le produit pharmaceutique utilisé contre le cancer de la prostate, le finastéride (inhibiteur de la srd5alpha de type 2 et srd5beta chez l'humain) a été utilisé pour des expositions à court terme (25, 50 et 100 µM) et à long terme (25 µM) chez *S. tropicalis*. Le

finastéride a inhibé l'activité de la *srd5beta* et de l'aromatase (*cyp19*) chez les larves de *S. tropicalis* exposées à court terme. Par contre, lorsque les têtards de *S. tropicalis* ont été exposée à long terme au finastéride (de l'œuf jusqu'à la métamorphose), aucun effet n'a été détecté sur l'activité de *cyp19* et une augmentation de l'activité hépatique de la *srd5beta* a été mesurée chez les mâles. De plus, le traitement à long terme au finastéride a induit le développement d'oocytes testiculaires chez les mâles, également nommés gonades intersexuelles. Finalement, des analyses de RT-PCR en temps réel ont démontrées que le finastéride a altéré l'expression génique de gènes impliqués dans la synthèse des stéroïdes sexuels et des hormones thyroïdiennes. Ces altérations de l'expression génique des hormones thyroïdiennes suivant l'inhibition de *srd5alpha* et *srd5beta* suggèrent une relation complexe entre les gènes responsables de la synthèse des hormones thyroïdiennes et du statut des hormones androgéniques au début du développement et lors de la métamorphose de la grenouille. En conclusion, les analyses de RT-PCR en temps réel, d'activités enzymatiques et d'histologie ont permises de démontrer que *srd5alpha* et *srd5beta* sont des enzymes importantes durant le développement de la grenouille et qu'elles sont de 'nouvelles' cibles pour les perturbateurs endocriniens.

ABSTRACT

Endocrine disrupting chemicals (EDCs) such as pesticides and pharmaceuticals can upset amphibian development and contribute to worldwide amphibian population declines. The first objective of this doctoral research was to investigate if the widely used herbicide atrazine (ATZ; used on corn and soy crops) alters *Lithobates (Rana) pipiens* (Northern leopard frog) development using concentrations of ATZ reported in Canadian ecosystems. Chronic exposures to 0.1 and 1.8 µg/L ATZ on *L. pipiens* tadpoles were performed in semi-controlled mesocosms. Atrazine reduced metamorphic success, induced female-biased sex ratio, altered the hepatic activity of steroid 5β-reductase (srd5beta; an enzyme involved in the conversion of testosterone into 5β-dihydrotestosterone) and affected the expression of estrogen receptor α in brain and deiodinase type 3 in tail. The second objective was to characterize the functions of the steroid 5α-reductase (srd5alpha; type 1, 2 and 3) and srd5beta in frogs. These enzymes represent a convergence in evolution: they share similar biological functions (e.g., testosterone reduction, bile acid biosynthesis and erythropoiesis), but srd5alpha and srd5beta do not have a common ancestor. Using real-time RT-PCR analysis, these enzymes were detected throughout *Silurana (Xenopus) tropicalis* (Western clawed frog) early development. The prostate drug finasteride (a 5α-reductase type 2 and srd5beta inhibitor in humans) was used in short-term (25, 50 and 100 µM) and chronic (25 µM) exposures of *S. tropicalis* under laboratory conditions. Finasteride inhibited srd5beta and aromatase (cyp19) activities in whole embryos after short-term exposures. However, chronically exposed *S. tropicalis* until metamorphosis, revealed no effects of finasteride on cyp19 activity and an increase in male hepatic srd5beta activity. Furthermore, chronic treatment with finasteride induced testicular oocytes in developing males (also referred to

as the intersex condition). In addition, real-time RT-PCR analysis showed that finasteride treatments altered sex steroid- and thyroid hormone-related gene expression. Alterations of thyroid hormone gene expression following the inhibition of *srd5alpha* and *srd5beta* suggest a complex relationship between the thyroid hormone-responsive genes and the androgen status in early frog development and at metamorphosis. In conclusion, real-time RT-PCR, enzymatic activity and histology analyses demonstrated that *srd5alpha* and *srd5beta* are important enzymes during frog development and are ‘new’ targets to EDCs.

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ABBREVIATIONS

$^3\text{H-A}$	1 β -3H-androstenedione
5 α -DHT	5 α -dihydrotestosterone
5 β -DHT	5 β -dihydrotestosterone
5 β -3 β -diol	5 β -androstan-3 β -17 β -diol
aa	Amino acids
ACTH	Adrenocorticotropin
AKR	Aldo-keto reductase
ANOVA	Analyse of variance
ANPCT	<i>Agencia Nacional de Promoción Científica y Técnica</i>
ar	Androgen receptor
ATM	Age to metamorphose
ATZ	Atrazine
BLAST	Basic local alignment search tool
C	Carbon
CaCl ₂	Calcium chloride
cDNA	Correspondent deoxyribonucleic acid
CNICT	<i>Consejo Nacional de Investigaciones Científicas y Técnicas</i>
CRH	Corticotrophin releasing hormone
CWQG	Canadian Water Quality Guideline for the protection of freshwater aquatic life
cyp19	Aromatase
DAPTF	Declining Amphibian Population Task Force
DDT	p,p-dichlorodiphenyltrichloroethane
DET2	De-etiolated 2
DIA	Deisopropylatrazine
dio1	Deiodinase type 1
dio2	Deiodinase type 2
dio3	Deiodinase type 3
dpf	Days post fertilization
dph	Days post hatching
dpm	Days post metamorphosis
E2	Estradiol
EC	Environment Canada
EDCs	Endocrine disrupting chemicals
EDTA	Ethylenediaminetetraacetic acid
EE2	17 α -ethinylestradiol
EPA	U.S. Environmental Protection Agency
eralpha	Estrogen receptor α
erbeta	Estrogen receptor β
EtOH	Ethanol
F	Female
FETAX	Frog embryo teratogenesis assay - <i>Xenopus</i>
FSH	Follicle-stimulating hormone
G	Gosner developmental stages
GI	Sequence identification number in NCBI

gpsn2	Synaptic glycoprotein 2
gpsn2-like	Synaptic glycoprotein 2-like
GSGA	Graduate Student Grants-in-Aid
GSI	Gonadosomatic index
HC	Health Canada
hCG	Human chorionic gonadotropin hormone
HF	Homeland Foundation
HHB	Howard Hughes Biology
hpf	Hour post-fertilization
HPLC-MS/MS	High performance liquid chromatography with dual mass spectrometry
I	Intersex
IWRC	Illinois Water Resource Center
KCl	Potassium chloride
KH ₂ PO ₄	Monopotassium phosphate
K _m	Michaelis-Menten constant
KT	Ketotestosterone
L	Laboratory exposure
LB	Luria-Bertani
LC50	Median lethal concentration
LH	Luteinizing hormone
M	Male
MECSSTJ	Ministry of Education, Culture, Sports, Science and Technology of Japan
MEJ	Ministry of the Environment of Japan
Meta	Metamorphosis
MgCl	Magnesium chloride
MI	Mesocosm located inside
MO	Mesocosm located outside
mRNA	Messenger ribonucleic acid
MRS	Modified Ringer's solution
MS-222	3-aminobenzoic acid ethyl ester
n/a	Not available
NaCl	Sodium chloride
NADP ⁺	Nicotinamide adenine dinucleotide phosphate oxidized
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NaHCO ₃	Sodium bicarbonate
NCBI	National Center for Biotechnology Information
NF	Nieuwkoop-Faber developmental stages
NIH	National Institutes of Health
NIHEHS	National Institutes of Health and Environmental Health Sciences
NSF	National Science Foundation
PCR	Polymerase chain reaction
RF	Rose Foundation
rpl8	Ribosomal protein subunit L8
RR	River Raisin

RT	Room temperature
RT-PCR	Reverse transcriptase - Polymerase chain reaction
SCPI	Syngenta Crop Protection Inc.
SD	Standard deviation
SEM	Standard error of the mean
srd5alpha1	5 α -reductase type 1
srd5alpha1/2	5 α -reductase subfamily containing type 1 and type 2 members
srd5alpha2	5 α -reductase type 2
srd5alpha3	5 α -reductase type 3
srd5beta β	5 β -reductase
SVL	Snout-vent length
T	Testosterone
T3	Triiodothyronine
T4	Thyroxine
TCA	Trichloroacetic acid
THs	Thyroid hormones
TK	Taylor-Kollros developmental stages
TLC	Thin layer chromatography
TO	Testicular oocytes
tralpha	Thyroid receptor α
trbeta	Thyroid receptor β
TSRI	Toxic Substance Research Initiative, Government of Canada
TU	Tufts University
UC	University of California
USDA	U.S. Department of Agriculture
USDI	U.S. Department of Interior
UV	Ultraviolet radiation
UW	University of Wisconsin
V _m	Maximum enzyme velocity
v/v	Volume/volume
W	Wild habitat
WAJF	W. Alton Jones Foundation
WDA-TCP	Wisconsin Department of Agriculture, Trade and Consumer Protection, USA
WHO	World Health Organization
WSU	Wichita State University
w/v	Weight/volume
WW	Wet weight
ypm	Years post metamorphosis

CHAPTER 1

General introduction

1.1 Problem identification

Worldwide populations of amphibians are facing a biodiversity crisis and populations are declining (Wake and Vredenburg 2008). In Canada, 14 out of 21 amphibian species are either extirpated, endangered or threatened (COSEWIC 2009). From the Greek *amphi* (two) and *bios* (life), amphibians have a bi-phasic life cycle. These vertebrates can be grazers and predators in aquatic and terrestrial ecosystems, which increases their risk of being exposed to environmental contaminants as an embryo, a larva and as an adult (reviewed in Boyer and Grue 1995). Amphibians possess also permeable skin, which allows the exchange of respiratory gases, water and electrolytes with the environment (Hofrichter 2000) and can readily absorb toxic substances (Blaustein and Johnson 2003). Exposure to pollution in early amphibian development can disrupt their normal growth, metamorphosis and sexual development, which consequently reduces the fitness of amphibian populations (Reeder et al. 2005). Thus, metamorphosis and sexual development are two classic endpoints that are frequently assessed in amphibian ecotoxicology studies.

The recent decline in amphibian populations is a complex issue with numerous potential causes. Many researchers are studying these possible causes, which range from infectious disease (e.g., chytridiomycosis; Voyles et al. 2009), habitat fragmentation (Dixo et al. 2009; Eigenbrod et al. 2009), climate change (e.g., increase in solar irradiation; D'Amen and Bombi 2009), invasive species (e.g., introduction of *Rana*

catesbeiana [American bullfrog] in California; D'Amore et al. 2009) to pollution (e.g., endocrine disrupting chemicals [EDCs], reviewed in Milnes et al. 2006; pesticides, Shenoy et al. 2009). Museum *Acris crepitans* (Northern cricket frog) specimens collected since 1852 have demonstrated that within the era of intensive industrial growth and initial use of pesticides and other chemicals such as p,p-dichlorodiphenyltrichloroethane and polychlorinated biphenyls, the percentage of frogs exhibiting testicular oocytes (also referred to as the intersex condition) was significantly higher than during the pre-organochlorine era (Reeder et al. 2005). Furthermore, lake-wide exposures with EDCs such as with the pharmaceutical 17 α -ethinylestradiol (EE2; synthetic estrogen) resulted in an induction of the intersex condition in the *Rana septentrionalis* (mink frog) population compared to those inhabiting control lakes (Park and Kidd 2005). Agricultural by-products and pharmaceuticals are contaminating drinking, surface and groundwater (Giroux 2004; Loper et al. 2007; Takacs et al. 2002), and some of these contaminants can affect aquatic and semi-aquatic wildlife by causing serious damage to reproductive capacities, sexual differentiation and behaviour in the wild (Cotton and Wedekind 2009; Milnes et al. 2006). Therefore, environmental contaminants that affect tadpole development are one of the contributing factors to the declining health of amphibian populations.

The aim of this thesis was to determine the effects of two model EDCs on frog development. The herbicide atrazine (ATZ) and the prostate cancer drug finasteride are considered. This first chapter introduces the concepts of frog metamorphosis, frog sexual development, cross-regulation between endocrine axes, EDCs and presents the organization of the thesis and the rationale behind each of the chapters.

1.2 Amphibian metamorphosis

Amphibian metamorphosis is a morphogenetic process that involves organogenesis (cell proliferation, cell differentiation and cell death), remodelling of the central nervous system, growth of appendages and resorption of the tadpole tail (reviewed in Kanamori and Brown 1996). Depending on the species, this tremendous morphological change can be stimulated by environmental factors including pond drying, temperature change, pH, and population density. These environmental signals impinge on the central control of the hypothalamus-pituitary-thyroid axis to initiate thyroid hormone (TH) release into the circulation, which drives the metamorphic cascade. Corticotropin-releasing hormone (CRH) is released from the hypothalamus, which activates the release of thyroid-stimulating hormone and adrenocorticotropin (ACTH) from the pituitary. Thyroid-stimulating hormone acts on the thyroid gland to synthesize, produce and store thyroglobulin, leading to the production of THs whereas ACTH is responsible of the release of corticosteroids from the adrenal glands, which have the capacity to enhance the effects of THs (Kikuyama et al. 1993; Shi 1999). The synthesis of THs involves the conversion of the prohormone thyroxine (T4) into the active form of THs, triiodothyronine (T3), which mostly occurs in peripheral tissues and involves the action of the deiodinase enzymes (dio; type 1, 2 and 3). Deiodinase type 2 (dio2) converts T4 to T3 (reviewed in St Germain et al. 2009). In contrast, the activity of the third type of dio (dio3) converts T4 to the inactive reverse-T3 and T3 into the inactive diiodothyronine, thereby reducing local TH availability (Brown 2005). Then, THs can also be activated or inactivated by dio1 activity (reviewed in St Germain et al. 2009). Thyroid hormones can bind to thyroid receptors (tr; alpha and beta), members of the nuclear receptor

superfamily (Tsai and O'Malley 1994). The complex TH-tr can dimerize with another TH-tr (homodimer) or with a retinoid-X receptor complex (RXR; heterodimer). These ligand-activated transcription factors regulate gene expression by interacting with cognate DNA sequences (TH response element) in target genes (Aranda and Pascual 2001). Thyroid hormone activation of tr can autoinduce the transcription of the TH receptor β (*trbeta*) and can negatively feedback on hypothalamic-pituitary control. Metamorphosis is mediated by the activation of *trbeta*. Finally, changes in the expression of *dio* and *tr* influence the T4 to T3 ratio, which in turn affects metamorphosis (Huang et al. 2001; Manzoni and Denver 2004).

1.3 Amphibian sexual development

In amphibians, sexual development is primarily determined by a genetic mechanism. However, it can also be overridden by epigenetic factors, such as temperature and exogenous sex steroids (reviewed in Wallace et al. 1999). Sex steroids (e.g., estrogens and androgens) play a crucial role in reproduction and in the development of secondary sex characteristics in amphibians (Hayes 2000). Sex steroid hormones are mainly secreted by the gonads (Hadley 1996). Their synthesis and release to the blood is controlled by follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are released from the anterior pituitary. The production of both FSH and LH is regulated by the hypothalamic neuropeptide gonadotropin-releasing hormone, and many other neuropeptides and neurotransmitters. Sex steroid hormones feedback on the hypothalamus and the pituitary to both inhibit and stimulate gonadotrophin production (Licht 1979; Trudeau 1997).

Estradiol (E2) is the most biologically active estrogen involved in female and male reproductive development and functions (reviewed in Li and Rahman 2008). Estrogen action is also mediated by members of the nuclear receptor family, the estrogen receptors α (*eralpha*) and β (*erbeta*; Evans 1988). Upon binding, the estrogen-receptor complex activates estrogen-responsive gene transcription by interacting with the estrogen response element in the promoter region of the genes (Katzenellenbogen 1996). The expression of *eralpha* mRNA has been linked to differentiation and development of amphibian ovaries whereas the involvement of *erbeta* in sexual differentiation remains unclear (Takase and Iguchi 2007). In amphibians, estrogens are involved in vitellogenin (egg yolk precursor protein) production and regulate oviduct growth in females (Hayes 2000).

The dominant androgen of many vertebrates is the steroid 5 α -dihydrotestosterone (5 α -DHT). Both testosterone and 5 α -DHT can activate the androgen receptor (ar) and regulate androgen-responsive gene transcription through the androgen response element, although 5 α -DHT has a stronger affinity for ar than testosterone (Chang 2002; Normington and Russell 1992). Early studies demonstrated that testes implanted in genetically female tadpoles induced testis development (phenotypic male) and that this sex reversal only occurred when implanted during the larval period (Chang 1953). Therefore, androgens participate in the regulation and maintenance of several male characteristics such as thumb-pad, larynx development and enlargement and differentiation of the Wolffian ducts in male amphibians (Hayes 2000). Testosterone can also be enzymatically converted to a β -configuration and produce the steroid 5 β -dihydrotestosterone (5 β -DHT). This hormone was long believed to be biologically

inactive (Kokontis and Liao 1999), although it has been associated with sexual behaviour and reproductive functions in birds, mice and rats (Lafayette et al. 2008; Steimer and Hutchison 1981; Yanai et al. 1977). However, the roles of 5 β -DHT on amphibian development remain unknown.

1.3.1 Steroidogenic enzymes

Many steroidogenesis enzymes are involved in amphibian sex steroid production. However, for the purpose of this doctoral thesis, we will focus on the enzymes that are involved in the last biosynthesis step of these three sex steroids: E2, 5 α -DHT and 5 β -DHT. The aromatase cytochrome P450 enzyme (cyp19; EC 1.14.14.1) is involved in estrogen production. It aromatizes testosterone into E2 (Simpson et al. 1994) and androstenedione into estrone (Lephart 1996). Aromatase has been identified in the brain, gonads and oviduct of amphibians (Di Fiore et al. 1998). The alteration of *cyp19* expression or activity consequently results in abnormal amphibian sexual development (Chardard and Dournon 1999; Olmstead et al. 2009).

Other steroidogenic enzymes synthesize sex steroids from testosterone. The NADPH-dependent 5 α -reductase (srd5alpha; EC 1.3.1.22) and 5 β -reductase (srd5beta; EC 1.3.1.3) catalyze the reduction of the Δ^4 -ene of testosterone and yield the respective 5 α -DHT or 5 β -DHT (Drury et al. 2009). The similarities and differences between srd5alpha and srd5beta are reviewed and discussed in detail in Chapter 4. Briefly, three types of 5 α -reductase have been identified. The steroid 5 α -reductase type 1 (srd5alpha1) is mostly associated with non-androgen targeted tissues; while 5 α -reductase type 2 (srd5alpha2) is distributed only in androgen targeted tissues (Urbatzka et al. 2007a). In contrast, the recently discovered steroid 5 α -reductase type 3 (srd5alpha3) has only been

recognized in human prostate cancer cell lines (Uemura et. 2008); therefore its presence in amphibians has not yet been reported. Only one type of *srd5beta* has been identified in vertebrates and it has only been detected in gonads (Callard and Leathem 1966) and liver (Lisboa et al. 1972) of amphibians. Human *SRD5α* and *SRD5β* deficiencies can lead to pseudohermaphroditism, prostate cancer, polycystic ovarian syndrome, hirsutism and hepatic dysfunction (Andersson et al. 1991; Clayton et al. 1996; Goodarzi et al. 2006; Thomas et al. 2009). However, few studies exist on the consequences of the inhibition of these enzymes in amphibians. Zaccanti and colleagues demonstrated that depletion of *srd5alpha* activity accelerates ovarian development in female *Rana dalmatina* gonads (Agile frog; Zaccanti et al. 1994), suggesting that an alteration of sex steroid biosynthesis is changing the normal estrogen/androgen ratio. Therefore, alterations of steroidogenic enzymes can lead to changes in sex hormone levels and perturb normal frog sexual development.

1.4 Cross-regulation between the reproductive and thyroid hormone axes

The TH system has been shown to interact with estrogens during vertebrate development. There is *in vitro* evidence for a cross-regulation at the genomic level between estrogen and TH receptors (e.g., in frog hepatocytes, Ulisse and Tata 1994; mammalian kidney fibroblast, Vasudevan et al. 2002; 2001; mammalian neuroblastoma cells, Zhao et al. 2005). This crosstalk between the TH/E2 systems has also been observed *in vivo* in amphibians. For example, studies on the *Lithobates pipiens* (Northern leopard frog) have shown that T3 induced *eralpha* mRNA and decreased *cyp19* expression in the brain (Hogan et al. 2007), EE2 decreased hepatic *dio3* mRNA (Hogan

2006) and sex steroids (e.g., testosterone and E2) altered the development of TH-responsive tissues (Gray and Janssens 1990). Furthermore, inhibition of TH synthesis by perchlorate favoured a female-biased sex ratio (*X. laevis*, Goleman et al., 2002 and *Danio rerio* [zebrafish], Mukhi et al. 2007) and induced the presence of intersex in *Gasterosteus aculeatus* (three-spine sticklebacks; Bernhardt et al. 2006). There is also evidence in the literature for a cross-regulation between the TH and androgen axes. Waterborne exposure of developing frogs to T3 induced *srd5alpha1* and *ar* expression in whole larvae (Duarte-Guterman et al. 2010) and injections of T4 to hypophysectomised and hypothyroid rats stimulated hepatic *srd5alpha* mRNA and activity (Ram and Waxman 1990). These findings demonstrate that there is a need to further assess the interactions between the TH and androgen systems.

1.5 Endocrine disrupting chemicals

As aforementioned, sex steroids are central hormones controlling normal sexual development in vertebrates. However, at low concentrations, some chemicals *i.e.* EDCs, can impair sex steroid biosynthesis and actions (Hayes 2005; Scholz and Kluver 2009). These EDCs (e.g., pesticides and pharmaceuticals) can be estrogenic, anti-estrogenic, androgenic or anti-androgenic and can induce biased sex ratios, increase the presence of the intersex condition and reduce fertility in fish and amphibians (reviewed in Milnes et al. 2006). For example, chronic exposures to picomolar concentrations of EE2 skewed sex ratios toward the female phenotype in *Silurana tropicalis* (Western clawed frog) tadpoles (Gyllenhammar et al. 2009). Moreover, these sex reversed-male-to-female animals exhibited higher proportions of oviduct defects than normal genotypic females

(Gyllenhammar et al. 2009). In contrast, exposures to the cyp19 inhibitor fadrozole led to male-biased sex ratios and induced intersex gonads (Olmstead et al. 2009). Olmstead and colleagues also demonstrated that when *S. tropicalis* are exposed to fadrozole during the larval period and are then transferred to fresh water until adulthood, frogs still exhibit the intersex condition (Olmstead et al. 2009). This finding suggests that short-term inhibition of cyp19 by EDCs during the critical period of gonadal development is sufficient to induce lasting gonadal defects. Understanding the effects and the mechanisms of action of EDCs on amphibian development is of particular importance since most amphibian larvae are confined to water bodies likely contaminated with EDCs during critical and hormonally sensitive periods of development.

1.6 The herbicide atrazine

Since the last decade, the agricultural herbicide ATZ has received particular scientific, public and political attention for its potential endocrine disrupting effects in vertebrates, especially in amphibians (reviewed in Hayes 2004). This herbicide is sprayed on agricultural fields during the spring season, coinciding with the time of the year when the frogs breed. There is evidence that ATZ alters frog development and induces a female-biased sex ratio (Hayes et al. 2002b; Hayes et al. 2006a; Oka et al. 2008).

Atrazine (2-chloro-4-ethyl-amino-6-isopropylamino-s-triazine) is a member of the triazine family and is one of the most used pesticides in the world (Fig. 1.1 a). This herbicide is applied for pre- and post-emergent broadleaf weed control. Its toxic mechanism of action is to interfere with plant chloroplast function by blocking the transport of electrons in photosystem II of targeted plants (Moreland 1980). The

herbicidal selectivity is based on the ability of tolerant crop plants (e.g., corn and soy) to reduce toxicity through hydroxylation or dealkylation pathways prior to chlorophyll destruction (Shimabukuro 1967). Atrazine is an organochlorinated compound that is extremely persistent in the environment, with a half-life ranging from days to years depending on its substrate (e.g., groundwater, freshwater, soil; Graymore et al. 2001). The persistence of ATZ in the environment explains its presence in air, rain, sediments, groundwater, rivers and lakes (Douglas et al. 1993; Hall et al. 1993; Readman et al. 1993; Solomon et al. 1996). Major metabolites of ATZ are also found in the environment, especially hydroxyatrazine, deethylhydroxyatrazine, deisopropylhydroxyatrazine and the chlorinated metabolites, deethylatrazine and deisopropylatrazine (DIA, Lin et al. 2008).

While the maximum acceptable concentrations of ATZ in drinking water range from 2 µg/L ATZ (WHO 2008), 3 µg/L (EPA 2009) to 5 µg/L ATZ (HC 2008), the Canadian Water Quality Guideline for the protection of freshwater aquatic life (CWQG) for ATZ is 1.8 µg/L (<http://ceqg-rcqe.ccme.ca/download/en/144/>). Reports on agricultural pesticide occurrence in Canadian waters have been published for Ontario (Struger et al. 2008; Graymore et al. 2001) and Québec (Giroux 2004). Maximum concentrations detected were 0.12 µg/L ATZ in groundwater, 4.2 µg/L in drinking wells, 74 µg/L in farm wells, 81 µg/L in rivers and 0.445 µg/L in rain. Although these concentrations appear to be low, some studies have shown biological effects on non-target species at these levels of ATZ.

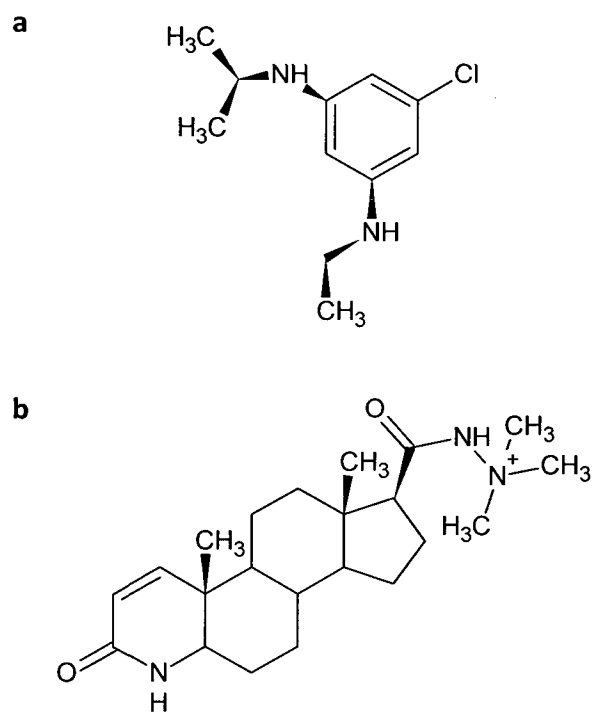


Figure 1.1 Molecular structures of ATZ (a) and finasteride (b).

1.6.1 Effects of atrazine on frog development

Laboratory, mesocosm and field studies have provided evidence that ATZ exposure may be associated with negative effects on frog development and gonadal differentiation. However, some authors believe that the existing data are neither consistent nor reproducible (reviewed in Solomon et al. 2008). There is also considerable controversy surrounding a possible correlation between sponsorship, authorship and study design with the outcomes of “no effects” that are reported in some of these studies (Hayes 2004). Therefore, an extensive and independent literature review of all studies (n = 38) measuring the effects of waterborne exposures to ATZ on frog development is presented (Table 1.1). These studies are classified by frog species, type of exposures (laboratory, mesocosm or in the wild), nominal concentrations used and exposed developmental stages. For each study, the solvent used to deliver ATZ, source of ATZ, concentration of ATZ in the control groups, duration of exposure, summarized outcomes and study’s sponsors are provided if applicable. To summarize the information, each exposure performed in the 38 publications was divided as low (less than 10 µg/L ATZ), medium (11 – 1000 µg/L ATZ) and/or high (1001 µg/L ATZ and higher) concentrations. However, studies performed in wild habitats (referred to as “W” in Table 1.1) were removed from this analysis since the historical exposure of these frog populations is unknown. Therefore, from these 38 publications, there were 95 exposures that were considered and that were separated into these two categories: endpoints related to frog general developmental (n = 62) and endpoints related to reproduction (n = 33). Of these 95 exposures, 60 % were performed on *X. laevis*, 13 % on *L. pipiens*, 6 % on *Rana clamitans* (green frog), 6 %, on *Bufo americanus* (American toad) and 15 % on other frog

species. More than half of these exposures (56 %) provided evidence that ATZ alters frog development.

For the general developmental endpoints, all exposures at high concentrations of ATZ affected frog development. Deformities (*L. pipiens*, *Rana sylvatica* [wood frog], *B. americanus* and *X. laevis*; n = 5 exposures), increased time to metamorphosis (*Hyla versicolor* [Gray tree frog] and *Rhinella arenarum* [South American toad]; n = 2 exposures) or mortality (*L. pipiens*, *B. americanus*, *R. arenarum* and *X. laevis*; n = 4 exposures) were the major observations obtained for more than one species of frogs.

At medium concentrations of ATZ, 64 % of studies also reported developmental defects. Increase in mortality (*R. catesbeiana*, *B. americanus*, *Pseudacris crucifer* [spring pepper], *R. arenarum* and *X. laevis*; n = 7 exposures), decrease in snout-vent-length and wet weight (*H. versicolor* and *X. laevis*; n = 4 exposures), increase in time to metamorphosis (*R. arenarum* and *X. laevis*; n = 5 exposures), alteration of gene expression related to growth (*X. laevis*; n = 1 exposure) and increase in abnormal swimming (*X. laevis*; n = 1 exposure) have been reported; however, no changes were found in *L. pipiens* (n = 2 exposures) or *R. clamitans* (n = 2 exposures). Interestingly, in *X. laevis*, 53 % of exposures resulted in an alteration of *X. laevis* development, while 47 % of the studies demonstrated no changes in developmental-related endpoints.

At low ATZ concentrations, only one exposure on *X. laevis* led to a change in frog development *i.e.* decrease in snout-vent length and wet weight, while nine other studies did not report any changes in *X. laevis* development. However, low concentrations of ATZ have induced developmental defects in other frog species. Increase in mortality (*B. americanus*, *P. crucifer* and *R. clamitans*; n = 3 exposures),

increase time to metamorphosis and decrease in snout-vent length (*R. clamitans* and *L. pipiens*; n = 2 exposures), and increase in the formation of thymic plaques (*L. pipiens*; n = 1 exposure) have been observed.

For reproduction-related endpoints, all of the exposures were performed at low or medium ATZ concentrations. Therefore, the effects of ATZ on reproductive functions at concentrations higher than 1001 µg/L are unknown. Most of the studies were performed on *X. laevis* (n = 27 out of 33 exposures). Of the endpoints studied, induction of abnormal gonads and decrease in plasma sex steroid concentrations were the most common endpoints reported by different groups (Hayes et al. 2002a; Hayes et al. 2003; Hecker et al. 2005a; Kloas et al. 2009b; Oka et al. 2008; Orton et al. 2006; Tavera-Mendoza et al. 2002a; b).

At low concentrations of ATZ in *X. laevis*, gonad abnormalities (n = 4 exposures), laryngeal deformities (n = 2 exposures), decrease in *cyp19* mRNA levels (n = 1 exposure), increase in gonadosomatic index (n = 1 exposure) and a decrease in plasma E2 concentration (n = 1 exposure) were observed. Seven other exposures did not report any significant changes. At medium concentrations of ATZ, gonad abnormalities including change in sex ratio (n = 6 exposures), decrease in plasma testosterone level (n = 2 exposures) and decrease of laryngeal size (n = 2 exposures) were recorded, while seven other exposures did not lead to any reproductive defects.

In *L. pipiens*, three out of four exposures demonstrated that ATZ altered gonadal development (e.g., alteration of germ cells count, increase in ovary size, induced testicular oocytes and underdeveloped gonads) at low or medium concentrations. However, for the only exposure that did not observe changes in *L. pipiens* gonadal

development, the authors argued that this was due to a population issue since all of the population (even the control animals) exhibited a delay in gonadal development, making it impossible to correctly assess the effects of the treatments (Hayes et al. 2006a). Only one exposure has been performed using *R. clamitans*, and the authors did not report any significant differences in gonadal histopathology between control and treated animals, at both low and medium ATZ concentrations (Coady et al. 2004).

Despite the lack of consensus among the research community regarding the effects of ATZ on frog development and reproduction, some patterns can be deduced. First, exposure of frogs to more than 1001 µg/L ATZ can affect normal growth. However, the consequences of high concentrations of ATZ on sexual development have not yet been studied. Second, from all the observed effects on general development, results indicate that ATZ is more likely to reduce than accelerate growth and metamorphosis. Third, the induction of abnormal gonadal development and decrease in plasma sex steroid concentrations are the two endpoints related to reproduction the most commonly reported in animals exposed to low and medium concentrations of ATZ. Taken all together, this analysis clearly shows that ATZ can affect development in some species of frogs and demonstrates the importance of investigating the mechanisms of disruption of this herbicide.

Table 1.1 Summary of the effects of ATZ on frog development

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Lithobates pipiens</i>	L	0, 10 [98.2 % ATZ Syngenta]*	n/a	TK2 to TK23-34	0, 10 µg/L: testicular oocytes 10 µg/L: alters testis cell numbers, ↑ ovary follicle size No effects: snout-vent length, wet weight, sex ratio	Declining Amphibian Population Task Force (DAPTF)	1
<i>Lithobates pipiens</i>	L	0, 20, 200 [99 % ATZ ChemService]	n/a	G25 to G42 (138 d)	No effects: mortality, snout-vent length, wet weight, hematocrit	University of Wisconsin (UW), Wisconsin Department of Agriculture, Trade and Consumer Protection, USA (WDA-TCP)	2
<i>Lithobates pipiens</i>	L	0, 2590, 4320, 7200, 12 000, 20 000 [99 % ATZ ChemService]	n/a	G10 to G12	4320 to 20 000 µg/L: ↑ deformed larvae No effects: hatchability, swimming speed	UW, WDA-TCP	3
<i>Lithobates pipiens</i>	L	0, 1560, 2590, 4320, 7200, 12 000, 20 000 [99 % ATZ ChemService]		Adult male frogs (4 d)	4320 to 20 000 µg/L: ↑ buccal ventilation 12 000 and 20 000 µg/L: ↑ thoracic ventilation 20 000 µg/L: stop feeding	UW, WDA-TCP	3

Table 1.1 Continued

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Lithobates pipiens</i>	L	0, 0.1, 25 (in 0.004 % ethanol) [ATZ source: n/a]	n/a	2 dph until tail resorption	0.1 and 25 µg/L: underdeveloped gonads, testicular oocytes and closed lobules, ↓ germ cells	W. Alton Jones Foundation (WAJF), World Wildlife Fund (WWF), Homeland Foundation (HF), Rose Foundation (RF), Howard Hughes Biology (HHB)	4
<i>Lithobates pipiens</i>	W	Ranged from 0.5 to 6.7	Ranged from 0.1 to 0.3	Young of the year	7/8 sites: testicular oocytes, underdeveloped gonads, ↓ germ cells	WAJF, WWF, HF, RF, HHB	4,5
<i>Lithobates pipiens</i>	MO	0, 0.1, 1.8 [97.1 % ATZ Syngenta]	Ranged from 0.003 to 0.028	G27 to G42	0.1 and 1.8 µg/L: ↓ success to meta. 1.8 µg/L: ↑ mortality, female sex ratio ↑ <i>eralpha</i> mRNA (brain) ^h ↓ <i>dio3</i> mRNA (tail) ^h Alters <i>srd5beta</i> activity No effects: time to meta., wet weight, snout-vent length, gonadal histopathology, <i>cyp19</i> activity, gene expression (<i>cyp19</i> , <i>dio2</i> , <i>tralpha</i> , <i>trbeta</i>) in brain, liver and tail	Environment Canada (EC), Health Canada, Natural Sciences and Engineering Research Council of Canada	6 ⁱ
<i>Lithobates pipiens</i>	W	Ranged from 0.068 to 3.13	Ranged from 0.045 to 0.39	Adult frogs	Control and ATZ contaminated sites: testicular oocytes	EC	7

Table 1.1 *Continued*

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Lithobates pipiens</i>	W	Ranged from 0.17 to 250	Ranged from 0.17 to 0.23	Adult and juvenile frogs	Control sites: testicular oocytes (adult frogs) Control and ATZ contaminated sites: testicular oocytes (juvenile frogs) No correlations: plasma T or E2 concentration, cyp19 activity, GSI, limb malformations	Syngenta Crop Protection Inc. (SCPI), National Institutes of Health and Environmental Health Sciences (NIHEHS)	8,9
<i>Lithobates pipiens</i>	L	0, 0.1 (in 0.0036 % ethanol) [$> 98\%$ ATZ ChemService]	0.1	From 2 dph until tail resorption	0, 0.1 µg/L: underdeveloped gonads (by histology) 0.1 µg/L: ↓ snout-vent length, wet weight, ↑ thymic plaques No effects: mortality, time to meta.	H. H. Wheeler and Park Water Company, NSF, HHSP	10
<i>Lithobates pipiens</i>	L	100, 1000, 10 000, 100 000, and 1 000 000 [field grade of 40.8 % ATZ]	n/a	G29, G40 (1 and 3d)	12 000 to 77 000 µg/L: LC50 at these stages	n/a	11
<i>Rana clamitans</i>	L	0, 10, 25 [97.1 % ATZ Syngenta]*	Ranged from 0.02 to 0.30	From G25 (506 d)	0, 10, 25 µg/L: gonadal abnormalities (by gross morphology), ↑ mortality 10 µg/L: ↑ time to meta., ↓ snout-vent length 0, 25 µg/L: intersex gonads (by histology) No effects: wet weight, sex ratio	SCPI, NIHEHS	12

Table 1.1 *Continued*

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Rana clamitans</i>	W	Ranged from 0.17 to 250	Ranged from 0.17 to 0.23	Adult and juvenile frogs	Control and ATZ contaminated sites: testicular oocytes (adult and juvenile frogs) ATZ contaminated sites: ↓ ratio 11KT/T (male adult frogs) No correlations: plasma T or E2 concentration, cyp19 activity, GSI	SCPI, NIEHS	8,9
<i>Rana clamitans</i>	W	Ranged from 0.068 to 3.13	Ranged from 0.045 to 0.39	Adult frogs	ATZ contaminated sites: ↓ GSI No correlations: gonadal histopathology	EC	7
<i>Rana clamitans</i>	L	0, 3, 30, 100 [85.5 % ATZ Syngenta]	n/a	G25-27 (30 d)	3 µg/L: ↑ mortality	National Institutes of Health (NIH)	13
<i>Rana sylvatica</i>	L	0, 2590, 4320, 7200, 12 000, 20 000 [99 % ATZ ChemService]	n/a	G10 to G12	4320 to 20 000 µg/L: ↑ deformed larvae	UW, WDA-TCP	3
<i>Rana sylvatica</i>	L	0, 3, 30, 100	n/a	G25-27 (30 d)	No effects: mortality	NIH	13
<i>Rana catesbeiana</i>	W	Ranged from 0.17 to 250 [85.5 % ATZ Syngenta]	Ranged from 0.17 to 0.23	Adult and juvenile frogs	ATZ contaminated sites: testicular oocytes (adult frogs) No correlations: plasma T or E2 concentration, cyp19 activity, GSI	SCPI, NIEHS	8,9

Table 1.1 *Continued*

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Rana catesbeiana</i>	L	51, 410, 6330, 14 800, 26 400, 45 800 [ATZ source: n/a]	51	4 dph	410 µg/L: LC50	NIH, National Science Foundation (NSF), Environmental Protection Agency (EPA) and U.S. Department of Interior (USDI)	14
<i>Rana catesbeiana</i>	L	4 813, 19 250, 77 000, 308 000 [AAtrex nine-O, 97.7 % ATZ Ciba-Geigy]	n/a	Tadpole stage (24h)	>4 813 µg/L: DNA damage	Great Lakes and Universities Research Fund, University of Windsor, Experience Development Program of Employment and Immigration (Canada)	15
<i>Bufo americanus</i>	L	58, 490, 5560, 10 800, 24 800, 48 200 [ATZ source: n/a]	51	4 dph	Less than 480 µg/L: LC50	NSF, NIH, EPA, USDI	14
<i>Bufo americanus</i>	L	0, 2590, 4320, 7200, 12 000, 20 000 [99 % ATZ ChemService]	n/a	G8 to G12	4320 to 20 000 µg/L: ↑ deformed larvae	UW, WDA-TCP	3

Table 1.1 *Continued*

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Bufo americanus</i>	L	0, 3, 30, 100 [85.5 % ATZ Syngenta]	n/a	G25-27 (30 d)	3, 30, 100 µg/L: ↑ mortality	NIH	13
<i>Bufo americanus</i>	L	100, 1000, 10 000, 100 000, and 1,000,000 [field grade of 40.8 % ATZ]	n/a	G29, G40	9 000 to 75 000 µg/L: LC50 at these stages	n/a	11
<i>Pseudacris crucifer</i>	L	0, 3, 30, 100 [85.5 % ATZ Syngenta]	n/a	G25-27 (30 d)	3, 30, 100 µg/L: ↑ mortality (G25) 3 µg/L: ↑ mortality (G27)	NIH	13
<i>Hyla versicolor</i>	MO	0, 20, 200, 2000 (in acetone) [technical grade ATZ, Ciba-Geigy]	1.64	From 11 dph until meta.	200, 2000 µg/L: ↓ snout-vent length, wet weight 2000 µg/L: ↑ time to meta. No effects: mortality	Max McGraw Wildlife Foundation	16
<i>Rhinella arenarum</i>	L	0, 1000, 2500, 5000, 7500, 10 000, 15 000, 20 000, 25 000, 30 000 [98 % ATZ ChemService]	20	From G4 (21 d)	7150 µg/L: LC50	Consejo Nacional de Investigaciones Científicas y Técnicas (CNICT), Agencia Nacional de Promoción Científica y Técnica (ANPCT)	17

Table 1.1 *Continued*

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Rhinella arenarum</i>	L	0, 1000, 2500, 5000, 7500, 10 000, 15 000, 20 000, 25 000, 30 000 [98 % ATZ ChemService]	20	From G25 (21 d)	2320 µg/L: LC50	CNICT, ANPCT	17
<i>Rhinella arenarum</i>	L	0, 0.1, 1, 10, 100, 1000 [98 % ATZ ChemService]	20	From G25 (21 d)	1000 µg/L : ↑ mortality	CNICT, ANPCT	17
<i>Rhinella arenarum</i>	L	0, 100, 1000, 5000, 10 000, 15 000, 20 000 [98 % ATZ ChemService]	20	From G38-39 until meta.	12 630 µg/L: LC50 100, 5000 µg/L: ↑ time to meta. 10 000 to 20 000 µg/L: ↓ success to meta.	CNICT, ANPCT	17
<i>Acris crepitans</i>	W	Ranged from 1 to 15	0.5	Adult frogs	No correlations: gonadal abnormalities (by gross morphology)	Midwest Society of Toxicology and the John G. Shedd Aquarium	18
<i>Litoria raniformis</i> , <i>Limnodynastes fletcheri</i> , <i>L. tasmaniensis</i> , <i>Crinia parinsignifera</i>	W	Ranged from 0.16 – 1.67	0.26	Adult frogs	No correlations: histopathology, sex ratio	Land and Water Australia	19

Table 1.1 *Continued*

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Xenopus laevis</i>	L	400 [99.4 % ATZ Sigma]	n/a	NF43 to NF62	400 µg/L: ↑ mortality, wet weight, snout to tail length, fat body size Altered 44 gene expressions in males and 77 in females (e.g., ↓ immune system genes, alters blood cell related- and growth and metabolism related-genes)	EPA	20
<i>Xenopus laevis</i>	L	0, 0.01, 0.1, 1, 25, 100 [ATZ source: n/a]	0.01	From 8 dpf until 83 dpf (75 d)	0.01, 1, 100 µg/L: ↓ snout-vent length, wet weight in females No effects: success to meta., sex ratio, gonadal histopathology	SCPI	21
<i>Xenopus laevis</i>	L	25 [ATZ source: n/a]	n/a	NF48 (4 d) NF58 (4 d)	No effects: <i>brain cyp19</i> , <i>srd5alpha1</i> , <i>srd5alpha2</i> , <i>LH</i> , <i>FSH</i> and <i>gonad cyp19</i> , <i>srd5alpha1</i> , <i>srd5alpha</i> gene expression	n/a	22
<i>Xenopus laevis</i>	MO	0, 1, 10, 25 (in 0.025 % methanol) [ATZ source: n/a]	0.1	From 4 dpf until meta. (70 to 133 d)	0, 1, 10, 25 µg/L: testicular oocytes No effects: sex ratio	SCPI	23
<i>Xenopus laevis</i>	MO until meta. MI/MO for 2 y	0, 1, 10, 25 (in 0.025 % methanol) [ATZ source: n/a]	0.1	From 4 dpf until 2 ypm	No effects: clutch size, hatching success, time to meta, mortality, wet weight, snout-vent length, sex ratio and gonadal histopathology of the second generation of frogs	SCPI	24

Table 1.1 *Continued*

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Xenopus laevis</i>	L	10 000, 25 000, 35 000 [98 % ATZ ChemService]	n/a	NF41 to NF46	10 000, 25 000, 35 000 µg/L: ↑ gut malformations 25 000, 35 000 µg/L: ↑ abnormal hearts, oedema 35 000 µg/L: ↑ visceral haemorrhaging	Tufts University (TU) and Graduate Student Grants-in-Aid (GSGA)	25
<i>Xenopus laevis</i>	L	10 000, 25 000, 35 000 [98 % ATZ ChemService]	n/a	NF42 to NF46	10 000, 25 000, 35 000 µg/L: ↑ abnormal hearts, gut malformations, oedema 25 000 µg/L: ↑ axis malformation 10 000, 35 000 µg/L: ↑ visceral haemorrhaging	TU, GSGA	25
<i>Xenopus laevis</i>	L	10 000, 25 000, 35 000 [98 % ATZ ChemService]	n/a	NF43 to NF46	10 000, 25 000, 35 000 µg/L: ↑ gut malformations, oedema 25 000, 35 000 µg/L: ↑ abnormal hearts 10 000, 35 000 µg/L: ↑ visceral haemorrhaging	TU, GSGA	25
<i>Xenopus laevis</i>	L	0, 0.1, 1, 10, 100 [97.4 % ATZ Sigma]	n/a	Wild-type males NF49 to NF66	0.1 µg/L: ↓ <i>cyp19</i> mRNA in females 10, 100 µg/L: ↑ female sex ratio No effects: success of meta., gonadal histopathology, vitellogenin levels	Ministry of the Environment of Japan (MEJ), Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT)	26

Table 1.1 *Continued*

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Xenopus laevis</i>	L	0, 0.1, 1 [97.4 % ATZ Sigma]	0.02	All ZZ-males NF49 to NF66	No effects: success of meta., gonadal histopathology, vitellogenin levels	MEJ, MECSSTJ	26
<i>Xenopus laevis</i>	L	0, 10, 100 [97.1 % ATZ Syngenta]*	0.07	Adult male frogs (49 d)	10 µg/L: ↑ GSI No effects: testicular cyp19 activity, germ cells, plasma T or E2 concentrations	SCPI	27
<i>Xenopus laevis</i>	L	0, 1, 25, 250 [97.1 % ATZ Syngenta]*	0.03	Adult male frogs (36 d)	250 µg/L: ↓ plasma T concentration No effects: mortality, GSI, <i>cyp19</i> mRNA, cyp19 activity, plasma E2 concentration, histopathology	SCPI, NIEHS	28
<i>Xenopus laevis</i>	L	0, 200, 400, 600, 800 [98 % ATZ ChemService]	n/a	NF47 (21 d)	No effects: flow cytometry	Illinois Water Resource Center (IWRC), U.S. Department of Agriculture (USDA)	29
<i>Xenopus laevis</i>	L	0, 800 [98 % ATZ ChemService]	n/a	NF47 (21 d)	800 µg/L: ↑ delay in development	IWRC, USDA	29
<i>Xenopus laevis</i>	L	0, 800 [98 % ATZ ChemService]	n/a	NF54 (21 d)	800 µg/L: ↑ delay in development	IWRC, USDA	29

Table 1.1 Continued

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Refs ^g
<i>Xenopus laevis</i>	L	0, 800 [98 % ATZ ChemService]	n/a	From NF40-42 (35 d)	800 µg/L: ↑ delay in development	IWRC, USDA	29
<i>Xenopus laevis</i>	L	0, 100, 450, 800 [98 % ATZ ChemService]	n/a	NF52-53 to NF66	100, 450, 800 µg/L: ↑ delay in development No effects: wet weight, total number of nuclei	IWRC, USDA	29
<i>Xenopus laevis</i>	L	0, 0.1, 1, 10, 25 (in FETAX medium) [97.1 % ATZ Syngenta]*	Ranged from 0.05 to 0.29	3 dph to NF66	0, 0.1, 1, 10, 25 µg/L: gonadal abnormalities (by gross morphology) 25 µg/L: hermaphrodite gonads (by gross morphology) No effects: mortality, success of meta., time to meta., snout-vent length, wet weight, laryngeal muscle size	SCPI	30
<i>Xenopus laevis</i>	L	0, 0.1, 1, 10, 25 (in FETAX medium) [97.1 % ATZ Syngenta]*	Ranged from 0.05 to 0.29	3 dph to 90 dpm (185 d)	0, 0.1, 1, 10, 25 µg/L: gonadal abnormalities (by gross morphology), testicular oocytes 0, 0.1, 1, µg/L: intersex gonads (by histology) 1 µg/L: ↓ plasma E2 concentrations in males 10 µg/L: hermaphrodite gonads (by gross morphology)	SCPI	30

Table 1.1 *Continued*

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Xenopus laevis</i>	L	0, 1, 10, 25 (in FETAX medium) [98.6 % ATZ Syngenta]*	0.05	From 2 dph to NF66 (78 d)	No effects: brain and gonadal cyp19 activity, plasma T concentrations 25 µg/L: intersex and discontinuous gonads (by gross morphology) ↑ abnormal swimming No effects: hatching success, mortality, forelimb emergence, tail resorption, body deformities, laryngeal muscle, wet weight, snout-vent length	SCPI	31
<i>Xenopus laevis</i>	L	0, 0.01, 0.1, 1, 10, 25 (in 0.004 % ethanol) [ATZ source: n/a]	n/a	NF48 to NF66	0.1, 1, 10, 25 µg/L: gonadal abnormalities (by gross morphology and histology) 1, 10, 25 µg/L: ↓ male laryngeal muscle No effects: mortality, snout-vent length, wet weight, time to meta.	University of California (UC) and NSF	32
<i>Xenopus laevis</i>	L	0.1, 0.4, 0.8, 1, 25, 200 (in 0.004 % ethanol) [ATZ source: n/a]	n/a	NF48 to NF66	0.1, 0.4, 0.8, 1, 25, 200 µg/L: gonadal abnormalities (by gross morphology and histology) 1, 25, 200 µg/L: ↓ male laryngeal muscle No effects: mortality, snout-vent length, wet weight, time to meta.	NSF, UC	32

Table 1.1 *Continued*

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Xenopus laevis</i>	L	0, 25 [ATZ source: n/a]	n/a	Adult male frogs (46 d)	25 µg/L: ↓ plasma T concentrations	NSF, UC	32
<i>Xenopus laevis</i>	L	0, 21 [99 % ATZ]	0.05	NF56 (2 d) Females only	21 µg/L: ↓ primary oogenesis ↑ secondary oogenesis, atretic oogenesis No effects: pituitary histopathology	Toxic Substance Research Initiative, Govt of Canada (TSRI)	33
<i>Xenopus laevis</i>	L	0, 21 [99 % ATZ]	0.05	NF56 (2 d) Males only	21 µg/L: testicular resorption, aplasia ↓ testicular volume, nursing cells, spermatogonial cell nests No effects: pituitary histopathology	TSRI	34
<i>Xenopus laevis</i>	L	0, 40, 320 [99 % ATZ ChemService]	n/a	NF46-48 to NF66	320 µg/L: ↑ mortality, time to meta. ↓ wet weight, snout-vent length, hematocrit	Wichita State University (WSU)	35
<i>Xenopus laevis</i>	L	0, 20, 40, 80, 160, 320 [99 % ATZ ChemService]	n/a	NF46-48 to NF66	20, 40, 80, 160, 320 µg/L: ↓ wet weight	WSU	35
<i>Xenopus laevis</i>	L	3000 to 2 000 000 (in FETAX or water) [40.8 % ATZ]	n/a	NF8 –NF11 (1 to 3 d)	< 8000 µg/L: EC50 100 000 µg/L: LC50 Teratogenic index: 3.03	n/a	36

Table 1.1 Continued

Species	Exposure ^a	Nominal concentrations of ATZ (µg/L) ^b	Concentration of ATZ in the control (µg/L) ^c	Length of the exposure ^d	Effects on frog development ^e	Sponsors ^f	Ref ^g
<i>Xenopus laevis</i>	W	Ranged from 0.12-1.23	0	Adult frogs	ATZ-contaminated sites: ↓ female weight wet No correlations: sex ratio, snout-vent length, age profile, population size	SCPI	37
<i>Xenopus laevis</i>	W	0.29-4.14	0.1-0.57	Adult frogs	ATZ-contaminated sites: ↓ T, E2 levels No correlations: laryngeal size, testicular cells, cyp19 activity, GSI	SCPI	38,39
↓: decrease ↑: increase cyp19: aromatase dio2: deiodinase type 2 dio3: deiodinase type 3 dpf: days post fertilization dph: days post hatching		dpm: days post metamorphosis E2: estradiol eralpha: estrogen receptor α FETAX: frog embryo teratogenesis assay - <i>Xenopus</i> FSH: follicle stimulating hormone G: Gosner stages GSI: gonadosomatic index		KT: ketotestosterone LC50: median lethal concentration LH: luteinizing hormone meta.: metamorphosis n/a: not available NF: Nieuwkwoop-Faber stages	srd5alpha1: steroid 5α-reductase type 1 srd5alpha2: steroid 5α-reductase type 2 T: testosterone TK: Taylor-Kollros stages tralpha: thyroid hormone receptor α trbeta: thyroid hormone receptor β ypm: years post metamorphosis		

^aType of exposure ranged between laboratory (L), wild habitat (W), mesocosm located inside (MI) and mesocosm located outside (MO).

^bNominal concentrations are reported in µg/L and the ATZ carrier is presented in brackets if used in the experimental design. Purity and manufacturer of ATZ is provided in square brackets.

^cConcentration of ATZ in the control (µg/L). If not measured, the minimal detection limit is used as the possible ATZ concentration in the control (if reported in the study).

^dStages of development during the length of the exposure are reported and the total number of days of the exposure is presented in brackets, if reported in the study.

^eThe nominal concentration at which effects of ATZ on frog development have been observed are reported in bold with associated effects on frog development. When no effects were found, the studied endpoints are listed.

^fSponsors are listed if mentioned in the study article. All the definitions of acronyms are found in the table.

^gReferences: 1. Orton et al. 2006; 2. Allran and Karasov 2000; 3. Allran and Karasov 2001; 4. Hayes et al. 2003; 5. Hayes et al. 2002b; 6. Langlois et al. 2009a; 7. McDaniel et al. 2008; 8. Murphy et al. 2006a; 9. Murphy et al. 2006b; 10. Hayes et al. 2006a; 11. Howe et al. 1998; 12. Coady et al. 2004; 13. Storrs and Kiesecker 2004; 14. Birge et al. 1983; 15. Clements et al. 1997; 16. Diana et al. 2000; 17. Brodeur et al. 2009; 18. Reeder et al. 1998; 19. Hyne et al. 2009; 20. Langerveld et al. 2009; 21. Kloas et al. 2009a; 22. Kloas et al. 2009b; 23. Jooste et al. 2005; 24. Du Preez et al. 2008; 25. Lenkowski et al. 2008; 26. Oka et al. 2008; 27. Hecker et al. 2005a; 28. Hecker et al. 2005b; 29. Freeman and Rayburn 2005; 30. Coady et al. 2005; 31. Carr et al. 2003; 32. Hayes et al. 2002a; 33. Tavera-Mendoza et al. 2002b; 34. Tavera-Mendoza et al. 2002a; 35. Sullivan and Spence 2003; 36. Morgan et al. 1996; 37. Du Preez et al. 2008; 38. Smith et al. 2005; 39. Hecker et al. 2004.

^hThese changes in gene expression were reported in a cohort of G34 tadpoles that did not metamorphose during the exposure period.

ⁱThis study was not part of the analysis since these are the results of this present doctoral thesis. We added these data to the table to allow comparison with the literature.

* ATZ donated or obtained directly from the manufacturer.

1.7 The 5 α - and 5 β -reductase inhibitor, finasteride

Anti-androgens are a class of EDCs capable of interfering with normal sexual development in amphibians (e.g., flutamide and vinclozolin; van Wyk et al. 2003). However, the consequence of disrupting *srd5alpha*, which catalyzes the synthesis of the potent androgen 5 α -DHT, has not yet been studied as a potential mechanism of action for endocrine disruption in any frog species. The 4-Aza-3-oxo-1-ene compounds are the major class of synthetic *srd5alpha* inhibitors and include the prostate cancer and benign prostatic hyperplasia drug, finasteride (17 β -[N-tert-butylcarbonyl]-4-aza-5 α -androst-1-en-3-one, MK-906; Stoner 1990). Finasteride is an irreversible inhibitor and its steroid-like structure (Fig. 1.1 b) allows it to act as an alternate substrate with higher affinity than testosterone to the human *srd5alpha2* (Andersson and Russell 1990; Bull et al. 1996). This inhibitor requires a structure similar to 3-oxo-4-ene with a secondary 17 β -substituent to successfully bind to the *srd5alpha*-NADPH or *srd5alpha*-NADP⁺ complexes (Rasmusson et al. 1986; Voigt and Hsia 1973). Finasteride has higher affinity to *srd5alpha2* than *srd5alpha1*, but its inhibiting potential on *srd5alpha3* is still unknown (Uemura et al. 2008). While designed to inhibit *srd5alpha* in humans, recent modelling studies succeeded to simulate an annealing omit electron density map of the *srd5beta*•NADP⁺•finasteride complex, allowed the discovery that finasteride also acts as a reversible competitive inhibitor of human *srd5beta* (Drury et al. 2009).

Finasteride has not yet been monitored in the Canadian environment; however, since this drug along with some of its metabolites (e.g., omega-hydroxyfinasteride) have been measured in human urine and semen (Constanzer et al. 1994; Lundahl et al. 2009), there is a potential for their presence in the environment. Therefore, understanding the

consequences of the inhibition of *srd5alpha* and *srd5beta* by finasteride in frogs would provide baseline studies on the action of EDCs acting like finasteride in the environment.

1.8 Thesis hypotheses and objectives

The aim of this thesis was to determine the effects of ATZ and finasteride on frog development. Firstly, we exposed *L. pipiens* tadpoles to concentrations of ATZ relevant to Canadian ecosystems, including the actual CWQG of 1.8 µg/L ATZ. We investigated the effects of this herbicide on developmental- and reproduction-related endpoints and further assessed possible mechanisms of disruption by ATZ using molecular biomarkers. Our general hypothesis was that ATZ alters both developmental- and reproductive systems in frogs by affecting TH- and sex steroid-related gene expression and altering the activity of steroidogenic enzymes. Secondly, we used finasteride as a model compound to assess the consequences of a disruption of *srd5alpha* and *srd5beta* on frog development. Exposures of *S. tropicalis* during early and larval development were performed to test our second general hypothesis, which states that the inhibition of both *srd5alpha* and *srd5beta* alters gonadal development and the expression of many genes involved in the TH and reproductive axes. The specific hypotheses of this thesis are listed in Table 1.2.

This doctoral research is of particular relevance considering that *srd5alpha* and *srd5beta* disruption has not been well studied in any frog species, especially during early development. Investigating mechanisms for endocrine disruption of environmental pollutants such as herbicides (e.g., ATZ) and pharmaceuticals (e.g., finasteride) during frog development may contribute to the understanding of amphibian population decline.

1.9 Model species

The first frog species used in this study, *L. pipiens*, was chosen for its relevance to Canadian ecosystems. This native frog species is likely to be exposed to ATZ contamination in Canada. *Lithobates pipiens* is indigenous to North America and it belongs to the Ranidae family. Its reproduction occurs in April where egg masses are laid under water in shallow ponds and hatching occurs 7-21 days after egg fertilization (Desroches and Rodrigue 2004). Recent molecular and phylogenetic work done on mitochondrial sequences of the genus *Rana*, separated some *Rana* species in the subgenus *Lithobates*; therefore this species changed names from *Rana pipiens* to *Lithobates pipiens* (Hillis and Wilcox 2005). Since 1998, *L. pipiens* has been designated as “Endangered” in British Columbia and under “Special Concern” in Manitoba, Saskatchewan, Alberta and in the Northwest Territories (COSEWIC 2009).

The second frog species used was the well-established laboratory frog *S. tropicalis*. *Silurana tropicalis* is a strictly aquatic frog, lays between 1,000 and 3,000 eggs at a time and has a short generation time that varies between four to five months (Amaya et al. 1998). Furthermore, the *S. tropicalis* genome is being sequenced, therefore there are extensive genomic resources available for this species (Bowes et al. 2009). This frog is native to West Africa and belongs to the *Pipidae* family (Tinsley and Kobel 1992). Phylogenetic analyses of morphological features have reclassified *S. tropicalis* from the *Xenopus* genus to the *Silurana* genus (Cannatella and Trueb 1988).

1.10 Thesis presentation

This doctoral thesis is a collection of six manuscripts (four published, one submitted and one in preparation). It is organized in five data chapters (Chapters 2 to 6). Chapters 2 and 3 were merged in the same publication (published in *Environmental Health Perspectives*). Chapter 4 was an invited publication for a special edition in *General and Comparative Endocrinology* for the 4th Comparative Neuroendocrinology Symposium on Evolutionary and Developmental Neuroendocrinology which was held during the 16th International Conference on Comparative Endocrinology, June 22-26, 2009 at the University of Hong Kong, Hong-Kong SAR. Chapter 5 is a collaborative manuscript with another doctoral candidate who joined part of her data (parallel exposure to fadrozole on *S. tropicalis*) with mine for comparison purposes, and that manuscript was also published in *General and Comparative Endocrinology*. Finally, Chapter 6 was separated into two publications, the first one has focused on histology and hepatic gene expression data (published in *Sexual Development*); while the other is based on brain gene expression data (submitted to *Aquatic Toxicology*). These two latter publications were also part of the collaborative work undertaken in Chapter 5. The manuscript in preparation will merge the Table 1.1 of Chapter 1 and the discussion related to ATZ in Chapter 7.

To facilitate the reading, an outline of the thesis' organisation is presented, showing the title, the hypotheses, the main outcomes and the risen questions for each chapter along with the chapter where that specific question is answered (Table 1.2). This table will guide the reader throughout the thesis and help to understand the linkages between the chapters.

Table 1.2 Overall progression of the thesis experimental approach

CHAPTER 1 : General introduction



CHAPTER 2 : Low concentrations of the herbicide atrazine alter sex ratio and reduce metamorphic success in *Lithobates pipiens* tadpoles raised in outdoor mesocosms

Hypotheses

Chronic exposure to ATZ during sexual development:

1. induces female-biased sex ratio and the formation of testicular oocytes in *L. pipiens*
2. reduces *L. pipiens* metamorphic success

Outcomes

Low concentrations of ATZ feminize frogs and affect metamorphosis in an outdoor mesocosm experimental design.

New questions

What are the mechanisms of action for the disruption of frog sexual development and metamorphosis by ATZ? (**Chapter 3**)



CHAPTER 3 : Mechanisms of action of the herbicide atrazine on the disruption of *Lithobates pipiens* metamorphosis and gonadal development

Hypotheses

Chronic exposure to ATZ during sexual development:

1. induces *cyp19* and *eralpha* mRNA levels
2. alters the expression of TH-related genes involved in the activation of THs
3. induces *cyp19* activity and alters *srd5beta* activity

Outcomes

ATZ did not affect *cyp19* mRNA level or activity; however, ATZ altered *eralpha* and *dio3* expression and *srd5beta* activity.

New questions

1. Given that the *srd5alpha* (type 1, 2 and 3) are involved in 5 α -DHT production (main androgen in vertebrates), what are the similarities and differences between *srd5alpha* and *srd5beta* in frogs? (**Chapter 4**)
2. Are *srd5alpha* and *srd5beta* important during amphibian development? (**Chapters 5 and 6**)



Table 1.2 Continued

CHAPTER 4 : Evolution of the steroid 5 α -reductases and comparison of their functions with the steroid 5 β -reductase

Hypotheses

1. The third type of srd5alpha is the most ancient form of srd5alpha
2. srd5alpha and srd5beta are present in same tissues
3. srd5alpha and srd5beta are involved in the same biological activities

Outcomes

The srd5alpha and srd5beta diverge in evolution, but share similar biological functions. Few studies have looked at these enzymes in amphibians; therefore, basic biological studies are needed.

New questions

1. Are these enzymes present and functional during frog embryogenesis and early larval development? (**Chapter 5**)
2. Are these enzymes targets for endocrine disruption by EDCs? (**Chapters 5 and 6**)
3. What are their roles in frog development? (**Chapters 5 and 6**)



CHAPTER 5 : Finasteride exposure modulates sex steroid- and thyroid hormone-related gene expression in *Silurana tropicalis* early larval development

Hypotheses

1. All srd5alpha and srd5beta are present during early frog development
2. Inhibition of srd5alpha and srd5beta during early larval development alters the expression of sex steroid- and TH-related genes

Outcomes

All srd5alpha and srd5beta were detected during early frog development and their inhibition affected the expression of androgen related-, estrogen related- and TH-related genes and steroidogenic enzyme activity.

New questions

1. Does srd5alpha and srd5beta inhibition affect sexual development in frogs? (**Chapter 6**)
2. What are the roles of srd5alpha and srd5beta in frog development past the early larval stages? (**Chapter 6**)



Table 1.2 Continued

CHAPTER 6 : Finasteride affects gonadal differentiation and both brain and hepatic gene expression in the frog *Silurana tropicalis*

Hypotheses

Chronic exposure to finasteride during sexual development:

1. induces female-biased sex ratio
2. affects the expression of sex steroid- and TH-related genes
3. reduces *srd5beta* mRNA levels and activity

Outcomes

Finasteride skewed the sex ratio, induced intersex animals and affected the expression of androgen related-, estrogen related- and TH-related genes.



CHAPTER 7 : General discussions and conclusions

1.11 Contribution of collaborations

This doctoral thesis was used as a source of projects for academic purposes to initiate undergraduate students to research and was also a basis of scientific publications; therefore it involved the collaboration of many undergraduate students and colleagues. The students who were involved in honours projects were co-authors of the publication related to that work. The contributions of all co-authors are stated on the first page of each chapter, while other contributions are recognized in the acknowledgment section.

CHAPTER 2

Low concentrations of the herbicide atrazine alter sex ratio and reduce metamorphic success in *Lithobates pipiens* tadpoles raised in outdoor mesocosms

Chapter adapted from **Langlois VS¹**, Carew AC², Pauli BD³, Wade MG⁴, Cooke GM⁵ and VL Trudeau⁶ (2009a) Environ Health Perspect *doi:10.1289/ehp.0901418*
(Chapters 2 and 3 were merged in the same publication)

Main contributions of each co-author:

¹Contributed to original ideas, conducted animal exposure, performed histology data analysis and manuscript preparation.

²Performed histology and animal care.

³Provided HPLC-MS/MS analysis, mesocosm material, site and financial support.

⁴Provided histological material and expertise.

⁵Revised the manuscript and collaborated on the study described in Chapter 3.

⁶Contributed to original ideas and revised the manuscript.

2.1 Introduction

There is controversial evidence that atrazine (ATZ) affects amphibian metamorphosis and development (Brodeur et al. 2009; Coady et al. 2004). Several studies report developmental defects in amphibians following ATZ exposures including tissue malformations (Lenkowski et al. 2008), body weight alterations (Sullivan and Spence 2003), gross developmental deformities (Allran and Karasov 2000), decreased growth (Diana et al. 2000) and survival (Rohr et al. 2004; Storrs and Kiesecker 2004), as well as disruption of metamorphosis (Brodeur et al. 2009; Coady et al. 2004; Freeman and Rayburn 2005; Larson et al. 1998; Sullivan and Spence 2003). However, several other studies have not found evidence to support that ATZ disrupts amphibian development, even in the same species (Carr et al. 2003; Coady et al. 2005; Diana et al. 2000; Oka et al. 2008; Orton et al. 2006). Under laboratory conditions, amphibians exposed to low levels of ATZ also exhibited skewed sex ratios toward females (Oka et al. 2008) and the presence of intersex gonads (Carr et al. 2003; Hayes et al. 2003). Short term exposure to ATZ (48 h) significantly reduced testicular development in *Xenopus laevis* (African clawed frog) tadpoles just prior to gonadal differentiation (Tavera-Mendoza et al. 2002a). Hayes and colleagues (2003) also reported a correlation between ATZ-impacted areas and the presence of intersex condition in frog populations (Hayes et al. 2003). However, numerous other studies report that ATZ does not alter sex ratios nor induce intersex in frogs (Carr et al. 2003; Coady et al. 2005; Hyne et al. 2009; Jooste et al. 2005; Kloas et al. 2009a; Oka et al. 2008). These differential responses to ATZ exposure during amphibian development remain to be explained. Metamorphosis and sexual differentiation are two critically important periods of development in amphibians and

disruption of these fundamental processes have negative effects on survival and adversely impact populations (Denver et al. 2002; Hayes et al. 2006b; Hogan et al. 2008).

In Canada, the Water Quality Guideline for the protection of freshwater aquatic life (CWQG) for ATZ is 1.8 µg/L; however, previous studies have found negative effects of this herbicide on amphibians at similar or lower levels of ATZ. It is therefore important to determine if concentrations of ATZ within the CWQG affect Canadian frog species. This study investigates the effects of ATZ on metamorphosis and gonadal development in *Lithobates pipiens* (Northern leopard frog), under environmentally-relevant conditions as simulated in an outdoor mesocosm system. We hypothesized that ATZ exposure at concentrations within the CWQG will 1) induce female-biased sex ratios and testicular oocyte formation in *L. pipiens*, and 2) reduce the success of *L. pipiens* metamorphosis. Survival, age, wet weight, snout-vent length, sex ratio and gonadal histology were assessed at metamorphosis. Complementary field surveys were undertaken to confirm relevant environmental ATZ concentrations in water and sex ratios in naturally metamorphosing tadpoles from the same population that was raised in captivity in the mesocosms.

2.2 Materials and Methods

2.2.1 Chemicals

The herbicide AAtrex Liquid 480 (Syngenta Crop Protection Canada Inc.; Registration # 18450; purity 97.1% ATZ, 2.9 % related triazines and 5 % of ethylene glycol w/v) was purchased locally and was used to mimic ATZ input into the environment. Ethanol (EtOH) was provided from in-house chemical suppliers (CAS number 64-17-5; purity $\geq$ 99.5 %) and 17 α -ethinylestradiol (EE2) was purchased from Sigma-Aldrich® (CAS 57-63-6, purity $\geq$ 98 %, Canada).

2.2.2 Animals

Animals were collected from the Raisin River region, Cornwall, Ontario, Canada (N45°09'58.9"; W074°47'41.9"; Fig. 2.1). For the mesocosm study, five fertilized egg masses were collected from the reference site (April 25, 2006; see 'R' on Fig. 2.1). The egg masses were maintained in the laboratory in 10-L tanks filled with activated carbon-filtered City of Ottawa water (pH 6.6; dissolved oxygen 8.4 – 10.0 mg/L; temperature 20–21 °C). After hatching, tadpoles were separated into extra tanks to allow a final density of approximately 1 g tadpole/L to avoid density effects (minimum recommended by the American Society for Testing Materials; ASTM 2000) and were maintained on a 12 h light/dark photoperiod with the light cycle occurring from 7 am to 7 pm. Water was aerated and 5-L of the water was renewed twice weekly. Tadpoles were fed daily *ad libitum* with Nutrafin fish flakes (Rolf C. Hagen, Inc., Montreal, Québec, Canada) and frog brittle (Carolina Biological Supply Co., Burlington, North Carolina, USA). The animals were kept in the laboratory until beginning of independent feeding (Gosner stage

25, G25; Gosner 1960). Thirty tadpoles (six tadpoles from each of five different egg masses) were added to each of the mesocosms, resulting in a final density of 0.1 g tadpole/L. Tadpoles were acclimatized to their new environment until G27, at which time the exposure began. 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.

For the field survey (summer 2006), newly metamorphosed frogs were caught ($n = 30$) from the reference site (July 13, 2006). Animals were brought to the University of Ottawa on ice and anaesthetised with a solution of 2 % tricaine methanesulfonate (MS-222; Sigma). Animals were sacrificed by cutting the spinal cord. The kidney-gonad complex was removed, fixed and decalcified in Cal-Ex II (Fisher) for 48 h. It was then preserved in 70 % EtOH until histological processing. Phenotypic sex was determined by visual inspection of the gonads during the dissection.

2.2.3 Mesocosm conditions

An outdoor mesocosm design was used in this study to mimic a natural pond setting. Each mesocosm consisted of a 378-L high density polyethylene (Rubbermaid®) tank that was washed many times with pressurized water and ‘aged’ for at least two years prior the beginning of the exposure. In each mesocosm, 300 L of groundwater, 50 g of rabbit pellets (Hagen®) and 100 g of dried leaves (collected from a non pesticide sprayed private property in Ottawa, ON) were added (Fig. 2.2 a). *Daphnia magna* originating from a local creek were introduced to each mesocosm to increase food diversity. Each mesocosm was aerated to avoid anoxic conditions and covered with a lid

made of nylon shade cloth netting to exclude predators. The plastic lids cut 73 % to 75 % of natural illuminance and ultraviolet (UV) radiation coming through each the mesocosms (Goldilux®, radiometer and photometer GRP-1). A removable roof system was built in order to prevent rainwater input which might contain ATZ (Giroux 2004). Two 50 m X 4.5 m X 2.4 m roof structures were built and covered by a polyethylene material in order to protect all mesocosms equally from precipitation (Fig. 2.2 b). The roofs were drawn over the tanks during rainy days only for the duration of the precipitation period and opened at all other times. During precipitation events, the roof material cut an additional 3 % of illuminance and from 3 % to 10 % of UV radiation. The resulting tadpole UV exposure realistically mimicked conditions in typical breeding ponds because natural ponds are covered by forest canopy and have high dissolved organic carbon volume which also decreased UV radiation penetration (Crump et al. 1999). Groundwater was added (30 L) to the mesocosms every two weeks to overcome water loss, which could result in changes in tadpole density and chemical concentrations.

2.2.4 Atrazine exposure

Tadpoles at G27 were exposed to five nominal treatments: 0, 0.1 or 1.8 µg/L ATZ, 0.0003 % EtOH and 1.5 µg/L EE2 dissolved in 0.0003 % EtOH (n = 5 mesocosms per treatment). In the experimental design, EE2 was used as a positive control for feminization (inducer of testicular oocytes in *L. pipiens*; Hogan et al. 2008). Due to the short half-life of EE2 in water (18.2 h, Mackenzie et al. 2003), two additions of 1.5 µg /L EE2 were added per week. Stock solutions of EE2 were made fresh prior to each addition. For ATZ, two applications were made on May 12th and May 19th, 2006 to achieve nominal concentrations. The mesocosms (n = 25) were randomly distributed to

minimize position effects. Dissolved oxygen (DO; mg/L), pH and temperature (°C) were recorded weekly using an YSI and a pH/K model probes. Ammonia levels remained below detection limits (< 250 µg/L; Lamotte® Ammonia Kit) throughout the experiment. All nets, containers and bubblers were treatment-coded to avoid cross-contamination. Rain samples collected on site were analyzed by high performance liquid chromatography with dual mass spectrometry (HPLC-MS/MS) for ATZ content and concentrations were below the detection limit (< 0.003 µg/L; data not shown).

2.2.5 Tissue collection

Animals reaching metamorphosis were removed at forelimb emergence, which is equivalent to G42 (metamorphic climax). Animals were anaesthetized by immersion in 1 % MS-222. Age to metamorphose (ATM; d), wet weight (WW; g) and snout-vent length (SVL; mm) were recorded. Animals were sacrificed by cutting the spinal cord. The kidney-gonad complex was removed, fixed and decalcified in Cal-Ex II (Fisher) for 48 h and then preserved in 70 % EtOH until histological processing (see section 2.2.6).

2.2.6 Sex ratio and histology

Upon dissection, all animals were classified as either male or female by visual inspection of gonadal gross morphology using a dissecting microscope (magnification of 4X; Fig. 2.3). A subset of male samples was randomly chosen for histology to determine the presence of testicular oocytes (referred to as intersex condition; Jooste et al. 2005). Fixed and decalcified gonad-kidney complexes were trimmed and prepared for histological processing, following the methods previously described in Hogan et al. (2008). Briefly, samples were dehydrated in increasing EtOH concentrations, cleared

with xylene and infiltrated with paraffin using Tissue-Tek® VIP™ Vacuum Infiltration Processor (Sakura E150/E300 Series). Each sample was then embedded in an individual paraffin block. Blocks were serially sectioned longitudinally at 5 µm intervals (Heidelberg Microm HM 350) and spread on a bath set at 49 °C containing a mix of water and STA-On tissue section adhesive (Surgipath, Richmond, IL). After the serial sections were expanded to normal size, they were mounted on glass slides (Surgipath). Sections were deparaffinised in xylene, rehydrated in EtOH, stained in hematoxylin and eosin-Y stains and covered with micro cover glasses (Surgipath). Samples were assigned a random number to perform blind analysis to avoid bias when analysing gonads. Digital pictures of the sections were taken by a Micro Publisher 3.3 Digital microscope camera (QImaging Corp., Burnaby, BC).

2.2.7 *Water analysis*

For the field survey, five different locations in the Raisin River (ON) were sampled every week during spring 2006 to obtain the ATZ signature from the spring spraying season (for details, see Fig. 2.4) and for the mesocosm study, water samples were also collected regularly (for details, see Fig. 2.5). Analysis of water samples for ATZ concentrations was performed by Environment Canada (Ottawa, ON) according to the protocol of Hua et al. (2006). Water samples (0.9 L) were spiked with 1 µg of internal standard (ATZ-d₅) and filtered on a 0.45 µm HATF membrane before being concentrated on SPE LC-18 cartridges (Supelclean®). The cartridges were frozen at -20 °C until all samples were extracted. Before elution, the cartridges were left to reach room temperature and ATZ was eluted from the SPE cartridges (Supelclean LC-18, 6 mL, 0.5 g, cat. no 57054 lot SP6891B) with 3 mL of methanol. Each sample was quantified using

a calibration curve with six standards ranging from 0.1 pg/μL to 10 pg/μL ($r^2 > 0.99$).

The HPLC-MS/MS detection limit was 0.003 μg/L.

2.2.8 Data analysis

Statistical analyses were performed using the software S-PLUS Version 8.0 (Insightful Corp. Seattle, Washington, USA). Pearson's chi-squared (χ^2) test was used to determine statistical differences for sex ratio, survival and success of metamorphosis. One-way analysis of variance (ANOVA) was used to analyze WW, SVL and ATM. The data analysed by ANOVA were tested for normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test). When data failed to meet the assumptions after transformation, the non-parametric Kruskal-Wallis one-way ANOVA on ranks was used, followed by the Mann-Whitney U test. ATZ and EtOH treatment data were compared to water control (0 μg/L ATZ); while EE2 treatment data were compared to the EtOH solvent control.

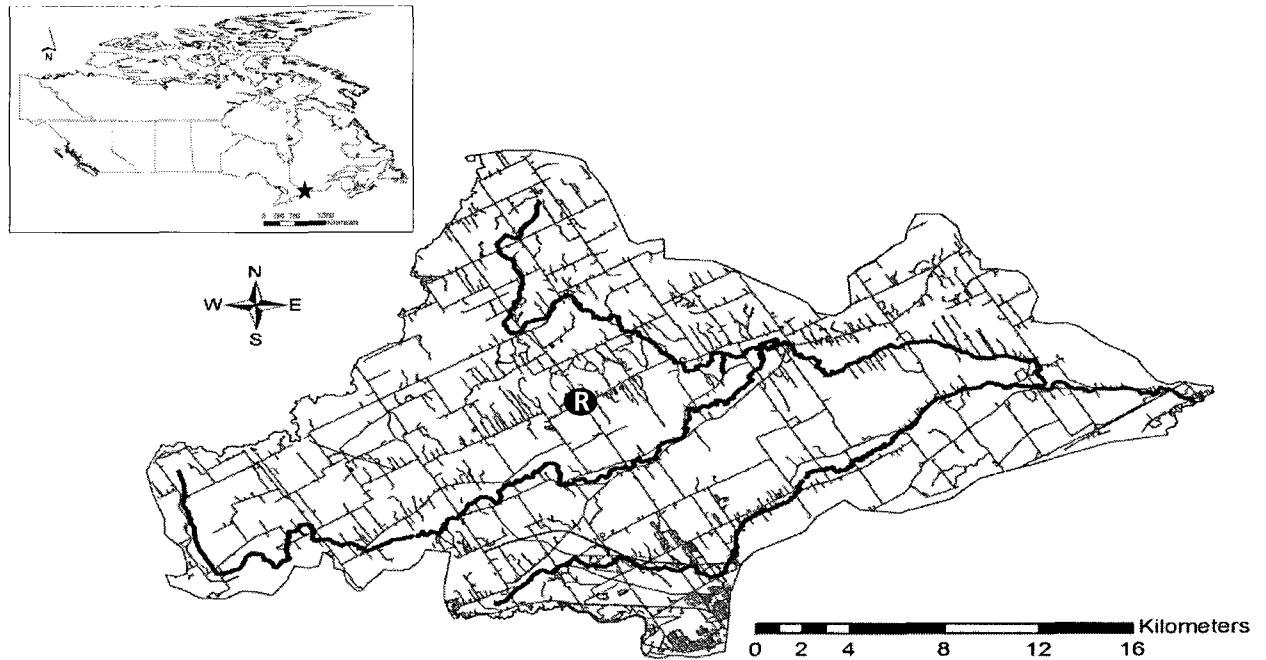


Figure 2.1 Localisation of the reference site in the Raisin River watershed (ON, Canada). The reference site, indicated by the letter R, represents the location of the pond where the egg masses and metamorphic frogs were collected for this study. On the map of Canada (inset), the star indicates the studied site region. Darker lines represent the five sections of the Raisin River.

a.



b.



Figure 2.2. Experimental design of the ATZ exposure. **a.** Each mesocosm contained dry leaves, Hagen® rabbit pellets and *Daphnia magna* and was equipped with a mesh lid to avoid tadpole predation. **b.** Removable roof systems were built to prevent rainwater input to the mesocosms.

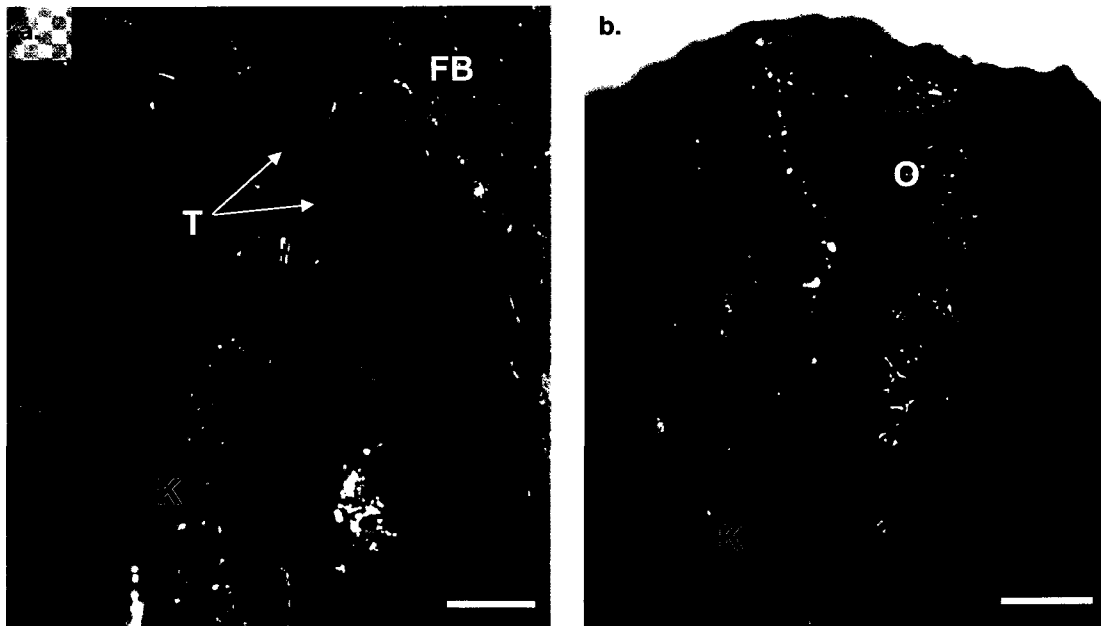


Figure 2.3 *Lithobates pipiens* male and female gonadal gross morphology at metamorphic climax. **a.** Normal male testes-kidney complex morphology from the 0.1 $\mu\text{g/L}$ ATZ treatment; scale bar, 0.5 mm. **b.** Normal female ovaries-kidney complex morphology from the 1.8 $\mu\text{g/L}$ ATZ treatment; scale bar, 1 mm. ATZ: atrazine; FB: fat body; G: Gosner stage; T: testes; K: kidney; O: ovary.

2.3 Results

2.3.1 *Atrazine is present in the Raisin River (ON)*

Atrazine was detected in every stream sampled on the Raisin River (ON), with concentrations ranging from 0.01 to 1.6 µg/L (Fig. 2.4). These concentrations are under the CWQG limit of ATZ (< 1.8 µg/L). The highest concentrations were measured on May 25 and June 2, 2006 and are associated with the spring signature from ATZ spraying activities in this agricultural area. Site 5, which was located downstream of all branches of the river, had the highest concentration of ATZ measured (1.6 µg/L) on June 2, 2006. The concentrations of ATZ obtained in the monitored streams confirm that the experimental levels used in the mesocosm exposures are environmentally relevant.

2.3.2 *Mesocosm physicochemical parameters were constant throughout the summer*

During the exposure, the concentrations of ATZ in the treatments ranged from 0.09 to 0.21 µg/L ATZ (nominal 0.1 µg/L ATZ) and between 1.6 to 3.7 µg/L ATZ (nominal 1.8 µg/L ATZ; Fig. 2.5). The ATZ concentrations in the control treatment ranged from the limit of detection (0.003 µg/L) to 0.028 µg/L ATZ (Fig. 2.5). To compensate for water loss through evaporation, groundwater was regularly added to mesocosm. A small input of ATZ to groundwater coming from peripheral agricultural fields after the spraying season could explain detectable ATZ in the water control on June 9, 2006; however, all control replicates were below 0.008 µg/L ATZ after July 21, 2006. No statistical differences were observed among tanks of the same treatment group for pH, DO and T°C measurements at every monitored event ($p > 0.05$; Fig. 2.6). Furthermore, at pre-treatment, the physicochemical parameters did not vary among treatments and

averaged 6.2 mg/L DO and 17.9 °C (data not shown). Note that results will be reported using the nominal concentrations to facilitate presentation.

2.3.3 Atrazine affects metamorphic success

High survival rates occurred in water (79 %) and EtOH (76 %) controls (Table 2.1). The survival rate in the 0.1 µg/L ATZ was 75 % and was not different from control. However, the 66 % survival rate in the 1.8 µg/L ATZ group was significantly lower than control ($p < 0.05$). The EE2 group also exhibited a significant decrease in survival rate (65 %) compared to its EtOH control (76 %; $p < 0.05$). Significantly fewer ATZ- and EE2-treated tadpoles reached metamorphosis at G42 (data were corrected for mortality). In the controls, 76 % (water control) and 85 % (EtOH control) of tadpoles reached metamorphosis; while only 45 %, 50 % and 55 % completed metamorphosis in the 0.1 µg/L ATZ; 1.8 µg/L ATZ and EE2 treatments, respectively ($p < 0.001$). Metamorphosis of all surviving animals occurred between the 4th and the 12th week of the exposure. There were no significant effects of ATZ treatments on ATM, SVL and WW, but EE2-exposed animals were on average 5.6 % smaller in length and 18 % lower in weight than EtOH control animals ($p < 0.05$).

2.3.4 Atrazine induces female-biased sex ratio

Sex ratios of surviving metamorphs were 1:0.6 (male:female) in both control groups (Table 2.2). These ratios are comparable to wild-caught metamorphosing animals from our reference site where we originally collected eggs for the mesocosm experiment. The natural sex ratio found in the *L. pipiens* young-of-the-year of the Raisin River was 1:0.5 (male:female) during the summer 2006. Only the highest ATZ exposure (1.8 µg/L

ATZ) exhibited a significantly altered sex ratio of 1:1.4; while the 0.1 µg/L ATZ treatments yielded a similar sex ratio (1:0.8) to the water control. Exposure to EE2 did not significantly change the sex ratio (1:0.9) compared to the EtOH control.

2.3.5 Atrazine did not induce intersex individuals

No intersex individuals were found in field collected, water control, EtOH control or ATZ-treated animals (Table 2.2); however 22 % of EE2-treated males expressed an intersex condition (Fig. 2.7). The presence of cortical alveoli was identified in testicular oocytes of EE2-induced intersex animals (Fig. 2.7).

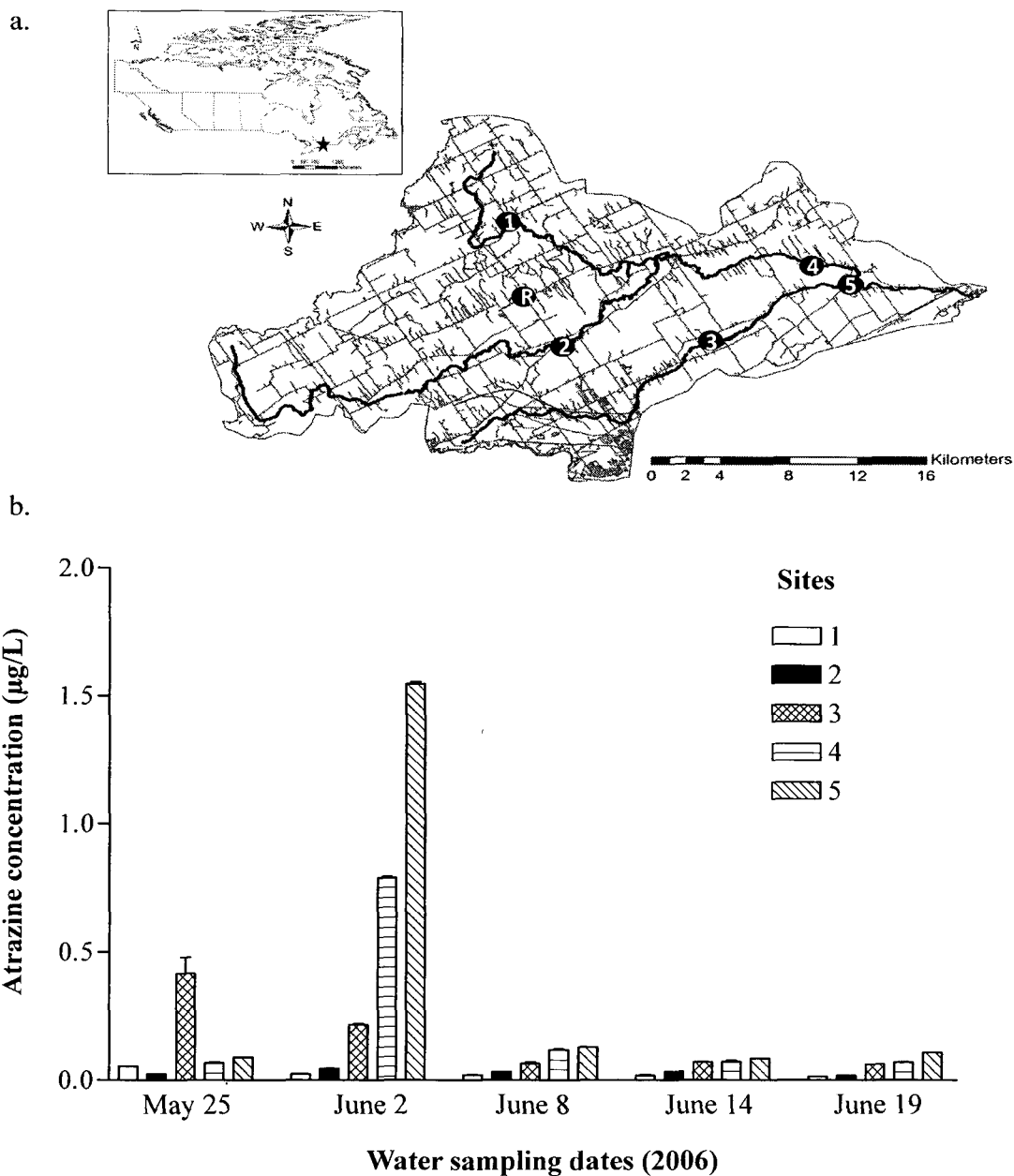


Figure 2.4 Concentrations of ATZ in water samples collected in the Raisin River (ON, Canada) during spring 2006. a. Water collection was performed between May 25th and June 19th, 2006 at five different sites on the Raisin River (numbered 1 to 5). b. The concentration of ATZ in the water at each site was quantified using HPLC-MS/MS (minimal detection limit was 0.003 µg/L). Bars represent the mean + SD (n = 2 / site per date). R: reference site.

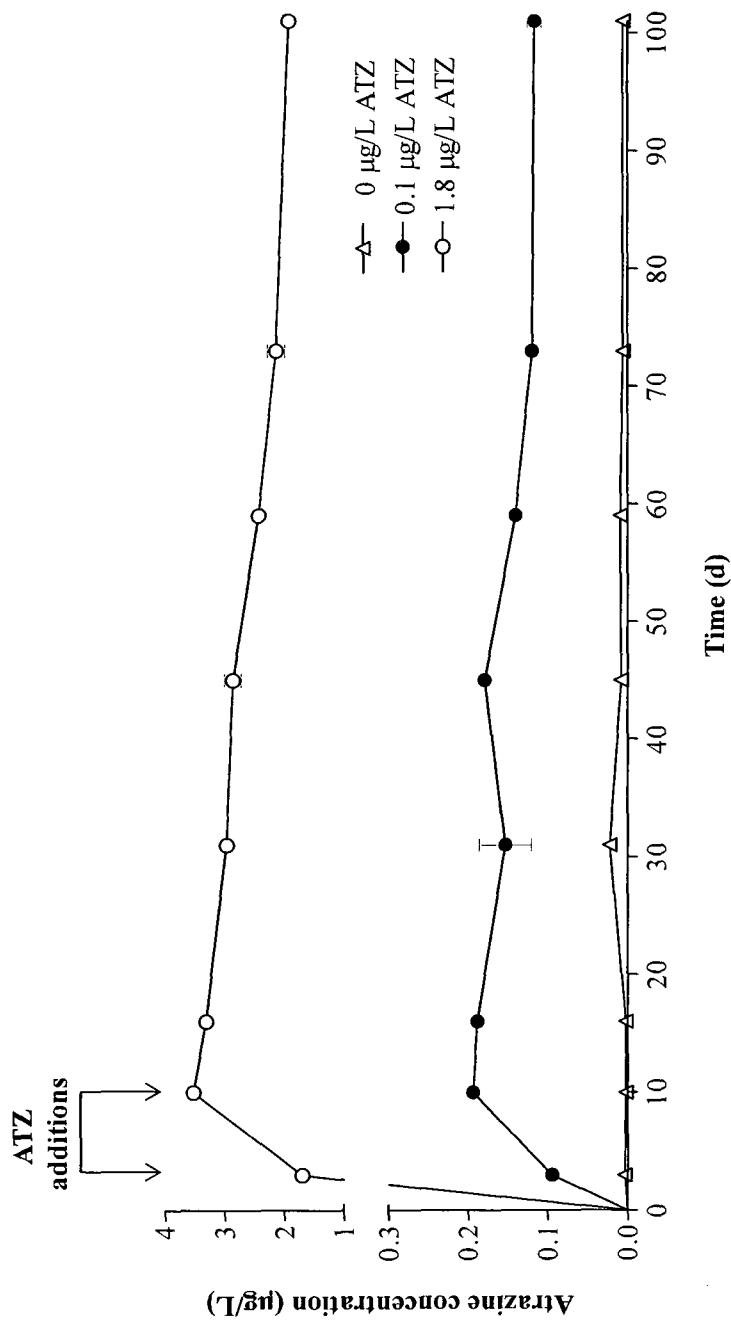


Figure 2.5 Concentrations of ATZ detected in mesocosms during the exposure period. Water collection ($n = 5/\text{mesocosm}$ per date) was performed between May 10th (day 1) and August 18th, 2006. Atrazine extractions were quantified using HPLC-MS/MS (minimal detection limit was 0.003 µg/L). Mean $\pm$ SD are presented. The arrows show the two successive additions of ATZ done on May 12th and 19th, 2006. No significant differences among tanks within a treatment were found (ANOVA; $p > 0.05$). ATZ: atrazine.

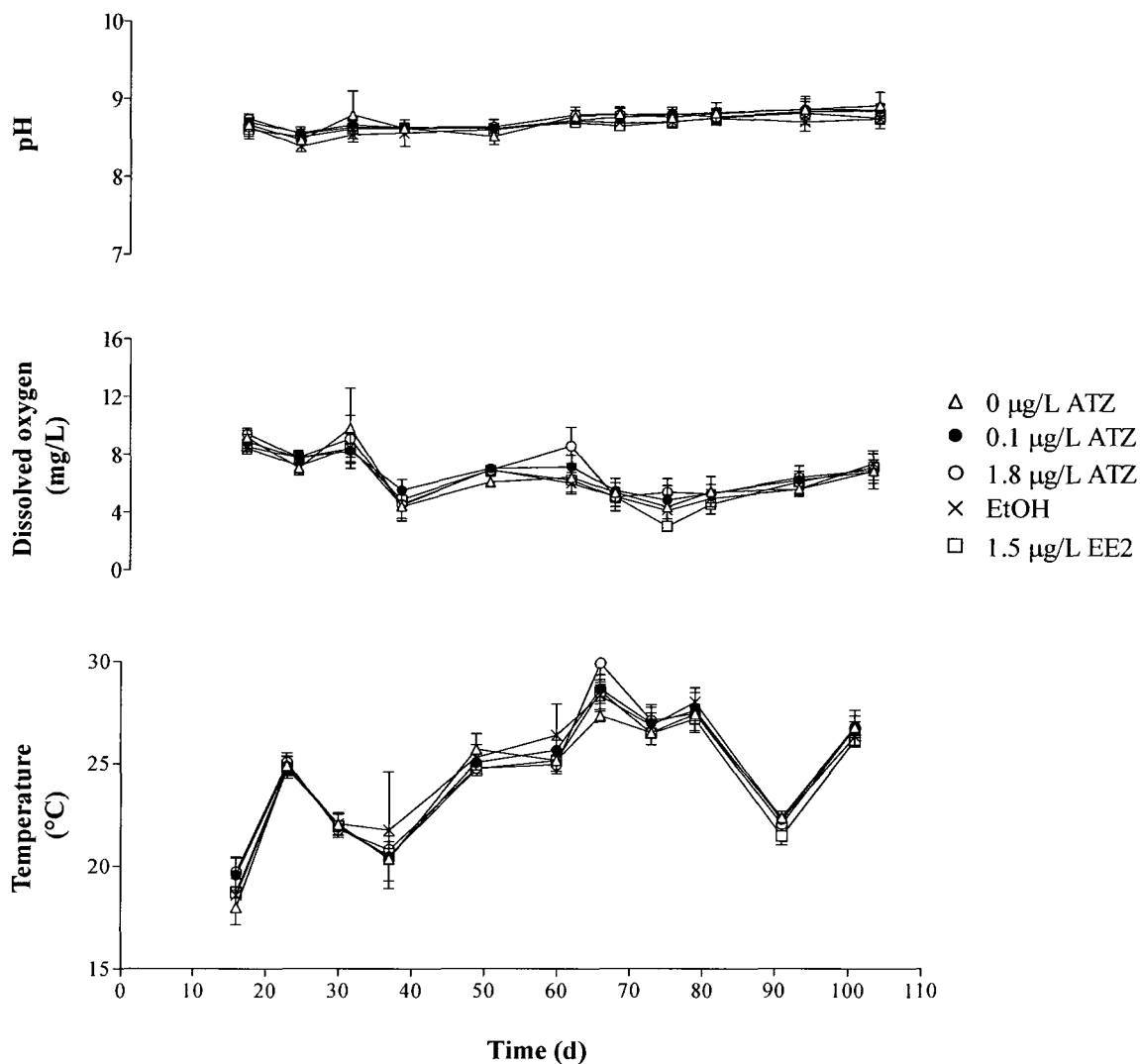


Figure 2.6 Monitoring of physicochemical parameters (pH, dissolved oxygen and temperature) in mesocosms during the exposure period. Monitoring ($n = 2\text{--}5$ mesocosms/treatment per date) was performed between May 25th (day 16) and August 18th, 2006. Temperature, dissolved oxygen and pH were recorded weekly using an YSI and a pH/K model probes. Mean $\pm$ SD are presented. No significant differences among tanks within treatment were found (ANOVA; $p \geq 0.85$). ATZ: atrazine, EtOH: ethanol; EE2: 17 α -ethinylestradiol.

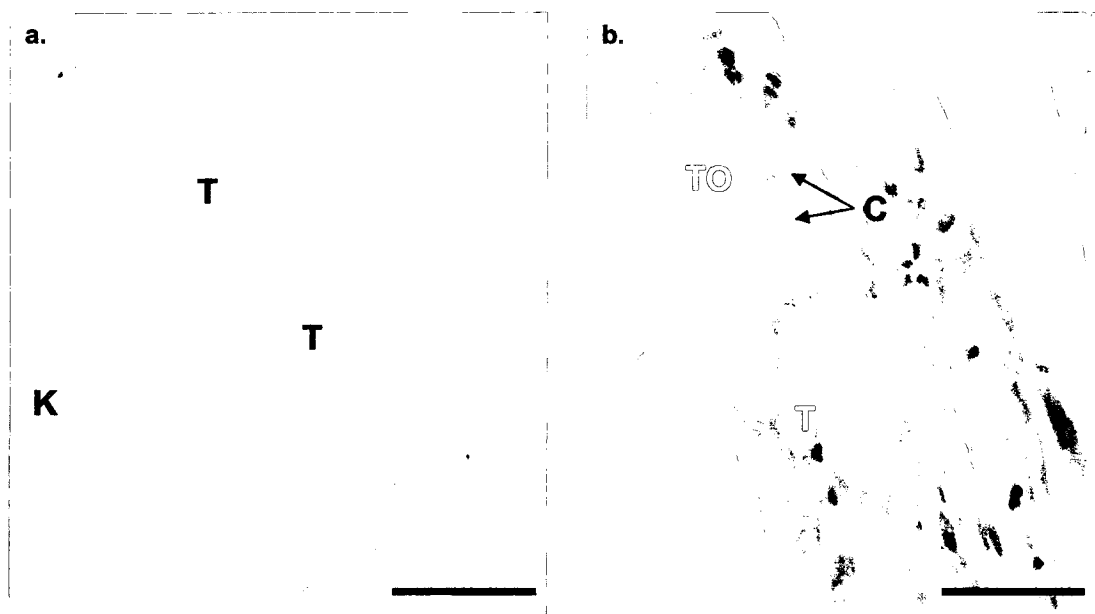


Figure 2.7 Histological sections of male and intersex gonads at metamorphic climax (G42). a. Longitudinal cross-section of normal male testes from the control treatment. Scale bar, 200 µm. b. Longitudinal anterior section of a testis from the 1.5 µg/L EE2 treatment showing a 30 µm oocyte with cortical alveoli. This animal was designed as an intersex. Scale bar, 30 µm. Both sections are 5 µM thick and stained with hematoxylin-eosin stain. C: cortical alveoli; EE2: 17α-ethinylestradiol; G: Gosner stage; T: testis; K: kidney; TO: testicular oocyte.

Table 2.1 Effects of ATZ and EE2 on *Lithobates pipiens* development and metamorphosis. *Lithobates pipiens* tadpoles were exposed from G27 to G42 to nominal ATZ concentrations (0, 0.1 and 1.8 µg/L) and to an EE2 positive control (1.5 µg/L) along with its EtOH carrier (0.0003 %). Sample size (n), survival success of all metamorphosed and remaining tadpoles (n), metamorphosis success corrected for mortality (n) and mean ± SEM are presented for age at metamorphosis (d), snout-vent length (mm) and wet weight (g). Data were analyzed using χ^2 -test.

Treatment	n	Survival ^a (n)	Metamorphosis success ^a (n)	ATM ^b (d ± SEM d)	SVL ^b (mm ± SEM mm)	WW ^b (g ± SEM g)
Control	150	118	90	72 ± 1	17.9 ± 0.3	0.96 ± 0.06
0.1 µg/L ATZ	150	113	50 *	79 ± 3	17.4 ± 0.1	0.89 ± 0.03
1.8 µg/L ATZ	150	99 *	47 *	75 ± 4	17.8 ± 0.5	0.98 ± 0.06
EtOH	150	114	99	76 ± 4	17.9 ± 0.4	1.01 ± 0.04
1.5 µg/L EE2	150	97 **	52 **	76 ± 4	16.9 ± 0.4 **	0.83 ± 0.05 **

ATZ: atrazine; EtOH: ethanol; EE2: 17 α -ethinyloestradiol; ATM: age at metamorphosis, SVL: snout-vent length, WW: wet weight, G: Gosner stage.

*indicates significant differences between the water control group and the ATZ treatments ($p \leq 0.05$)

**indicates significant differences between the EtOH group and the EE2 treatment ($p \leq 0.05$)

^aincludes all animals that reached or passed G42

^bincludes only the animals at G42

Table 2.2 *Lithobates pipiens* gonadal gross morphology and histology analysis of mesocosm and Raisin River animals. *Lithobates pipiens* tadpoles were exposed as described in Table 1. Sample size (n) and sex ratio (male : female ratio) of G42 frogs in all five treatments and in wild-caught metamorphs from the reference site are reported. Gross morphology was assessed by visual inspection of the gonads; while a subsample of males was used for gonadal histology to assess the presence of intersex condition. Percentage of intersex individuals is presented. Data were analyzed using χ^2 -test.

Treatment	n ^a	Gonadal Gross Morphology			Gonadal Histology		
		Males (n)	Females (n)	Sex ratio (M : F)	Males ^b (n)	TO (%)	
Control	60	37	23	1 : 0.6	20	0	
0.1 µg/L ATZ	34	19	15	1 : 0.8	17	0	
1.8 µg/L ATZ	31	13	18	1 : 1.4 *	12	0	
EtOH	66	42	24	1 : 0.6	17	0	
1.5 µg/L EE2	35	18	17	1 : 0.9	18	22 **	
RR reference site	30	20	10	1 : 0.5	10	0	

ATZ: atrazine; EtOH: ethanol; EE2: 17 α -ethinylestradiol; M: male; F: female; G: Gosner stage; RR: Raisin River; TO: testicular oocytes.

*indicates significant differences between the water control group and the ATZ treatments ($p \leq 0.05$)

**indicates significant differences between the EtOH group and the EE2 treatment ($p \leq 0.05$)

^afrom the metamorphosed animals, only G42 individuals were used for gonadal gross morphology

^brandomized sub-samples of males were chosen for gonadal histology

2.4 Discussion

This study demonstrates that exposures to environmentally relevant ATZ concentrations in outdoor mesocosms affect both amphibian metamorphosis and gonadal differentiation. Our findings provide evidence that low ATZ concentrations affect metamorphosis by decreasing the number of frogs reaching metamorphosis and that chronic exposure to this herbicide feminizes *L. pipiens* tadpoles.

Amphibian metamorphosis provides a unique opportunity to study the impact of anthropogenic chemicals on the thyroid hormone (TH) system. Our results demonstrate that ATZ and EE2 exposures significantly alter the success of metamorphosis in *L. pipiens* (only 45 and 55 % of treated frogs reached metamorphosis compared to 76 % and 85 % in controls, respectively). Metamorphic success is rarely reported in the literature and is not commonly used as an endpoint since most exposures are terminated when enough animals have metamorphosed to perform subsequent experiments. For example, Jooste et al. (2005) ended their exposure when they obtained 150 metamorphs (*X. laevis*) without reporting how many tadpoles did not metamorphose in the treatments. Age at completion of metamorphosis is a common endpoint used to assess disruptions in metamorphosis; however, it measures delays in developmental rate and not metamorphic success. A recent study performed by Brodeur and colleagues (2009) also measured the success of metamorphosis by cumulating (%) the number of animals reaching metamorphosis and demonstrated that ATZ decreases the metamorphic success in *Rhinella arenarum* (South American toad); however, only at concentrations of 10 mg/L and above (Brodeur et al., 2009). In amphibians, THs are essential for metamorphosis

and are involved in the remodelling of TH target tissues (Shi 1996). A disruption in TH production can result in metamorphic defects. Exposure of *X. laevis* to ammonium perchlorate (a known inhibitor of thyroidal iodide uptake) resulted in fewer tadpoles completing tail resorption, forelimb emergence and hindlimb development (Goleman et al. 2002b). Some studies have suggested that ATZ alters the TH axis in frogs and mammals (*X. laevis*; Freeman and Rayburn 2005; female albino rats, Kornilovskaya et al. 1996; male Wistar rats; Stoker et al. 2000). Here, we present evidence that ATZ exposure alters the metamorphic success of *L. pipiens* and we suggest that this occurs through the disruption of the TH axis.

In our experiment, ATZ exposures led to a female-biased sex ratio as the nominal 1.8 µg/L ATZ treatment produced 20 % more phenotypic female tadpoles compared to animals reared in untreated water. A recent study demonstrated that wild-type male *X. laevis* tadpoles exposed to 0.1-100 µg/L ATZ also displayed a concentration-dependent increase in phenotypic females (Oka et al. 2008). Conversely, other studies did not report any bias in *X. laevis* sex ratio after chronic exposure to ATZ (Carr et al. 2003; Kloas et al. 2009a). Early studies have shown that genetic male *L. pipiens* exposed to a high concentration of estradiol resulted in 100 % females (Chang and Witschi 1956), demonstrating that feminized individuals were sex-reversed-male-to-female animals. Therefore, we could speculate that some of the females obtained after the ATZ treatment in this current study are males sex reversed into females. Furthermore, Chang and Witschi (1956) bred sex reversed-male-to-female *L. pipiens* frogs with normal males and found a highly biased-male sex ratio. If some of the females obtained in the ATZ-

exposed treatment are indeed sex reversed-male-to-females (this study), it is possible that this could impact the survival and sex ratio of their progeny.

In addition to female-biased sex ratio, studies have demonstrated that ATZ increases the incidence of intersex condition in amphibians (*X. laevis*; Hayes et al. 2002a; *L. pipiens*; Hayes et al. 2003). However, we did not observe intersex gonads in ATZ-exposed *L. pipiens*. The intersex condition has been characterized as a transitional phase during gonadal differentiation and sex reversal (Hogan et al. 2008; Yeh et al. 2003); therefore, our results suggest that the intersex-transitional period was completed by G42. In contrast, our positive control EE2 displayed a proportion of testicular oocytes in treated male tadpoles. Thus, we cannot dismiss that ATZ may have induced intersex conditions prior to G42, and that any presumptive testicular oocytes had regressed by the time we sampled animals at metamorphosis.

2.5 Conclusions

Using an outdoor mesocosm design, we provide evidence that ATZ can affect amphibian development at levels measured in water across the distribution of *L. pipiens* in North America (Graymore et al. 2001). Much controversy surrounds the effects of ATZ on frogs; for example, at one extreme *X. laevis* exposed to low levels of ATZ under laboratory conditions exhibited gonadal dysgenesis (Hayes et al. 2006b; Oka et al. 2008), while at the other extreme no effects were observed following similar exposures (Coady et al. 2005; Kloas et al. 2009a). The potential reasons for such differences are numerous and have been discussed previously (reviewed in Hayes 2004; Solomon et al. 2008), for example, there are questions about the relevance of studies with non-native species to predict potential effects of indigenous species. Studying a North American native species, our results demonstrate that environmentally-relevant levels of ATZ can be biologically active in *L. pipiens* as we report female-biased sex ratios and disruption of metamorphosis. Feminization and disruption of metamorphosis are important physiological consequences that could potentially alter amphibian population fitness. Therefore, the examination of mechanisms of action of ATZ such as gene expression and sex steroid biosynthesis enzyme activity is warranted (Chapter 3).

CHAPTER 3

Mechanisms of action of the herbicide atrazine on the disruption of *Lithobates pipiens* metamorphosis and gonadal development

Chapter adapted from **Langlois VS¹**, Carew AC², Pauli BD³, Wade MG⁴, Cooke GM⁵
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Main contributions of each co-author:

¹Contributed to original ideas, performed animal dissection, real-time RT-PCR optimization and related-data analysis, cyp19 activity assay and srd5beta activity assay optimizations and related-data analysis and manuscript preparation.

²Involved in animal dissections.

³Revised the chapter using his expertise in amphibian toxicology.

⁴Provided the srd5beta activity assay material and expertise.

⁵Revised the chapter using his expertise on thyroid hormone disruption.

⁶Contributed to original ideas and revised the manuscript.

3.1 Introduction

In the last decade, the herbicide atrazine (ATZ) has been associated with disruptions in frog metamorphosis and sexual development (Chapter 2; Hayes et al., 2003; Brodeur et al., 2009), however the mechanisms of action of ATZ remain unknown. The dominant hypothesis in the literature regarding the disruption of gonadal development by ATZ is via the alteration of aromatase (cyp19) activity (Hayes et al. 2002a). Aromatase is a cytochrome P450 enzyme which converts testosterone into estradiol (E2; Lephart 1996) and androstenedione into estrone (Simpson et al. 1994). In numerous fish, amphibians and reptiles, cyp19 induction or inhibition produces female-biased or male-biased sex ratios, respectively (Chardard and Dournon 1999; Navarro-Martin et al. 2009; Richard-Mercier et al. 1995). Despite that induction of *in vitro* cyp19 activity has been reported in human cell lines after exposure to ATZ (Heneweer et al. 2004; Holloway et al. 2008), several other studies have not observed such responses in amphibians (Coady et al. 2005; Hecker et al. 2005a; Hecker et al. 2005b; Murphy et al. 2006a; Oka et al. 2008). The underlying reasons for these differences in findings and the mechanisms of action through which ATZ may disrupt vertebrate development remain unclear.

Here we investigated alternative mechanisms through which ATZ may induce estrogen-like effects in amphibians. This includes the induction of the expression of the nuclear estrogen receptor α (*eralpha*), which is activated upon estrogen binding and has been recognized as a biomarker of estrogenic exposure (Lutz et al. 2005). Studies have shown that following treatment with estrogenic substances, the expression of *eralpha* increases: in the brain of *Lithobates pipiens* (Northern leopard frog) tadpoles

(17 α -ethinylestradiol; EE2; Duarte et al. 2006), in whole tadpole bodies of *Xenopus laevis* (African clawed frog; bisphenol A; Levy et al. 2004) and in the liver of *Pimephales promelas* (Fathead minnow; EE2; Filby et al. 2007). The steroid 5 β -reductase (srd5beta) pathway is also potentially involved in feminization of developing amphibians (Chapter 6). Member of the aldo-keto reductase superfamily, srd5beta can regulate androgen bioavailability by catalyzing the conversion of testosterone to 5 β -dihydrotestosterone (5 β -DHT; Chapter 4). Therefore, we hypothesized that ATZ exposure alters *eralpha* expression and srd5beta activity in the target tissues of exposed tadpoles.

There is also evidence that ATZ affects amphibian development and metamorphosis (Chapter 2; Coady et al. 2004; Freeman and Rayburn 2005). Metamorphosis is a morphogenetic process that involves organogenesis, remodelling of the central nervous system, the growth of appendages and tadpole tail resorption. This transformation is stimulated by environmental signals that trigger the central control of the hypothalamus-pituitary-thyroid axis to initiate thyroid hormone (THs; thyroxine [T4] and triiodothyronine [T3]) release into the circulation. Conversion of T4 to T3 and subsequent degradation mainly occurs in peripheral tissues and involve deiodinase enzymes (dio). Then, THs act through TH receptors (tr), which regulate gene expression by interacting with the TH response element in target genes (Aranda and Pascual 2001). Changes in the expression of *dio2*, *dio3*, *tralpha* and *trbeta* could influence the T4 to T3 ratio, which in turn will affect metamorphosis (Manzon and Denver 2004).

We investigated the potential mechanisms of action of ATZ on sexual development and metamorphosis in *L. pipiens*. We hypothesized that ATZ induces *cyp19* and *eralpha* mRNA expression, induces cyp19 activity, alters srd5beta activity and alters TH-related gene expression. To our knowledge, this is the first study to use a chronic ATZ exposure in amphibians and evaluate changes in (1) *cyp19* expression and activity simultaneously; (2) *eralpha* expression; (3) srd5beta activity; and (4) *dio* and *tr* expression in prometamorphic and metamorphic tadpoles.

3.2 Materials and Methods

3.2.1 Experimental design

This project is a follow-up study of the previous chapter; therefore details on the experimental design can be found in Chapter 2 (section 2.2). Briefly, *L. pipiens* tadpoles were chronically exposed (from G27 to G42) to five nominal treatments in outdoor mesocosms. The treatments were: 0 µg/L ATZ (ATZ control; n = 5 mesocosms), 0.1 µg/L ATZ (n = 5), 1.8 µg/L ATZ (n = 5), 1.5 µg/L EE2 dissolved in EtOH (0.0003 %; n = 5) and 0.0003% EtOH (EE2 control; n = 5). The herbicide AAtrex Liquid 480 (purity 97.1% ATZ, 2.9 % related triazines and 5 % of ethylene glycol w/v) was used as a source of ATZ. The synthetic estrogen EE2 was used in the experimental design as a positive control for feminization. Each mesocosm consisted of a 378-L high density polyethylene Rubbermaid® tank to which 300 L of groundwater, 50 g of Hagen® rabbit pellets, 100 g of dried leaves and a *Daphnia magna* population. 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.

3.2.2 Tissue collection

Animals reaching metamorphosis were removed at metamorphic climax (Gosner stage 42; G42; Gosner 1960). At the end of the exposure, a cohort of prometamorphic G34 tadpoles was sampled from the remaining non-metamorphosed tadpoles for gene expression analysis. The developmental stage G34 was chosen because it is during sexual differentiation (Hogan 2006) and represents also the

developmental period when tadpoles begin to respond to THs (Shi 1999). A schematic of tissue collection is presented in Fig. 3.1. Animals were anaesthetized by immersion in 1 % tricaine methanesulfonate (MS-222; Sigma) and were sacrificed by cutting the spinal cord. Brain, liver and tail were removed and all samples were immediately frozen on dry ice and stored at -80 °C.

3.2.3 RNA isolation and cDNA synthesis

Samples were homogenized using a MM301 Mixer Mill (Retsch, Newton, PA, USA) at 20 Hz for 2 min. Total RNA was obtained from whole brain and liver of G34 and G42 animals using the QIAGEN RNeasy Mini Kit (including the RNase-free DNase treatment) and the QIAGEN RNeasy Micro Kit (Qiagen, Mississauga, ON, Canada), respectively. TRIzol reagent (Invitrogen Canada Inc., Burlington, ON, Canada) was used to isolate total RNA from the tails of both G34 and G42 stages, which was followed by DNase treatment (Promega, Madison, WI, USA). All procedures were performed as described by the manufacturers. RNA was resuspended in DNase and RNase free water (Invitrogen) and stored at -80 °C. Concentrations of RNA were determined using GeneQuant RNA/DNA calculator spectrophotometry (Amersham Pharmacia Biotech). Total cDNA was prepared from 1 µg and 2 µg (G34 and G42 tissues, respectively) of total RNA and 0.2 µg random hexamer primers (Invitrogen) using Superscript II RNase H⁻ reverse transcriptase as described by the manufacturer (Invitrogen).

3.2.4 *Gene and protein nomenclature*

To be consistent throughout this thesis, the amphibian gene nomenclature used followed Xenbase guidelines (Bowes et al. 2009). Guidelines state that frog genes should be written in small letters and in italics and that proteins should carry the same name as the gene but should not be in italic. Furthermore, if Greek letters are present in a gene or protein name (e.g., α), they must be written out (e.g., alpha).

3.2.5 *Real-time RT-PCR*

Real-time RT-PCR simplex (SYBR Green detection) and triplex assays (dual labelled fluorescent probes) as described in Hogan et al. (2007) were used to detect transcripts for *cyp19* (coding sequence accession no. DQ449025), *eralpha* (accession no. DQ398027), *tralpha* (accession no. DQ398026), *trbeta* (accession no. AY049025), *dio2* (accession no. L42815), *dio3* (accession no. DQ398025) and the ribosomal protein L8 (*rpl8*; accession no. AY452063). The stress neuropeptide corticotropin-releasing hormone (*crh*; accession no. DQ779202) was also analyzed by real-time RT-PCR in tadpole brains as described in Croteau (2009) to control for stress in the experiment design. Gene expression analyses were performed for all targeted genes in brain, liver and tail tissues. Briefly, each 25- μ L reaction performed in simplex contained a mix of SYBR Green I dye (Molecular Probes, Eugene, OR), PCR buffer (Qiagen), $MgCl_2$ (Qiagen), dNTPs (Invitrogen), ROX reference dye (Stratagene), DNase and RNase free water (Invitrogen), HotStarTaq (Stratagene) and a set of custom made primers (forward and reverse) for each targeted gene (Invitrogen). The thermocycle program began with an enzyme activation step at 95 °C (15 min) followed

by 40-45 cycles of 95 °C (15 s, denaturation), gene specific annealing temperature (between 58-62 °C for 5 s) and 72 °C (30 s, elongation). Triplex strategies were used to simultaneously detect 1) *tralpha*, *trbeta* and *rpl8* and 2) *dio2*, *dio3* and *rpl8*. The triplex 25-μL reaction contained gene specific labelled fluorescent probes (Cy5, FAM and HEX dyes; Integrated DNA Technologies, Coralville, IA), PCR buffer (Qiagen), MgCl₂ (Qiagen), dNTPs (Invitrogen), ROX reference dye (Stratagene), DNase and RNase free water (Invitrogen), HotStarTaq (Stratagene) and a set of custom made primers (forward and reverse) for each targeted gene (Invitrogen). The thermocycle program consisted of an enzyme activation step at 95 °C (15 min) followed by 40-45 cycles of 95 °C (15 s, denaturation) and gene specific annealing/elongating temperature between 60-62 °C (1 min). In every real-time RT-PCR assay, samples were run in duplicate along with a negative template control (RNase-free water instead of cDNA template) and a negative reverse transcriptase control (cDNA template for which water was added instead of Superscript II). The standard curve method was used to interpolate mRNA concentration; therefore, serial dilutions of G34 and G42 cDNA mix (brain, liver or tail) were used as standard curve templates. The *rpl8* was used as a housekeeping gene since its mRNA level did not change significantly between treatments. Data are presented as fold change relative to respective controls. Neither estrogen-related genes (*cyp19* and *eralpha*) were measured in the tail since it is not a reproductive tissue. Furthermore, the expression of *cyp19* was not performed in the liver since *cyp19* was not detectable in *L. pipiens* liver (Hogan 2006).

3.2.6 Assay for the activity of *cyp19*

Aromatase activity was measured in the brain of *L. pipiens* using a modified radiometric method (Du et al. 2001). Aromatase activity was determined by measuring the tritiated water released from the C-1 β carbon atom of 1 β -³H-androstenedione (³H-A) during its conversion to estrogen (Du et al. 2001). We determined the specificity of the assay using the aromatase inhibitor fadrozole (also used as a breast cancer drug in Japan; Novartis Pharma AG) on a brain homogenate of a *L. pipiens* adult female. Fadrozole (0, 0.5, 1.0, 1.5, 2.0 and 2.5 μ M) inhibited *cyp19* activity in a concentration-dependent manner with increasing levels of fadrozole (Fig 3.2).

Using the optimized assay, *cyp19* activity was measured in G42 animals. Pools of two brains of the same sex animals (n = 4-6 pools per treatment per sex; all pools weighed 21.2 to 58.3 mg) were homogenized in 200 μ L of potassium phosphate buffer (100 mM KCl, 10 mM KH₂PO₄, 1 mM EDTA, 10 mM dithiothreitol, pH 7.4) and centrifuged at 1,000 g for 10 min at 4 °C. Then, 50- μ L cofactor (100 mM KCl, 10 mM K₂HPO₄, 1mM EDTA, 10 mM dithiothreitol, 5 mM glucose-6-phosphate, 1 mM NADPH, 10 U glucose-6-phosphate dehydrogenase; pH 7.4) and 0.3 μ M ³H-A (specific activity 23.5 Ci/mmol; Dupont Co., NEN Research Products, Boston, MA) were incubated for 80 min in a shaking water bath at 37 °C. The tissue homogenate (50 μ L) was added to the mix of the cofactor and ³H-A, and incubated for 80 min in a shaking water bath at 25 °C. The reaction was stopped by adding 200 μ L of ice-cold charcoal solution (80 mg charcoal/ml in 10 % trichloroacetic acid, TCA) to the samples. Samples were incubated on ice for 10 min and centrifuged at 1620 g for 10 min at 4 °C. The supernatant was extracted twice with the charcoal solution. Samples

were run in triplicate and each assay included negative substrate controls (sample containing buffer instead of tissue) and tritiated water controls (sample containing tritiated water instead of $^3\text{H-A}$) to calculate the assay recovery. The isotope ^3H was measured as disintegrations per minute using a liquid scintillation counter (Beckman Coulter LS 6500) since it is correlated to cyp19 activity. Aromatase activity is expressed as fmol/h·mg protein. Total protein concentration was measured using the Bio-Rad protein assay kit as described by the manufacturer (Bio-Rad Co.).

3.2.7 Assay for the activity of the steroid 5 β -reductase

The activity of srd5beta was measured in *L. pipiens* liver using a modified assay of the mammalian steroid 5 α -reductase (srd5alpha) activity (Robaire 1979). Activity was determined by the conversion of testosterone into 5 β -reduced metabolites (5 β -DHT and 5 β -androstane-3 β -17 β -diol [5 β -3 β -diol]). We determined the specificity of the assay using the srd5alpha inhibitor finasteride (Sigma), also known to inhibit srd5beta (Drury et al. 2009) on a liver homogenate of a *L. pipiens* adult female. Finasteride (10, 30 and 100 μM) inhibited srd5beta activity in a concentration-response manner (Fig 3.3).

Using the optimized assay, srd5beta activity was measured in metamorphic tadpoles (G42). Individual livers ($n = 6$ livers per treatment per sex; all livers weighed 5.7 to 35.1 mg) were sonicated in 0.4 mL of Tris-buffer (100 mM tris, 250 mM sucrose, 25 mM KCl, 5 mM MgCl_2 , 7 mM mercaptoethanol, 100 mM HCl and 500 μM of NADPH; pH 7.4) and a 50- μL aliquot of the homogenate was incubated with 2×10^{-5} μM of 4- ^{14}C -testosterone (specific activity 2.0 GBq/mmol; Amersham Biosciences)

in 3 mL of Tris-buffer. Incubations were performed in borosilicate glass tubes held in a model 50 reciprocal shaking bath at 37 °C. Reactions were terminated at 30, 60 and 90 min by adding 1 mL of the incubated homogenates to 5 mL of ethyl acetate containing 30 µL of a mixture of unlabelled steroids (0.1 mM each; Steraloids Inc., Newport, RI, USA; Table 3.1). Steroids were extracted by vortexing and centrifuging the samples at 1000 g for 10 min. The organic fractions were transferred to conical tubes after being separated from the aqueous phases in an acetone-dry ice bath and evaporated to dryness using a SavantTM Speedvac®Plus Evaporator connected to a SavantTM refrigerated vapour trap. Residues were resuspended in chloroform-methanol (1:1) and applied on thin layer chromatography plates (TLCs; Partisil® K6 Silica Gel 60Å; Whatman International Ltd, England). Steroid metabolites were extracted using a two-dimensional TLC system; the first dimension was developed in a chloroform-acetone (85:15) solvent system, while the second dimension was developed twice in ethyl acetate-hexane (4:6). Every TLC was placed with a tritium storage phosphor screen (General Electric, Uppsala, Sweden) overnight. Tritium screens were scanned using a Storm 820 phosphoimager (General Electric) and analyzed with Image Quant TL 3.0 software (Amersham Bioscience). Unlabeled steroids serve as standard reference points and as carrier material in this assay to locate ¹⁴C-labelled metabolites, and were individually developed in the same aforementioned TLC solvent systems. Then, TLCs were exposed to iodine vapour, ultraviolet radiation (254 nm) and were placed in the oven (110 °C) for 2 min after being sprayed with Allen reagent (sulphuric acid-ethanol-water 80:18:2; v/v/v; Allen et al. 1950) carried in nitrogen gas. Comparisons between standards and testosterone metabolite samples allowed for metabolite

identification. Unknown metabolites were recrystallized to confirm their identity. Successive recrystallizations from methanol, EtOH and acetone demonstrated that the ^{14}C -labelled steroids co-crystallized with the 5β -DHT and 5β - 3β -diol (Axelrod et al. 1965). For recrystallization purposes, unknown testosterone metabolites were scrapped individually and mixed with a few drops of water to form a paste to liberate the radioisotope from the silicone. The radioisotopes were extracted from the water by vortexing the samples with 2 mL of hexane and centrifugating these samples three times at 1000 g for 10 min (IEC Centra-7, International Equipment Company; Chattanooga, TN). The 6-mL extracts were evaporated using the SavantTM Speedvac[®]Plus evaporator connected to a SavantTM refrigerated vapour trap (Fisher), received 300 mg pure 5β -DHT or 300 mg pure 5β - 3β -diol and were dissolved in ethanol (resulting in supersaturated solutions). Samples were left at RT, 4 °C or -20 °C for crystallization overnight. The supernatant was removed and the crystals were washed with methanol. Aliquots of first mother liquors (50 μL) and 10 mg of crystals were measured for radioactivity in 5 mL of aqueous or non-aqueous scintillation fluid (Dupont, MA). The same dissolution and recrystallization procedures were repeated for the EtOH and acetone solvents and the radioactivity in the mother liquors and crystals were counted (Table 3.2). The activity of *srd5beta* is expressed as the sum of 5β -DHT and 5β - 3β -diol/h/mg protein. Total protein concentration was measured using the Bio-Rad protein assay kit as described by the manufacturer (Bio-Rad Co.).

3.2.8 Data analysis

For gene expression analysis, the threshold for each target gene was automatically calculated by the MxPro software and was corrected when necessary to

be in the linear range of the amplification curve (MXPro 4.0; Strategene). The relative standard curve method was used to calculate relative mRNA concentration of genes (Applied Biosystems 1997).

Statistical analyses were performed using the software S-PLUS Version 8.0 (Insightful Corp. Seattle, Washington, USA). One-way analysis of variance (ANOVA) was used for G34 tadpole gene expression data (factor: treatments) whereas two-way ANOVA was used to analyze G42 tadpole gene expression and enzymatic activity data (factors: treatments and sex). Data were tested for normality (Kolmogorov-Smirnov's test) and homoscedasticity (Levene's test). The Bonferroni post-hoc test was used to evaluate significant differences ($p \leq 0.05$) in treatment and control group data. When data failed to meet parametric assumptions after transformation, the non-parametric Kruskal-Wallis one-way ANOVA on ranks was used, followed by the Mann-Whitney U test. ATZ and EtOH treatment data were compared to water control; EE2 treatment data were compared to the EtOH solvent control.

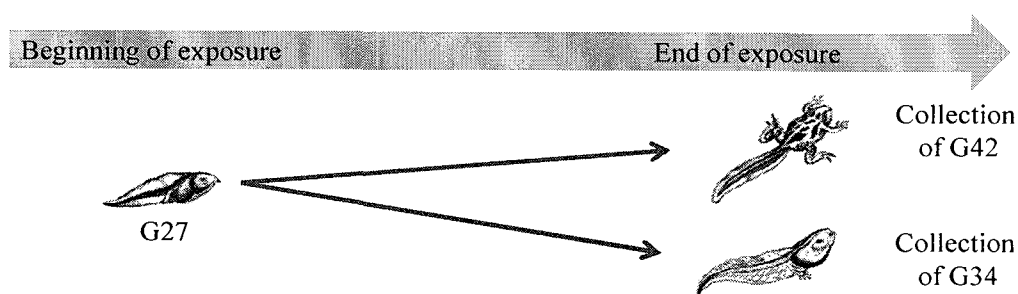


Figure 3.1 Experimental design of tissue collection for the ATZ exposure. *Lithobates pipiens* tadpoles were exposed from G27 to G42 to nominal ATZ concentrations (0, 0.1 and 1.8 $\mu\text{g/L}$) and to EE2 1.5 $\mu\text{g/L}$ along with its EtOH carrier (0.0003 %). From the remaining non-metamorphosed tadpoles at the end of the exposure, a cohort of prometamorphic G34 tadpoles were also sampled. ATZ: atrazine; EE2: 17 α -ethinylestradiol; EtOH: ethanol; G : Gosner stage.

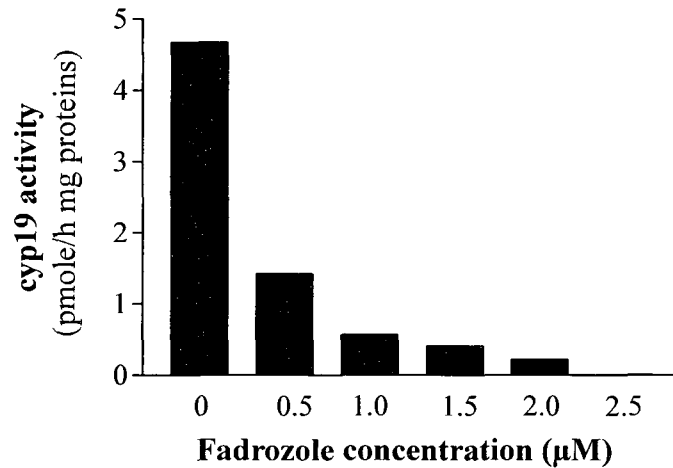


Figure 3.2 Effects of fadrozole, a cyp19 inhibitor, on cyp19 activity *in vitro* in the brain of *Lithobates pipiens* female. Activity of cyp19 was inhibited in a concentration-dependent manner (0, 0.5, 1.0, 1.5, 2.0 and 2.5 μM) with increasing levels of fadrozole in the brain (section 3.2.6). A brain homogenate of a *L. pipiens* adult female (n = 1) was used to assess cyp19 activity for each fadrozole concentration. The activity of cyp19 is expressed in pmole/h normalized to total protein content (mg). cyp19: aromatase.

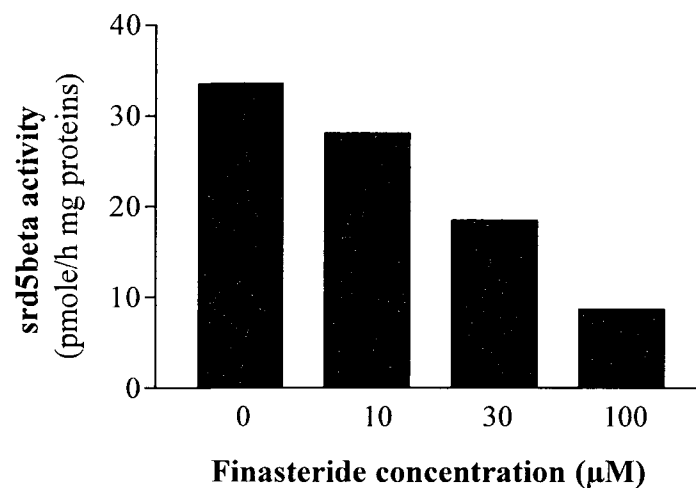


Figure 3.3 Effects of finasteride on srd5beta activity *in vitro* in the liver of *Lithobates pipiens* female. The activity of srd5beta was inhibited by finasteride (0, 10, 30 and 100 μM; section 3.2.7). A liver homogenate of a *L. pipiens* adult female (n = 1) was used to assess the srd5beta activity for each finasteride concentration (n = 1 / treatment). The activity of srd5beta is expressed in pmole/h and normalized to total protein content (mg). srd5beta: steroid 5β-reductase.

Table 3.1 Authentic standards used in the *srd5beta* activity assay. Complete list of the 18 unlabelled steroids (0.1 μ M each) used to identify and validate radioactive metabolites obtained after sample incubation (section 3.2.7).

Scientific appellations	
4-androsten-17 β -ol-3-one	4-androsten-7 α , 17 β -diol
5 α -androstan-17 β -ol-3-one	5 α -androstan-3 α , 16 α -diol-17-one
5 β -androstan-17 β -ol-3-one	5 α -androstan-3 β , 16 α -diol-17-one
5 α -androstan-3 α -17 β -diol	5 α -androstan-3, 17-diol-one
5 α -androstan-3 β -17 β -diol	5 α -androstan-3 α -ol-17-one
5 β -androstan-3 α -17 β -diol	5 α -androstan-3 β -ol-17-one
5 β -androstan-3 β -17 β -diol	5 β -androstan-3 α -ol-17-one
4-androsten-3,17-dione	4-androsten-2 α , 17 β -diol-3-one
4-androsten-6 β , 17 β -diol	4-androsten-16 α , 17 β -diol-3-one
<i>srd5beta</i> : steroid 5 β -reductase	

Table 3.2 Specific activity of 5 β -DHT and 5 β -3 β -diol after recrystallization method. Validation of the srd5beta activity assay was assessed by recrystallizing 5 β -DHT and 5 β -3 β -diol radioactive metabolites obtained following the srd5beta activity protocol (section 3.2.7). The metabolites were recrystallized three times in three different solvents (methanol, ethanol and acetone) and their activity is expressed in CPM/mg.

Steroids	Specific activity (CPM/mg)/crystallization		
	Methanol	Ethanol	Acetone
5 β -DHT	493	408	631
5 β -3 β -diol	418	507	458

5 β -DHT: 5 β -dihydrotestosterone; 5 β -3 β -diol: 5 β -androstan-3 β -17 β -diol; srd5beta : steroid 5 β -reductase

3.3 Results

3.3.1 Method validations

Both enzyme activity methods (cyp19 and srd5beta) were successfully optimized using *L. pipiens* tissues and were validated after *in vitro* analysis using specific inhibitors, fadrozole and finasteride, respectively (Fig. 3.2 and 3.3). The main metabolites obtained after the srd5beta activity assay, 5 β -DHT and 5 β -3 β -diol, were confirmed by a series of recrystallizations since each metabolite had three recrystallizations with similar CPM/mg values (Table 3.2). The mean CPM/mg $\pm$ SD for 5 β -DHT was 510.7 $\pm$ 112.5 CPM/mg and was 461.0 $\pm$ 44.6 CPM/mg for 5 β -3 β -diol.

For gene expression analyses, a standard curve test was performed for each gene prior to conducting a complete run, to ensure optimal real-time RT-PCR conditions. Each real-time RT-PCR reaction had an efficiency of 100 % $\pm$ 10 % and $r^2 > 0.985$.

3.3.2 Atrazine did not induce aromatase gene expression and activity

To determine the current hypothesis in the literature which suggests that the feminizing mechanism of ATZ is through the induction of cyp19 activity, we measured both *cyp19* mRNA level and cyp19 activity after a chronic exposure to ATZ. Atrazine did not significantly induce *cyp19* expression and/or activity (Fig. 3.4). In G34 brains, despite a slight concentration-response increase in *cyp19* mRNA levels after ATZ treatment, no statistically significant changes were observed for both ATZ and EE2

treatments (Fig. 3.4 a). In addition, no changes were observed in *cyp19* mRNA level and *cyp19* activity in the brain of G42 animals (Fig. 3.4 b). In contrast, the positive control EE2 significantly increased both *cyp19* mRNA level ($p < 0.01$) and activity ($p < 0.001$) in the brain of G42 females and males (Fig. 3.4 b).

3.3.3 *Effects of atrazine on the estrogen receptor α gene expression*

To test our hypothesis that ATZ is feminizing frogs via the induction of *eralpha* mRNA, we measured gene expression of *eralpha* in brain and liver of G34 and G42 *L. pipiens* tadpoles. Brain *eralpha* mRNA level in G34 tadpoles was increased by 2.5-fold in the 1.8 $\mu\text{g/L}$ ATZ treatment compared to water control ($p < 0.01$; Fig. 3.5 a). No changes in expression were reported for *eralpha* mRNA in the brains of G42 tadpoles or in the livers from G34 and G42 tadpoles (Fig. 3.5 b-d). EE2 did not significantly affect the expression of *eralpha* in any tissue regardless of sex or stage.

3.3.4 *Effects of atrazine on thyroid hormone-related gene expression*

To determine if the lower metamorphic success obtained after ATZ exposure (Chapter 2) was accompanied by a disruption of the TH axis; TH related-gene expression was measured. No changes were detected for *dio2* mRNA in brain, liver or tail for both stages of development (Fig. 3.6). Similarly, no *dio3* mRNA changes were identified in brain and liver of G34 and G42 animals (Fig. 3.7); however a 70 % decrease in *dio3* was detected in tail tissue of G34 animals exposed to 1.8 $\mu\text{g/L}$ ATZ ($p < 0.05$; Fig. 3.7 e). Atrazine and EE2 did not affect the expression of either TH receptors (*tralpha* and *trbeta*) in any of the tissues and stages examined (Fig. 3.8 and 3.9). Furthermore, no changes in *crh* expression were detected (Fig. 3.10).

3.3.5 Atrazine affected a sex difference in the steroid 5 β -reductase activity

A clear sexual dimorphism in srd5beta activity was identified between the animals in the water control group, where the livers of the females expressed 43 % more activity than males ($p < 0.001$; Fig. 3.11). Interestingly, this sex difference was abolished in animals exposed to either ATZ treatment ($p < 0.01$).

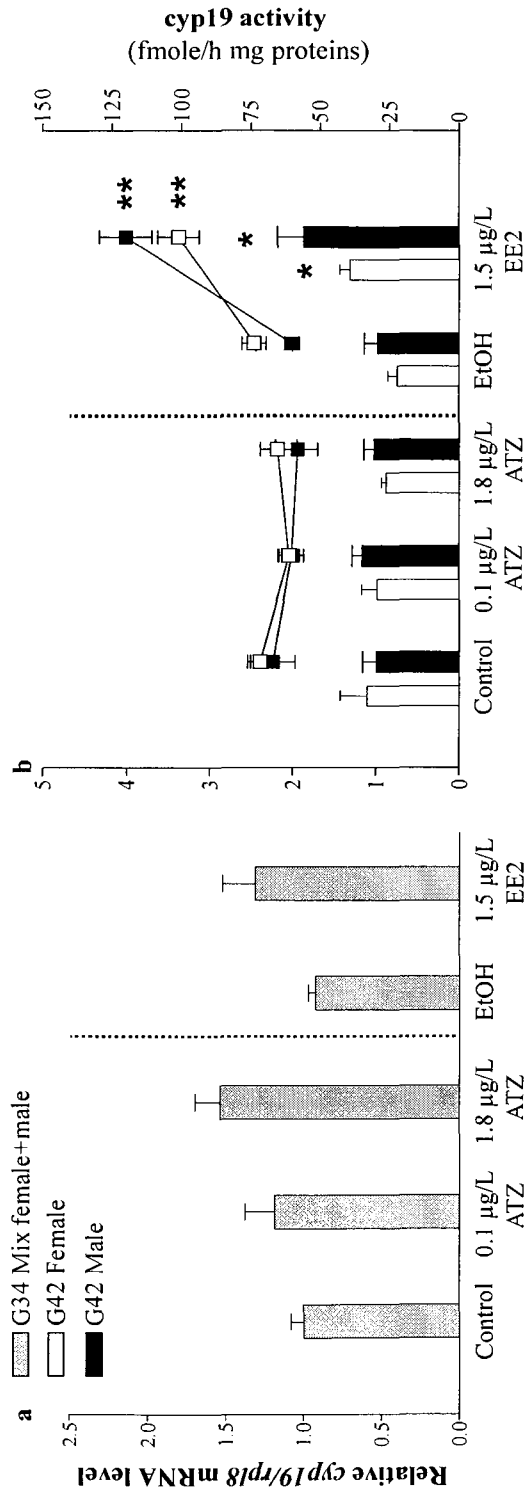


Figure 3.4 Effects of chronic ATZ and EE2 exposures on brain *cyp19* expression and activity in *Lithobates pipiens* tadpoles were exposed as described in Fig 3.1. Effects of ATZ and EE2 on *cyp19* mRNA in brain of G34 prometamorphic tadpoles (a) and G42 metamorphic tadpoles (b) were determined by real-time RT-PCR. The levels of *cyp19* mRNA are expressed relative to the water control group and are normalized to the expression of *rpl8* (bars; left Y-axis). Effects of ATZ and EE2 on *cyp19* activity in the brain of G42 animals were assessed using a radiometric method. The activity of *cyp19* is expressed in fmole/h normalized to protein content (lines; right Y-axis). Bars and lines represent the mean $\pm$ SEM ($n = 4-8$). Data were analyzed using one-way ANOVA (G34) and two-way ANOVA (G42) followed by Bonferroni post-hoc comparisons if applicable. Significant differences from the control group are indicated if $p \leq 0.05$, to report mRNA differences (*) and activity differences (**). Dashed lines separate the water and the EtOH controls. ATZ: atrazine; *cyp19*: aromatase; EE2: 17 α -ethinylestradiol; EtOH: ethanol; G: Gosner stage; *rpl8*: ribosomal protein L8.

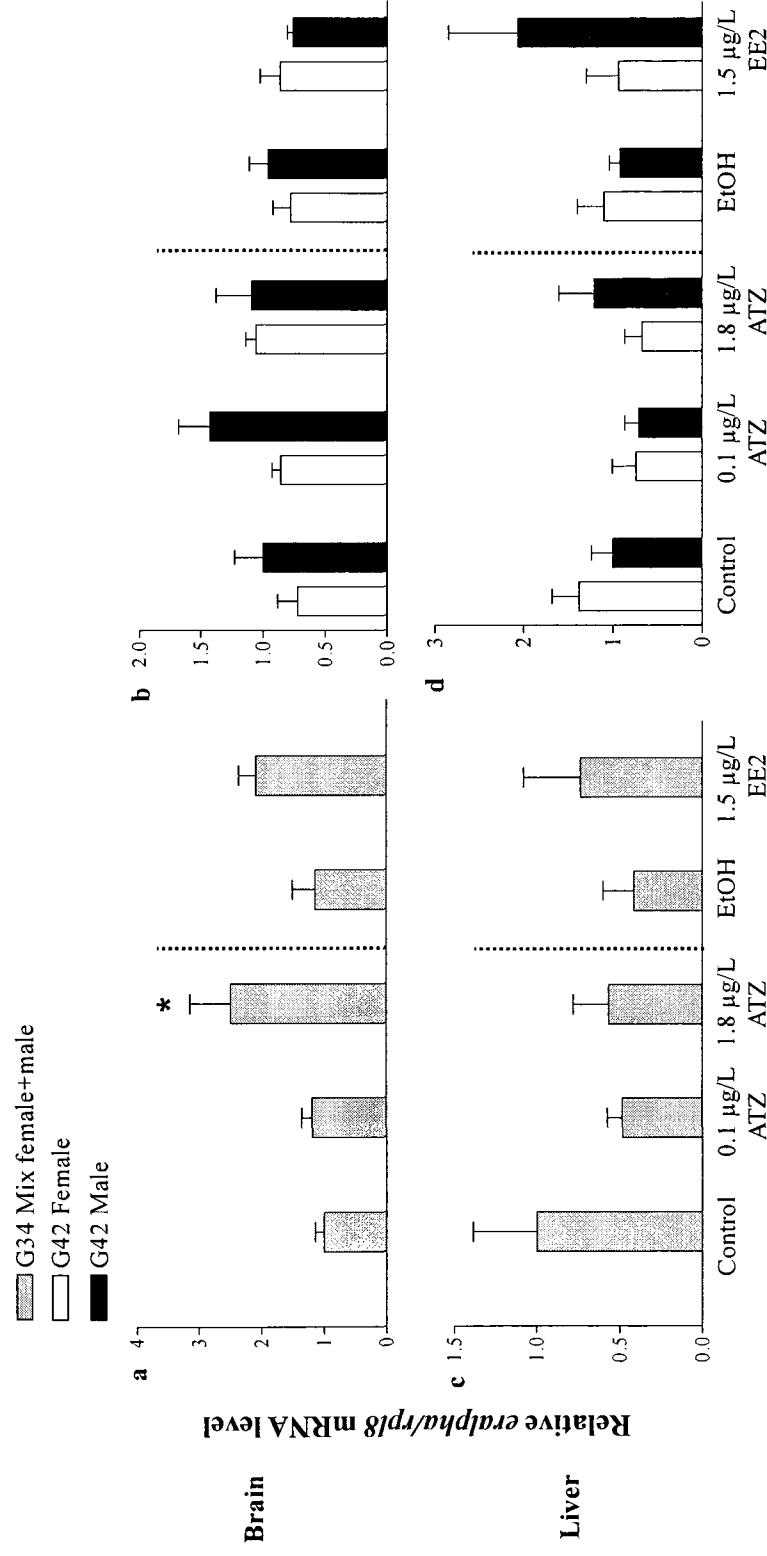


Figure 3.5 Effects of chronic ATZ and EE2 exposures on the expression of *eralpha* in the brain and liver of *Lithobates pipiens*. *Lithobates pipiens* tadpoles were exposed as described in Fig 3.1. Effects of ATZ and EE2 were determined by real-time RT-PCR on *eralpha* mRNA in the brain of G34 prometamorphic (a) and G42 metamorphic (b) tadpoles and in the liver of G34 (c) and G42 (d) tadpoles. The levels of *eralpha* mRNA are expressed relative to the water control group and are normalized to the expression of *rpl8*. Bars represent the mean $\pm$ SEM ($n = 4-8$). Data were analyzed using one-way ANOVA (G34) and two-way ANOVA (G42) followed by Bonferroni post-hoc comparisons if applicable. Asterisks (*) were used to report significance differences from the control groups, if $p \leq 0.05$. The scales of the Y-axis vary between graphs. Dashed lines separate the water and the EtOH controls. ATZ: atrazine; EE2: 17 α -ethinylestradiol; EtOH: ethanol; *eralpha*: estrogen receptor *alpha*; G: Gosner stage; *rpl8*: ribosomal protein L8.

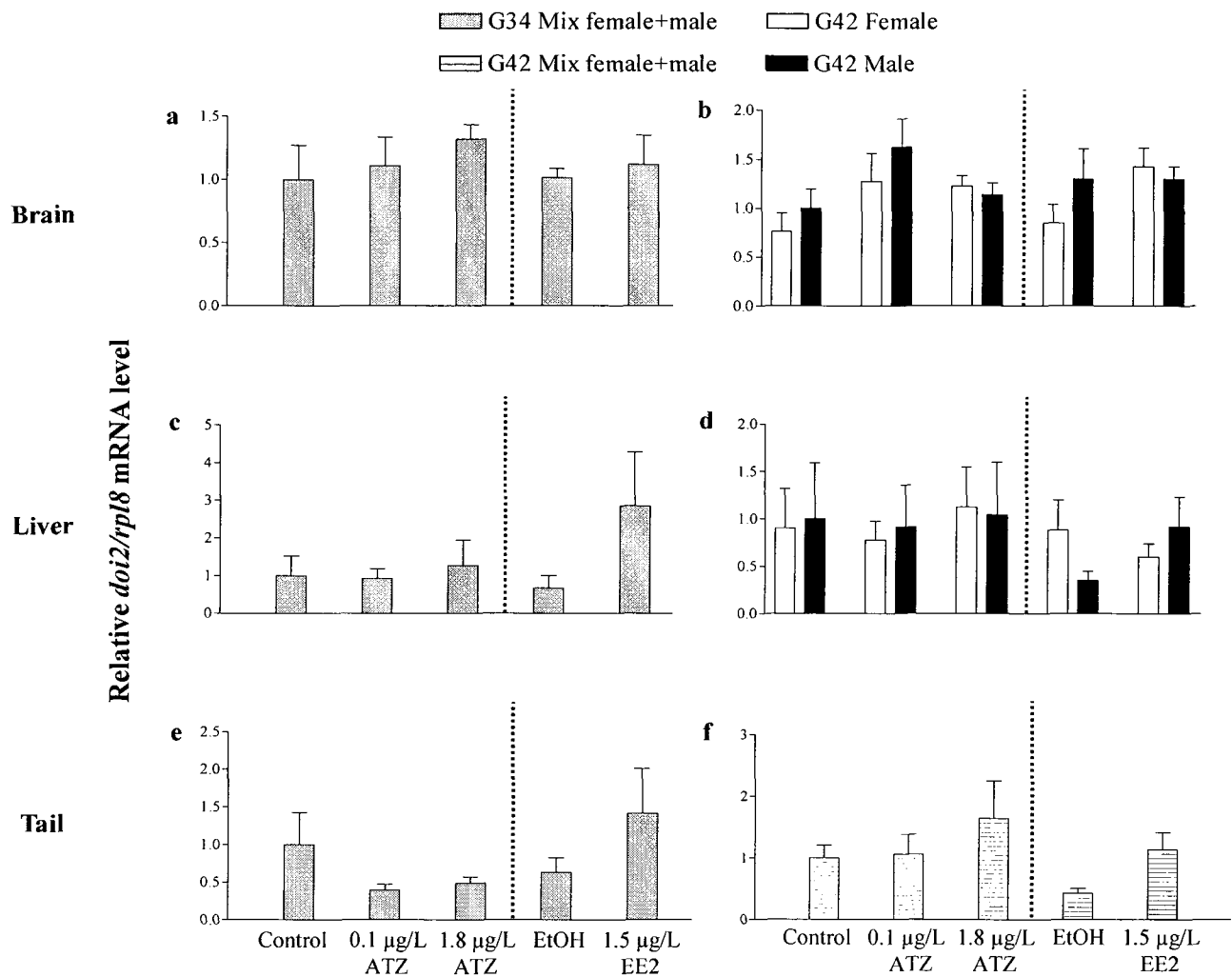


Figure 3.6 Effect of chronic ATZ and EE2 exposures on the expression of *dio2* in the brain, liver and tail of *Lithobates pipiens*. *Lithobates pipiens* tadpoles were exposed as described in Fig 3.1. Effects of ATZ and EE2 were determined by real-time RT-PCR on *dio2* mRNA in the brain of G34 prometamorphic (a) and G42 metamorphic (b) tadpoles, in the liver of G34 (c) and G42 (d) tadpoles and in the tail of G34 (e) and G42 (f) tadpoles. The levels of *dio2* mRNA are expressed relative to the water control group and are normalized to the expression of *rpl8*. Bars represent the mean $\pm$ SEM (n = 4-8). Data were analyzed using one-way ANOVA (G34) and two-way ANOVA (G42). No significant differences in any gene expression were found. The scales of the Y-axis vary between graphs. Dashed lines separate the water and the EtOH controls. ATZ: atrazine; *dio2*: deiodinase type 2; EE2: 17 α -ethinylestradiol; EtOH: ethanol; G: Gosner stage; *rpl8*: ribosomal protein L8.

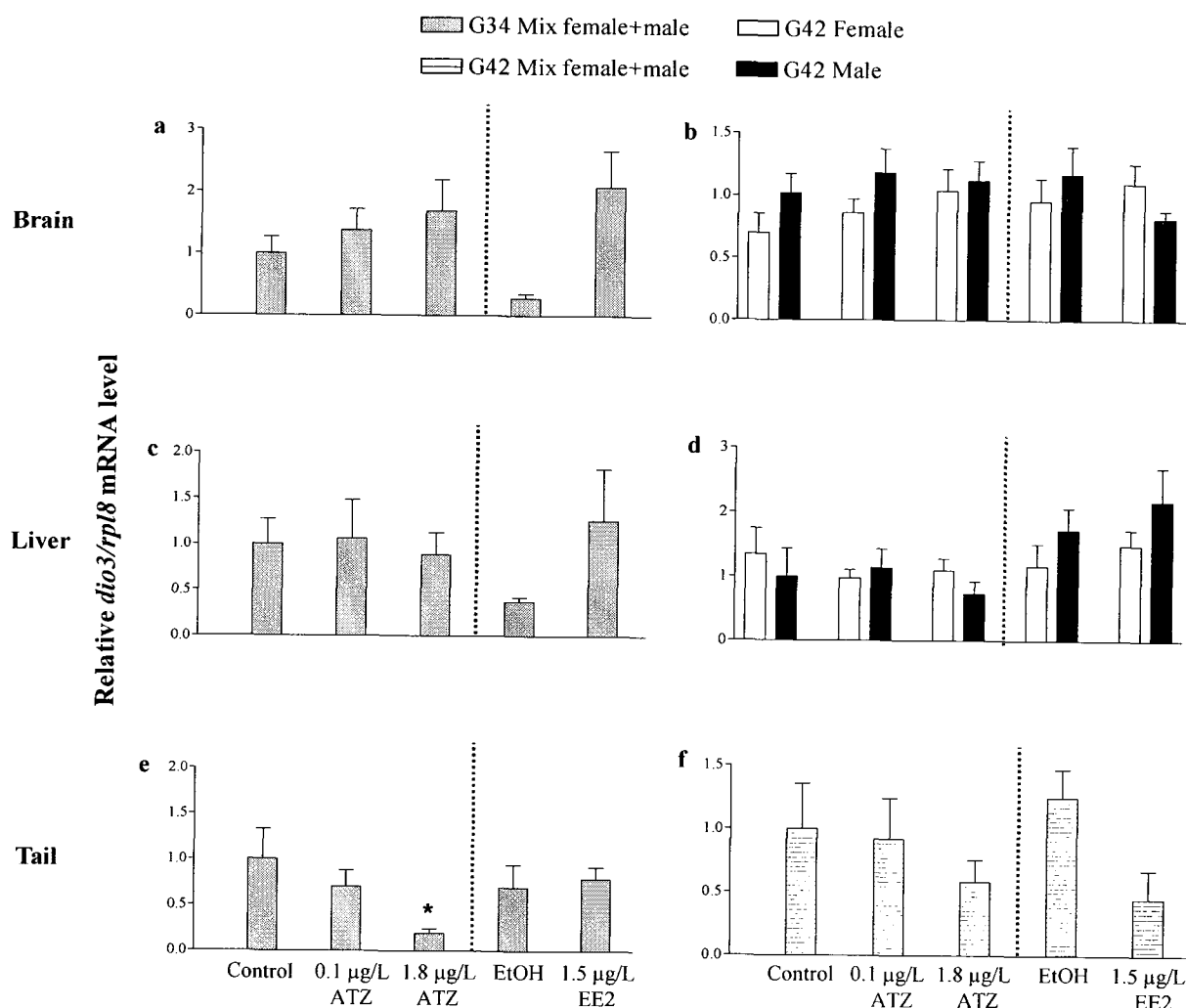


Figure 3.7 Effect of chronic ATZ and EE2 exposures on the expression of *dio3* in brain, liver and tail of *Lithobates pipiens*. *Lithobates pipiens* tadpoles were exposed as described in Fig 3.1. Effects of ATZ and EE2 were determined by real-time RT-PCR on *dio3* mRNA in the brain of G34 prometamorphic (a) and G42 metamorphic (b) tadpoles, in the liver of G34 (c) and G42 (d) tadpoles and in the tail of G34 (e) and G42 (f) tadpoles. The levels of *dio3* mRNA are expressed relative to the water control group and are normalized to the expression of *rpl8*. Bars represent the mean $\pm$ SEM (n = 4-8). Data were analyzed using one-way ANOVA (G34) and two-way ANOVA (G42) followed by Bonferroni post-hoc comparisons if applicable. Asterisks (*) were used to report significance differences from the control groups, if $p \leq 0.05$. The scales of the Y-axis vary between graphs. Dashed lines separate the water and the EtOH controls. ATZ: atrazine; *dio3*: deiodinase type 3; EE2: 17 α -ethinylestradiol; EtOH: ethanol; G: Gosner stage; *rpl8*: ribosomal protein L8.

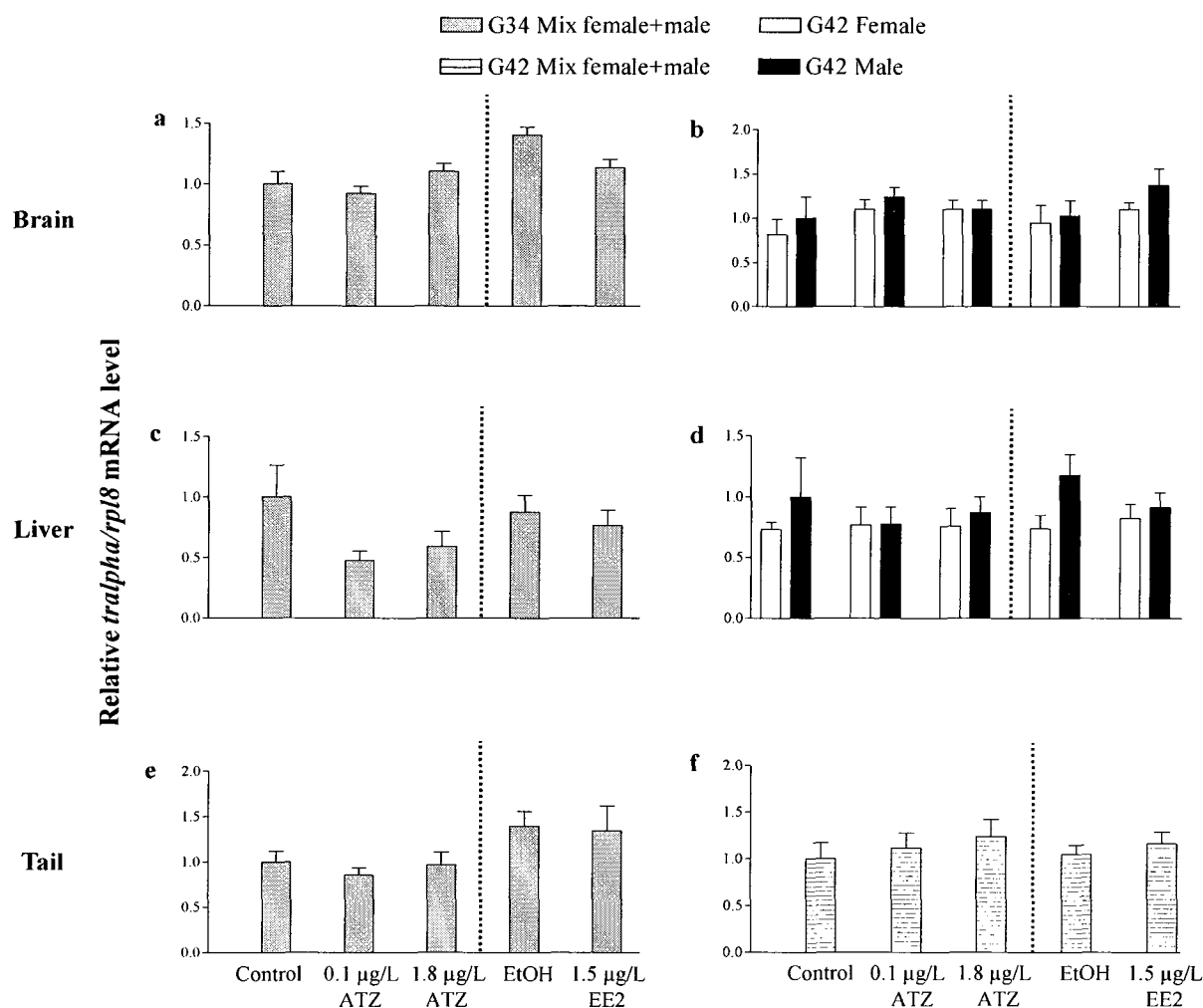


Figure 3.8 Effect of chronic ATZ and EE2 exposures on the expression of *tralpha* in the brain, liver and tail of *Lithobates pipiens*. *Lithobates pipiens* tadpoles were exposed as described in Fig 3.1. Effects of ATZ and EE2 were determined by real-time RT-PCR on *tralpha* mRNA in the brain of G34 prometamorphic (a) and G42 metamorphic (b) tadpoles, in the liver of G34 (c) and G42 (d) tadpoles and in the tail of G34 (e) and G42 (f) tadpoles. The levels of *tralpha* mRNA are expressed relative to the water control group and are normalized to the expression of *rpl8*. Bars represent the mean $\pm$ SEM (n = 4-8). Data were analyzed using one-way ANOVA (G34) and two-way ANOVA (G42). No significant differences in any gene expression were found. The scales of the Y-axis vary between graphs. Dashed lines separate the water and the EtOH controls. ATZ: atrazine; EE2: 17 α -ethinylestradiol; EtOH: ethanol; G: Gosner stage; *rpl8*: ribosomal protein L8; *tralpha*: thyroid hormone receptor alpha.

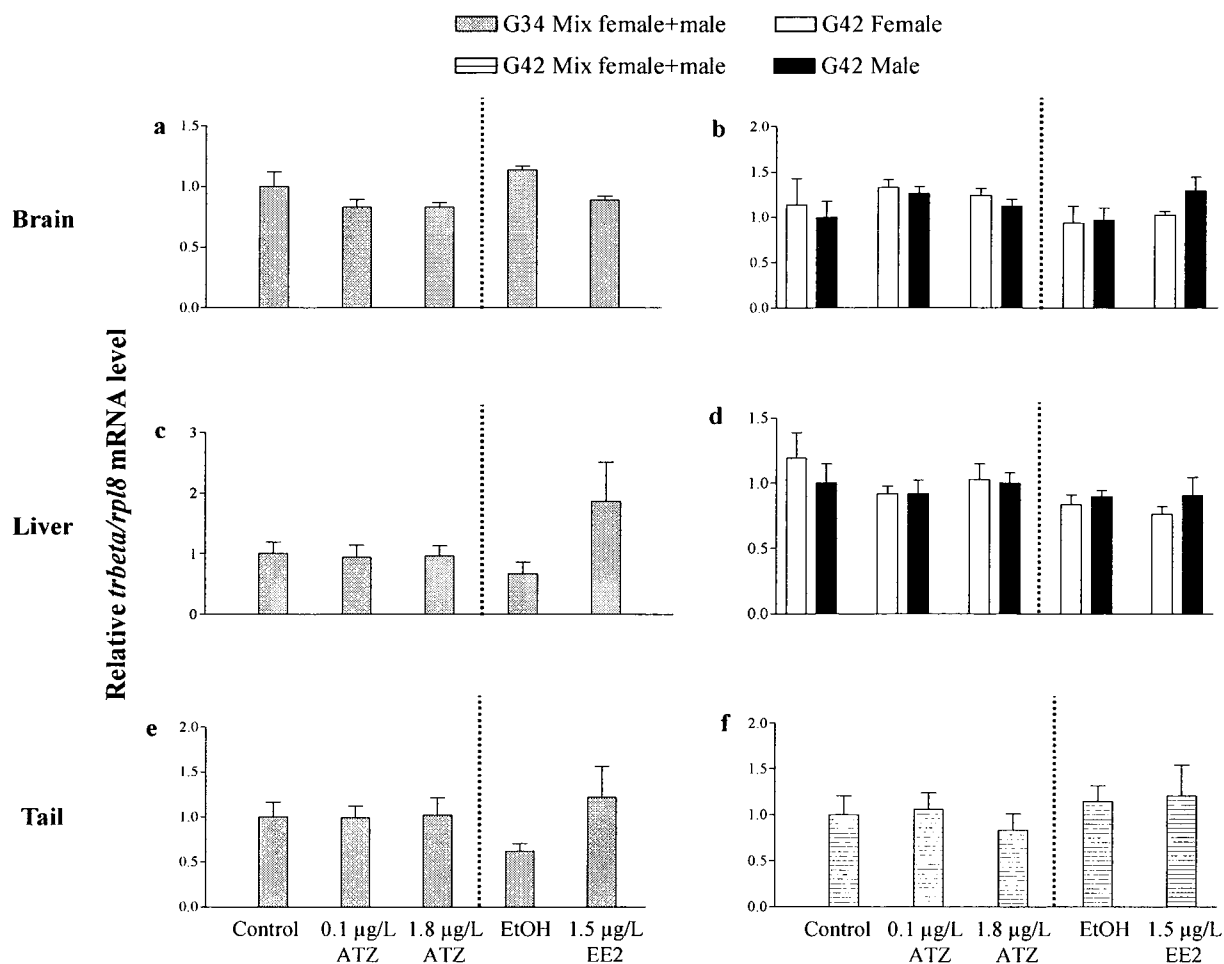


Figure 3.9 Effect of chronic ATZ and EE2 exposures on the expression of *trbeta* in the brain, liver and tail of *Lithobates pipiens*. *Lithobates pipiens* tadpoles were exposed as described in Fig 3.1. Effects of ATZ and EE2 were determined by real-time RT-PCR on *trbeta* mRNA in the brain of G34 prometamorphic (a) and G42 metamorphic (b) tadpoles, in the liver of G34 (c) and G42 (d) tadpoles and in the tail of G34 (e) and G42 (f) tadpoles. The levels of *trbeta* mRNA are expressed relative to the water control group and are normalized to the expression of *rpl8*. Bars represent the mean $\pm$ SEM (n = 4-8). Data were analyzed using one-way ANOVA (G34) and two-way ANOVA (G42). No significant differences in any gene expression were found. The scales of the Y-axis vary between graphs. Dashed lines separate the water and the EtOH controls. ATZ: atrazine; EE2: 17 α -ethinylestradiol; EtOH: ethanol; G: Gosner stage; rpl8: ribosomal protein L8; tralpha: thyroid hormone receptor beta.

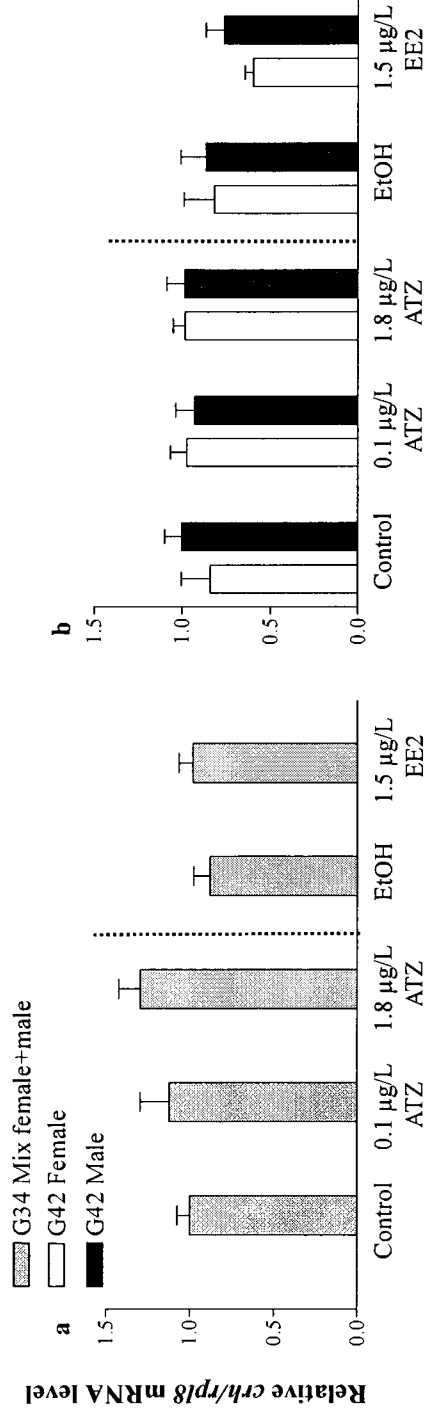


Figure 3.10 Effects of chronic ATZ and EE2 exposures on the expression of *crh* in the *Lithobates pipiens* brain. *Lithobates pipiens* tadpoles were exposed as described in Fig 3.1. Effects of ATZ and EE2 were determined by real-time RT-PCR on *crh* mRNA in the brain of G34 prometamorphic (a) and G42 metamorphic (b) tadpoles. The levels of *crh* mRNA are expressed relative to the water control group and are normalized to the expression of *rpl8*. Bars represent the mean $\pm$ SEM (n = 4-8). Data were analyzed using one-way ANOVA (G34) and two-way ANOVA (G42). No significant differences in any gene expression were found. The scales of the Y-axis vary between graphs. Dashed lines separate the water and the EtOH controls. ATZ: atrazine; crh: corticotropin-releasing hormone; EE2: 17 α -ethinylestradiol; EtOH: ethanol; G: Gosner stage; rpl8: ribosomal protein L8.

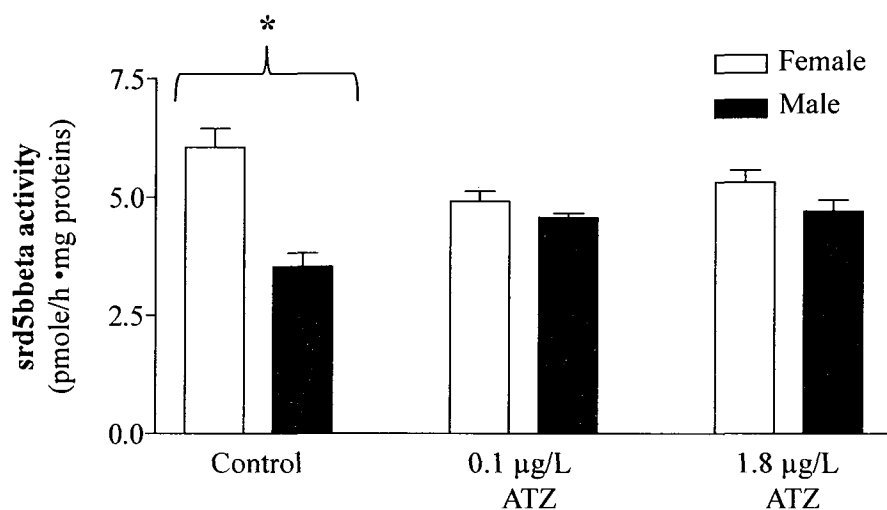


Figure 3.11 Effects of chronic ATZ exposure on srd5beta activity in the liver of *Lithobates pipiens*. *Lithobates pipiens* tadpoles were exposed as described in Fig 3.1. The activity of srd5beta was determined in G42 animals using a radiometric method and is expressed in pmole/h normalized to protein content. Bars represent the mean + SEM (n = 6). Data were analyzed using two-way ANOVA followed by Bonferroni post hoc comparisons. Significant differences are indicated by an asterisk (*) when $p \leq 0.05$. ATZ: atrazine; srd5beta: steroid 5β-reductase.

3.4 Discussion

This study demonstrates that exposure to environmentally relevant levels of ATZ in outdoor mesocosms affects both amphibian gonadal differentiation and metamorphosis. We present evidence that changes in brain *eralpha* mRNA and hepatic *srd5beta* activity may be involved in the mechanism(s) by which ATZ feminizes *L. pipiens* when tadpoles are chronically exposed to low concentrations of this herbicide. We also investigated for the first time the effects of ATZ on TH-related gene expression in amphibians and demonstrated that *dio3* mRNA level was affected by this herbicide.

Many attempts have been made to investigate the possible estrogenicity of ATZ, and this, in several vertebrate models. Atrazine failed to induce estrogen-mediated responses in the uterus of immature female Sprague-Dawley rats, in the estrogen-responsive MCF-7 human breast cancer cell line, or in the estrogen-dependant recombinant yeast strain PL3 (Connor et al. 1996). Moreover, ATZ failed to induce vitellogenin production *in vivo* in *X. laevis* liver and *in vitro* in *X. laevis* hepatocyte cultures after exposures to levels ranging from 0.1 to 100 µg/L ATZ (Oka et al. 2008). Since ovarian differentiation in amphibians is mediated by estrogens, the dominant hypothesis in the literature remains that ATZ induces *cyp19* activity (Hayes et al. 2002a; Hayes et al. 2003; Tavera-Mendoza et al. 2002a; b). There is evidence that *cyp19* activity is induced indirectly through phosphodiesterase inhibition (Roberge et al. 2004) and through binding to the steroidogenic factor 1 (Fan et al. 2007a; Fan et al. 2007b). Whether the induction of *cyp19* activity is the estrogenic mechanism of ATZ is still a matter of debate. Several studies using amphibians have refuted the *cyp19* induction

hypothesis (Coady et al. 2005; Murphy et al. 2006a; Oka et al. 2008). Our data also supports that ATZ action is not mediated via *cyp19* activity induction in the brain since no changes in *cyp19* mRNA level or *cyp19* activity were detected in the brain of *L. pipiens* tadpoles. Taken together, the *cyp19* hypothesis for the disruption of sexual development by ATZ is not consistently supported, and we therefore investigated other potential mechanisms of action.

We studied *eralpha* expression and *srd5beta* activity, which are two other endpoints that, if altered by ATZ, could produce an estrogen-like response. Our data confirms that G34 tadpoles exposed to 1.8 µg/L ATZ expressed higher *eralpha* mRNA level in the brain compared to control animals. Similar increases in *eralpha* mRNA has been reported in the brain of *L. pipiens* after EE2-treatment under laboratory conditions (Duarte et al. 2006). In our mesocosm study, EE2 also increased G34 brain *eralpha* mRNA, although this result was not statistically significant. This suggests that estrogenic compounds have the ability to increase *eralpha* mRNA level. Hogan et al. (2007) reported that expression of brain *eralpha* mRNA was similar at G30 and G36 – a period that coincides with gonadal differentiation in *L. pipiens*. Therefore, a 2.5-fold increase in *eralpha* expression at G34 following ATZ exposure could alter the sensitivity of the developing brain and lead to physiological changes. It has been demonstrated in *L. pipiens* tadpoles that the most sensitive period to feminization occurs during early development prior to G30 (Hogan et al. 2008; Richards and Georger 1978); therefore, amphibians exposed to ATZ early in development may be more sensitive to ATZ-induced feminization.

In our study, we also demonstrated that ATZ alters hepatic *srd5beta* activity in *L. pipiens* tadpoles. Recent studies in our laboratory have shown that inhibiting *srd5beta*

activity results in a female-skewed sex ratio, suggesting that this enzyme could be involved in amphibian gonadal development (Chapter 6). Hepatic sexual dimorphism in *srd5beta* activity are reported in *X. laevis* and in rats, where female expressed higher *srd5beta* activity than males (Lisboa et al. 1972; Cooke GM, unpublished data). In the current study, a similar dimorphic pattern was observed in the livers of *L. pipiens* tadpoles from the control treatment. However, after exposure to ATZ this sex difference in *srd5beta* activity disappeared. There is a lack of data regarding the importance of this sexual dimorphism, but it is likely the result of the differential androgen status between the developing males versus females. In addition to a possible role in gonadal development, *srd5beta* is also involved in other biological functions such as erythropoiesis (Garavini and Cristofori 1984) and bile biosynthesis (Kondo et al. 1994). Thus, an alteration in *srd5beta* could also lead to other physiological defects. Future studies should explore these new endpoints for ATZ action.

In amphibians, THs are essential for metamorphosis as they are involved in the remodelling of TH-target tissues such as brain, hindlimb, intestine and tail (Shi 1999). A disruption in TH production can result in important physiological defects. Numerous studies have suggested that ATZ alters TH in vertebrates (Freeman and Rayburn 2005; Kornilovskaya et al. 1996; Stoker et al. 2002). Here, we present a mechanism by which ATZ exposure affects the TH axis by disrupting TH-related gene expression in *L. pipiens*. Real-time RT-PCR results demonstrated a reduction in *dio3* mRNA in G34 tadpole tails. This decrease in mRNA level is most likely caused by a compensatory mechanism to trigger metamorphosis by reducing T3 breakdown into inactive metabolites. This indirect

mechanism suggests an imbalance in THs in ATZ-treated animals compared to control animals. Further investigations of TH levels could validate this hypothesis.

3.5 Conclusions

In this study, we developed and optimized two enzymatic assays to assess *cyp19* and *srd5beta* activities in *L. pipiens* tissues. We investigated the mechanisms of action of the herbicide ATZ on gonadal development and metamorphic disruption by measuring changes in gene expression and enzymatic activity. Our data do not support that ATZ induces *cyp19* mRNA level and activity in the frog brain; however we have observed two other physiological alterations induced by this chemical: 1) induction of *eralpha* mRNA level in prometamorphic tadpole brains - during the gonadal differentiation period and, 2) alteration of hepatic *srd5beta* activity - eliminating the natural sex dimorphism between males and females at metamorphic climax. For the latter, there is a lack of data regarding the roles of *srd5beta* and the importance of this sexual dimorphism in frogs. Since *srd5beta* is involved in similar biochemical processes as *srd5alpha*, which is involved in the production of the main androgen 5 α -dihydrotestosterone in vertebrates, future studies should compare the biological activities of these enzymes (Chapter 4) and investigate possible functions of *srd5beta* in frogs (Chapters 5 and 6).

CHAPTER 4

Evolution of the steroid 5 α -reductases and comparison of their functions with the steroid 5 β -reductase

Chapter adapted from **Langlois VS¹**, Zhang D², Cooke GM³ and VL Trudeau⁴ (2009b)

Gen Comp Endocrinol *doi: 10.1016/j.ygcen.2009.08.004*

Main contributions of each co-author:

¹Contributed to original ideas, reviewed the literature, performed phylogenetic analyses and prepared the manuscript.

²Taught phylogenetics to V.S.L. and revised the phylogenetic analyses and manuscript.

³Revised the manuscript with his expertise on the steroid 5 α -reductase.

⁴Contributed to original ideas and revised the manuscript.

4.1 Introduction

Endocrine disrupting chemicals (EDCs) can upset amphibian development and may contribute to worldwide amphibian population declines. Although many sexual developmental defects favouring the female phenotype (e.g., shift towards female biased sex ratio and presence of oocytes in male testes) have been observed after exposure to certain EDCs (Milnes et al. 2006), little is known concerning the specific mechanism underlying these effects. We have suggested that androgen synthesis enzymes, steroid 5-alpha-reductase (srd5alpha) and steroid 5-beta-reductase (srd5beta) are possible targets for EDC action in amphibians (Chapters 3, 5 and 6). Research efforts have focused on srd5alpha and srd5beta in mammalian model organisms because of their implication in numerous human diseases such as prostate carcinoma, benign prostate hyperplasia, male pattern baldness, pseudohermaphrodisism and hepatic dysfunction (Clayton et al. 1996; Kokontis and Liao 1999; Peters and Sorkin 1993; Sinclair 1998; Wilson et al. 1993); however, little is presently known about these enzymes in most non-mammalian species. The aim of this chapter is to compare and contrast the evolution, tissue distribution and functions (particularly regarding the androstane biosynthesis) of srd5alpha and srd5beta.

4.2 Materials and methods

4.2.1 Protein comparison

Protein sequences of srd5alpha1, srd5alpha2, srd5alpha3 and srd5beta were obtained by sequence searching using the National Center for Biotechnology Information (NCBI) BLAST system (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the GI numbers (notation system of NCBI) were used to report the sequences analyzed in this study. The following sequences were used for amino acid comparisons: *Homo sapiens* (human; GI:4507201, GI:39812447, GI:13375785 and GI:5174695), *Rattus norvegicus* (rat; GI:77404421, GI:12083683, GI:14149035209 and GI:20302063), *Gallus gallus* (chicken; no srd5alpha1 was found, GI:118088122, GI:11809512 and GI:11808290), *Silurana tropicalis* (Western clawed frog; GI:55742446, GI:62859365, GI:118405100 and GI:71896073) and *Danio rerio* (zebrafish; GI:115496282, GI:62955375, GI:133778743; no srd5beta was found). Total numbers and percentage of identity of amino acids were compared to the *H. sapiens* srd5alpha and srd5beta sequences. The online ClustalW2 software was used to calculate percent of homology (<http://www.ebi.ac.uk/Tools/clustalw2/>).

4.2.2 Phylogenetic analyses

From our first attempt to elucidate the evolution between srd5alpha and srd5beta, we recognized that these enzymes do not share a common evolution. Therefore, we analyzed their phylogenies separately.

4.2.2.1 *Steroid 5 α -reductases*

To determine the evolution of *srd5alpha*, we further collected *srd5alpha* sequences from many of the main eukaryote species and conducted the first phylogenetic analysis of the *srd5alpha* family. The phylogenetic analysis was performed using PSI-BLAST (<http://blast.ncbi.nlm.nih.gov/>) to retrieve *srd5alpha* homologous protein sequences in Eukaryota. The sequences used and their species abbreviation are presented in Fig. 4.1. Sequence alignment was generated by the Muscle program (Edgar 2004) and converted into a PHYLIP format using READSEQ 1.0 prior to analysis by Maximum-likelihood analyses using the PhyML program 3.0 ([http:// www.atgc-montpellier.fr/phyml/](http://www.atgc-montpellier.fr/phyml/); Guindon and Gascuel 2003; Guindon et al. 2005). The analyses used a JTT amino acid substitution model with a discrete gamma distribution and 100 bootstraps were performed to assess the significance of the phylogenetic grouping (Jones et al. 1992).

4.2.2.2 *Steroid 5 β -reductase*

While *srd5alpha* are members of the oxidoreductase superfamily (pfam ID: PF02544; Jenkins et al. 1991), *srd5beta* is a member of the aldo-keto reductase (AKR) superfamily (pfam ID: PF00248; Charbonneau and The 2001). Exhaustive phylogenetic analyses of the AKR superfamily have been published (Jez et al. 1997; Jez and Penning 2001); however, the *srd5beta* branch of the AKR phylogeny does not include non-mammalian sequences. Therefore, we enhanced the *srd5beta* phylogenetic analysis. A human *srd5beta* protein sequence was used as a query via PSI-BLAST to obtain *srd5beta*-related sequences in other mammals, birds, frogs and fish. Maximum-likelihood phylogenetic analyses were performed as described in section 4.2.2.1. The analyses used

a MtREV substitution model with a discrete gamma distribution and 500 bootstraps were performed to assess the significance of the phylogenetic grouping (Yang et al. 1998).

4.3 Results

4.3.1 Protein comparison

Although srd5alpha and srd5beta have similar functions in reducing testosterone, they have different full length amino acid sequences (Table 4.1) and they do not have significant sequence similarities (4-7 %; Table 4.2). Steroid 5 α -reductase type 3 possesses on average more amino acids (310 aa) than the two other types of srd5alpha (255 and 251 aa, respectively), but less than the srd5beta, which contains 342 aa on average. Furthermore, the first two types of srd5alpha are closer in protein similarity (46 %) than they are with the third type (20-25 %). All of the srd5alpha forms are relatively well conserved among vertebrates, with an average of 53 to 65 % identity; however, srd5beta is more conserved (79-81 %). *Silurana tropicalis* shares 51, 54, 56 and 80 % of identity with the human sequences for srd5alpha types 1, 2 and 3 and srd5beta, respectively.

4.3.2 Phylogenetic analyses

We attempted to understand the evolutionary relationships between the subtypes of srd5alpha. The srd5alpha family can be classified into three main subfamilies: the srd5alpha subfamily containing type 1 and type 2 members (srd5alpha1/2; highlighted by green and orange lines on Fig. 4.1), the srd5alpha type 3 subfamily (highlighted by pink lines) and the srd5alpha-like proteins (highlighted by red and blue lines). These srd5alpha-like proteins are named differently, for example, glycoprotein synaptic 2 (gpsn2) in fish (*D. rerio*, GI:189516842 and 220672879), frogs (*S. tropicalis*, GI: 60551245; *Xenopus laevis*, GI:48231534), rat (*R. norvegicus*, GI: 19924091) and human

(*H. sapiens*, GI: 24475816); glycoprotein synaptic 2-like (SPSN2-like) in fish (*D. rerio*, GI:123232867); trans-2,3-enoyl-CoA reductase (TER) in fish (*D. rerio*, GI:125853747); steroid 5 alpha-reductase 2-like 2 in rat (*R. norvegicus*, GI:157819815), steroid alpha reductase family member in nematoda (*Caenorhabditis elegans*, GI: 17531783); enoyl reductase or Tsc13p in yeast (*Schizosaccharomyces pombe*, GI: 19112157); 3-oxo-5-alpha-steroid 4 dehydrogenase/fatty acid elongase/trans-2-enoyl-CoA reductase in plants (*Arabidopsis thaliana*, GI:15233250). For the purpose of this chapter, we will refer to these various proteins as gpsn2 and gpsn2-like.

Besides these, other additional lineage- or species-specific duplications result in multiple copies of some members in different kingdoms (represented by bold black lines on Fig. 4.1), for example, plants (*Physcomitrella patens subsp. patens* and *A. thaliana*), fungi (*Aspergillus oryzae*), protists (*Entamoeba dispar* and *Trichomonas vaginalis*) and Animalia (e.g., *C. elegans*).

The partial srd5beta phylogenetic branch shows that mammals are closely related among themselves (98-100 % amino acid similarities) followed by birds, amphibians and fish (Fig. 4.2).

Table 4.1 Numbers of amino acids contained in full srd5alpha and srd5beta coding sequences of different vertebrates.

Species	srd5alpha1	srd5alpha2	srd5alpha3	srd5beta
<i>Homo sapiens</i>	259	254	318	326
<i>Rattus norvegicus</i>	255	254	278	326
<i>Gallus gallus</i>	n/a	255	310	448
<i>Xenopus tropicalis</i>	257	239	308	326
<i>Danio rerio</i>	265	252	309	n/a
Average	255	251	310	342

n/a: not available, srd5alpha1: steroid 5 α -reductase type 1, srd5alpha2: steroid 5 α -reductase type 2, srd5alpha3: steroid 5 α -reductase type 3, srd5beta: steroid 5 β -reductase.

Table 4.2 Percentages of amino acid similarities of different vertebrate srd5alpha and srd5beta sequences. Percentages of identity of amino acids were compared to the *H. sapiens* srd5alpha and srd5beta sequences. *Homo sapiens* sequences were further compared to each other with the compared enzyme in brackets. The online ClustalW2 software was used to calculate percent of homology.

Species	<i>Homo sapiens</i>			
	srd5alpha1	srd5alpha2	srd5alpha3	srd5beta
<i>Homo sapiens</i>	46 (srd5alpha2)	25 (srd5alpha3)	20 (srd5alpha1)	4-7 (srd5alpha1,2,3)
<i>Rattus norvegicus</i>	60	77	77	79
<i>Gallus gallus</i>	n/a	67	58	81
<i>Xenopus tropicalis</i>	51	54	56	80
<i>Danio rerio</i>	53	54	44	n/a
<i>Average</i>	53	65	61	80

n/a: not available, srd5alpha1: steroid 5 α -reductase type 1, srd5alpha1: steroid 5 α -reductase type 1, srd5alpha2: steroid 5 α -reductase type 2, srd5alpha3: steroid 5 α -reductase type 3, srd5beta: steroid 5 β -reductase.

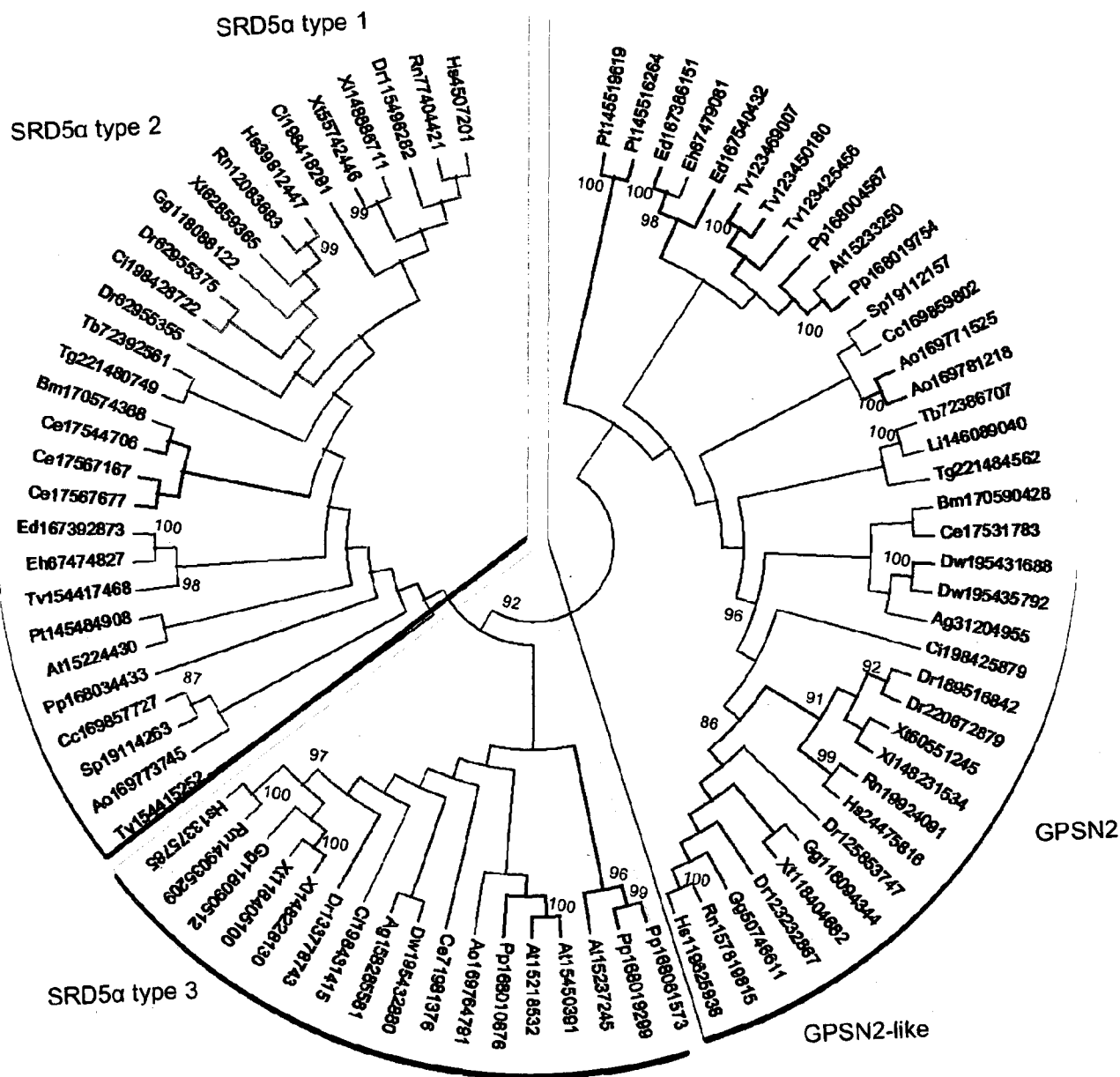


Figure 4.1 Unrooted phylogenetic tree of the *srd5alpha* family. PSI-BLAST was used to retrieve *srd5alpha* homologous sequences and analyses were performed with a JTT amino acid substitution model. The sequences are presented by its species abbreviation followed by its NCBI GI number. Support values higher than 85% are shown next to their nodes. Colored segments present gene-specific duplication and bolded segments represent species-specific duplication. Ag, *Anopheles gambiae*; Ao, *Aspergillus oryzae*; At, *Arabidopsis thaliana*; Bm, *Brugia malayi*; Cc, *Coprinopsis cinerea*; Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis*; Dr, *Danio rerio*; Dw, *Drosophila willistoni*; Ed, *Entamoeba dispar*; Eh,

Entamoeba histolytica; Gg, *Gallus gallus*; GPSN2, glycoprotein synaptic 2; Hs, *Homo sapiens*; Li, *Leishmania infantum*; Pp, *Physcomitrella patens* subsp. *Patens*; Pt, *Paramecium tetraurelia*; Rn, *Rattus norvegicus*; SRD5 α , steroid 5 α -reductase; Sp, *Schizosaccharomyces pombe*; Tb, *Trypanosoma brucei*; Tg, *Toxoplasma gondii*; Tv, *Trichomonas vaginalis*; Xl, *Xenopus laevis*; Xt, *Xenopus (Silurana) tropicalis*.

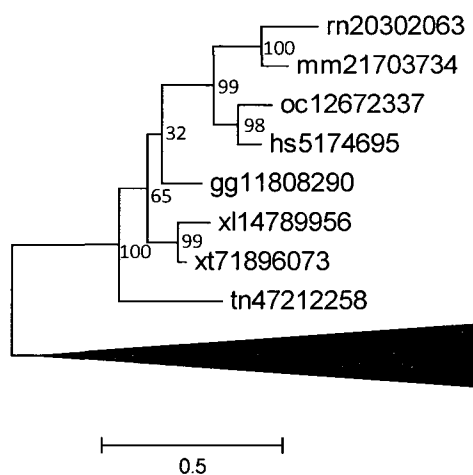


Figure 4.2 Phylogenetic branch of the *srd5beta* from the aldoketo-reductase superfamily tree. PSI-BLAST was used to retrieve *srd5beta* homologous sequences and analyses were performed with an MtREV substitution model. Support values are shown next to their nodes. The black triangle represents the rest of the aldoketo-reductase superfamily tree. The scale bar represents 0.5 amino acid substitution per amino acid site. gg, *Gallus gallus*; hs, *Homo sapiens*; mm, *Mus musculus*; oc, *Oryctolagus cuniculus*; rn, *Rattus norvegicus*; *srd5beta*, steroid 5 β -reductase; tn, *Tetraodon nigroviridis*; cl, *Xenopus laevis*; xt, *Xenopus (Silurana) tropicalis*.

4.4 Discussion

We attempted to understand the evolutionary and functional relationships between the subtypes of *srd5alpha* and the *srd5beta*. We established the first phylogenetic analysis of the *srd5alpha* family and demonstrated that three subfamilies of enzymes resulted from the evolution of a common ancestor. Furthermore, we enhanced the *srd5beta* phylogeny adding fish, frogs and chicken, and showed that *srd5beta* is highly conserved among vertebrates. Since *srd5alpha* and *srd5beta* diverge in evolution but converge in function, we further compared their gene ontology, tissue distribution and biological functions.

The present study demonstrates that the *srd5alpha* family can be classified into three main subfamilies : the *srd5alpha* subfamily containing type 1 and type 2 members (*srd5alpha1/2*), the *srd5alpha* type 3 subfamily and the *gpsn2* subfamily containing *gpsn2* and *gpsn2*-like. All primary species (from plant, amoeba, yeast, to vertebrate) in Eukaryota contain all three subfamilies, indicating that the duplication event that generated these subfamilies may have occurred in early Eukaryota.

Interestingly, our *srd5alpha* phylogeny demonstrated that plants possess a member of the *srd5alpha1/2* subfamily, the de-etiolated 2 (DET2, GI:15224430), which is involved in brassinosteroid biosynthesis by catalyzing the campesterol to campestanol conversion (Li et al. 1997; Li et al. 1996). In contrast to human *srd5alpha1* and *srd5alpha2*, which reduces the $\Delta^{4,5}$ double bond in ring A, it has been suggested that DET2 reduces $\Delta^{5,6}$ in ring B. However, studies have established that DET2 is a functional homolog of the mammalian *srd5alpha1* and *srd5alpha2* since human embryonic kidney cells overexpressing *det2* could catalyze the reduction of testosterone to 5 α -

dihydrotestosterone (5 α -DHT; a vertebrate biochemical reaction; Li et al. 1997).

Similarly, human *srd5alpha1* and *srd5alpha2* expressed in *det2* mutant plants can replace DET2 action and participate in brassinosteroid biosynthesis by converting campesterol into campestanol (a plant biochemical reaction; Li et al., 1997). However, DET2 was not inhibited by the human *srd5alpha* inhibitor, finasteride, in this study. Our phylogenetic analysis suggests that many gene duplication events occurred in plants (e.g., *A. thaliana*) indicating the existence of more than one type of *srd5alpha*-like protein. On the other hand, some plants also have a '5 β -reductase' enzyme, which like vertebrates, can catalyze the stereospecific reduction of the $\Delta^{4,5}$ double bond in ring A of several steroids (Gavidia et al. 2007). This enzyme is known as progesterone 5 β -reductase and is involved in the biosynthesis of the plant steroid cardenolide (GI: 45758665; Stuhlemmer and Kreis 1996). Cardenolide was suggested to be involved in defence mechanisms against predators such as against the larvae of *Danus plexippus* (monarch butterfly; Malcolm and Zalucki 1996; Zalucki et al. 1990). The toxic property of cardenolide is used in the treatment of heart failure in humans (reviewed in Bagrov et al. 2009) and also in the defence mechanisms of certain butterflies that eat and accumulate cardenolide to prevent predation (reviewed in Malcolm and Brower 1989). Gavidia et al. (2007) demonstrated that plant progesterone 5 β -reductase and mammalian *srd5beta* do not share structural homology and suggested an independent 5 β -reductive enzyme evolution.

Despite that the three types of *srd5alpha* are not all members of the same subfamily according to our phylogenetic analyses, some studies have demonstrated similarities between their gene oncology and protein characteristics. In humans, the C-terminal regions of *srd5alpha* are highly conserved among all types of *srd5alpha* (Uemura

et al. 2008) and some residues, such as H232, are likely responsible for the catalytic activity of these enzymes (Wang et al. 1999). It has been suggested that the N-terminal region is hydrophobic and could react with aliphatic and aromatic side chains of substrates (Wigley et al. 1994). In addition, some authors suggested that the N-terminal region and the H296 residue could have critical roles in *srd5alpha* activity since mutations at both sites abolished *srd5alpha* activity (Uemura et al. 2008). Even though the *srd5alpha* isoforms share high sequence similarity and are integral membrane proteins, some important chemical differences exist among them. Steroid 5 α -reductase type 1 has a broad pH range (6.0-8.5), while *srd5alpha2* has a sharp pH optimum (5.0-5.5; Chang 2002) and *srd5alpha3* appears to be efficient at pH 6.9 (Uemura et al. 2008). Under optimal conditions, *srd5alpha2* has a higher V_m/K_m and a 1000-fold greater affinity for steroid substrates than *srd5alpha1* (Andersson et al. 1991; Chang 2002; Normington and Russell 1992). However, no such comparisons exist yet for *srd5alpha3* because of its recent discovery (Tamura et al. 2007). Steroid 5 α -reductases also have different sensitivities to inhibitors (reviewed in Shimazaki 2002). It has been shown that *srd5alpha2* is more sensitive to finasteride than *srd5alpha1*, although both enzymes respond similarly to dutasteride; both drugs are *srd5alpha* inhibitors and normally used for prostate cancer and benign prostatic hyperplasia treatments (Shimazaki 2002). No inhibition studies have yet been performed on any mammalian *srd5alpha3* (Uemura et al. 2008).

Another ancient duplication event also generated two types of *gpsn2* subfamilies in vertebrates. While the functions of the *gpsn2* subfamily are not fully understood, several reports have shown that *gpsn2* members are involved in the fourth reaction of

fatty acid elongation by reducing a fatty chain double bond in fungi (Fig. 4.3; Han et al. 2002; Kohlwein et al. 2001; Paul et al. 2007), plants (Gable et al. 2004; Song et al. 2009) and mammals (Moon and Horton 2003). Although the substrate (fatty acid) of *gpsn2* members is structurally different from the other two *srd5alpha* subfamilies (e.g., testosterone), all three subfamilies of *srd5alpha* share a similar biochemical activity of reducing a double bond of the specific substrate. This may reflect a functional divergence of the activity of the protein families in the evolution. The inferred evolutionary history for the *srd5alpha* family is presented in Fig. 4.4. We propose that the early eukaryotic ancestor underwent duplication events to generate three subfamilies (type 1/2, type 3 and *gpsn2*). Thereafter, associated with speciation, three ancestors were duplicated into three subfamilies. At an early stage of chordate and vertebrate evolution, ancestors of *srd5alpha* type 1/2 and *gpsn2* subfamilies underwent duplication events, which resulted in type 1 and type 2 within the type 1/2 subfamily and in *gpsn2* and *gpsn2*-like subfamilies, in the *gpsn2* subfamily.

Steroid 5 β -reductase is more highly conserved among vertebrates than all the types of *srd5alpha*. Steroid 5 β -reductase is a member of the ancient superfamily AKR, which included 3 α -hydroxysteroid dehydrogenase and 17 β -hydroxysteroid dehydrogenase (Charbonneau and The 2001; Jez et al. 1997) and includes members from archaeobacteria to vertebrates (reviewed in Jez et al. 1997). The AKR members are monomeric (α/β)₈-barrel proteins, NADP(H)-dependant and share a conserved nicotinamide-cofactor-binding pocket. It has been suggested that a single amino acid change in the AKR active site may modify the catalysis from a carbonyl oxidoreduction, for example the hydroxysteroid dehydrogenase function, to a carbon-carbon double-bond

reduction, for example the *srd5beta* function (Jez et al. 1997). Steroid 5 β -reductase catalyzes double-bond reduction by transferring a hydride ion located on the NADPH 4-*pro*-R position to the 5 β -position on its steroid substrate (Berseus and Bjorkhem 1967) and adding a proton to the 4 α -position (Bjorkhem 1969). Mouse adrenal tissue characterization determined that the pH optimum of *srd5beta* was 6.5 (Collins and Cameron 1975). Steroid 5 β -reductase can catalyze the reduction of the Δ^4 group in testosterone, progesterone, 4-androstenedione, epitestosterone, 17 α -hydroxyprogesterone and 20 α -hydroxyprogesterone (Li et al. 1997; Schumacher et al. 2003). Interestingly, finasteride, which was believed to be a specific *srd5alpha* inhibitor in mammals, has recently been shown to also inhibit *srd5beta* activity *in vitro* in human recombinant cells (Drury et al. 2009). Furthermore, *in vitro* inhibition of *srd5beta* in human prostate cells that overexpress *srd5beta* supports the idea that finasteride is effective at inhibiting *srd5beta* activity (pers. comm. Dr. Van Luu-The, Université Laval). Despite a poor protein sequence similarity between the *srd5alpha* and *srd5beta*, their proteins must at least share some similarities in their substrate binding domains to have similar responses to finasteride (Chapters 5 and 6). Inhibition studies and proteomic analyses are required to help understand the difference between the three subtypes of *srd5alpha* and to elucidate any protein functional similarity between *srd5alpha* and *srd5beta* (e.g., their substrate binding domains).

Tissue distribution surveys have also established the presence of both *srd5alpha* and *srd5beta* in vertebrates. Immunoblotting analysis identified *srd5alpha* in many mammalian tissues such as prostate, seminal vesicles, epididymis, skin, liver, fat, sebaceous gland, brain, scalp and hair follicles (Killinger et al. 1990; Normington and

Russell 1992; Olsen et al. 2005; Thigpen et al. 1993; Vallarino et al. 2005). In non-mammalian species, *srd5alpha* activity has been mostly reported in gonad, brain and liver tissues (Balthazart 1991; Lisboa and Breuer 1966; Pasmanik and Callard 1985; 1988; Soma et al. 1999). The activity of *srd5alpha1* is mainly highest in non-androgen target tissues (e.g., skin) whereas *srd5alpha2* is mainly associated with androgen target tissues (e.g., prostate and gonads; Normington and Russell 1992; Thigpen et al. 1993). In contrast, *srd5alpha3* has only been identified in human prostate cancer cell lines (Uemura et al. 2008). The tissue distribution of *srd5beta* is broad since it has been detected in the gonad, brain and liver tissues of many vertebrates (e.g., fish, frogs, birds and mammals; Hutchison and Steimer 1981; Lisboa et al. 1974; Sugimoto et al. 1990; Terada et al. 1980; Yeung and Chan 1985).

The wide and co-localized distributions of *srd5alpha* and *srd5beta* among vertebrate tissues suggest some similarity or relationship in biological function. Androstane biosynthesis is one of the main functions exerted by *srd5alpha* and *srd5beta*. A stereospecific hydrogenation of the testosterone double bond at C-4 to C-5 by either *srd5alpha* or *srd5beta* yields asymmetric structures that are 5 α -DHT and 5 β -dihydrotestosterone (5 β -DHT), respectively. Both reduced steroids are expected to show differential chemical properties and binding affinities due to their stereospecific configuration; 5 α -DHT has a “chair” configuration whereas the 5 β -DHT has a “boat” configuration.

The steroid 5 α -DHT is known to be the dominant androgen in many vertebrates and its functions are mostly associated with development and regulation of male secondary characteristics (Russell and Wilson 1994). Mutations in *srd5alpha2* during

human embryogenesis result in abnormal external genitalia and prostate development (Griffin and Wilson 1989). However, in another study, some *srd5alpha2* deficient males still exhibited a slight virilisation that was explained by peripheral *srd5alpha1* activity (Thigpen et al. 1993) and possibly *srd5alpha3* activity. In amphibians, 5 α -DHT also acts as a potent androgen, since exposures of *Rana clamitans* (green frog) eggs until metamorphosis resulted in a 97.7 % male phenotype (Coady et al. 2004). Therefore, *srd5alpha* activity is necessary for 5 α -DHT formation, which leads to normal male development and function in many species.

In marked contrast, the reproductive functions of *srd5beta* are largely unexplored because of the long held view that 5 β -DHT does not have androgenic effects (Davies et al. 1980; Kokontis and Liao 1999; Massa et al. 1980; Mori et al. 1974). However, there are numerous reports that indicate reproductive functions of *srd5beta* and 5 β -DHT. For example, sexual behaviour studies indicate important roles of *srd5beta*, including the regulation of steroid production by testosterone inactivation and/or direct inhibition of aromatase (*cyp19*) by 5 β -reduced metabolites of testosterone in the brain of birds (Balthazart et al. 1981; Hutchison and Steimer 1981; Steimer and Hutchison 1981). Hutchison and Steimer (1981) suggested that inactivation of testosterone by conversion to 5 β -DHT by *srd5beta* regulates brain sensitivity to androgens under changing hormonal conditions. Long-term castrated doves showed increased levels of 5 β -DHT in the preoptic area (androgen sensitive) compared to intact males (Steimer and Hutchison 1981), suggesting that 5 β -reduction regulates low androgen levels during non-breeding seasons. The 5 β -DHT has also been shown to exhibit direct biological activities. Exposure to 5 β -DHT inhibited mammary development in mice (Yanai et al. 1977) and induced

vasorelaxation in the thoracic aorta (Montano et al. 2008) and vas deferens (Lafayette et al. 2008) in rats. Even though 5 β -DHT does not directly affect male secondary sexual development (compared to 5 α -DHT), 5 β -DHT can induce other androgen-related functions and 5 β -reduction can regulate testosterone availability.

In addition to participation in reproductive functions, both *srd5alpha* and *srd5beta* are involved in many other biological processes. Both 5 α -androstanes and 5 β -androstanes are involved in erythropoiesis in mammals (Gordon et al. 1970), birds (Irving et al. 1976) and amphibians (Garavini and Cristofori 1984). The 5 α -androstanes increase the production of the erythropoietin hormone in the kidneys whereas 5 β -androstanes stimulate heme production in the liver (Kokontis and Liao 1999). The 5 β -reduced steroids have a stronger erythropoietin-inducing activity than the 5 α -reduced metabolites in *Triturus cristatus camifex* (crested newt; Garavini and Cristofori 1984). Furthermore, both *srd5alpha* and *srd5beta* are involved in bile biosynthesis, where they catalyze the conversion of 7 α ,12 α -dihydroxy-4-cholesten-3-one into 7 α ,12 α -dihydroxy-5 α -cholestan-3-one and 7 α ,12 α -dihydroxy-5 β -cholestan-3-one, respectively. However, only 7 α ,12 α -dihydroxy-5 β -cholestan-3-one has been shown to be biologically active and is further reduced into bile. The 5 α -reduction is suggested to be an inactivation pathway for bile biosynthesis regulation in humans (Kondo et al. 1994). While speculative, another common function could be proposed for *srd5alpha* and *srd5beta* because they can both regulate biosynthesis by inactivating substrates. For example, *srd5alpha* can inactivate bile biosynthesis and *srd5beta* can inactivate 5 α -DHT biosynthesis.

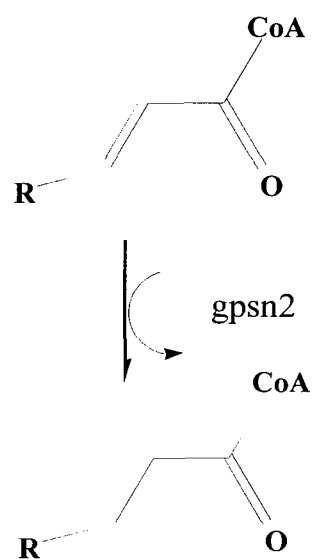


Figure 4.3 The fourth reaction of the fatty acid chain elongation reaction occurring in plants and animals and requiring the action of gpsn2. gpsn2: glycoprotein synaptic 2.

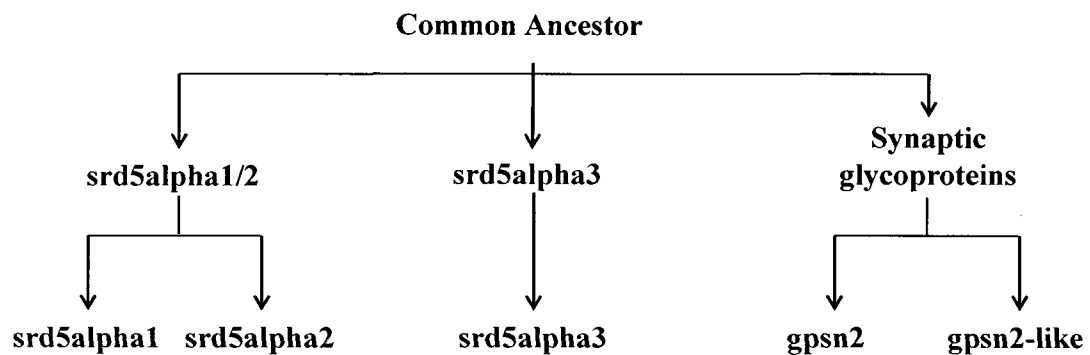


Figure 4.4 Schematic of the *srd5alpha* family phylogeny. This schematic is based on the proposed *srd5alpha* phylogenetic analysis. *srd5alpha*: steroid 5 α -reductase; *gpsn2*: glycoprotein synaptic 2.

4.5 Conclusions

We established the first phylogenetic analysis of all subtypes of *srd5alpha*, where we identified major gene duplication events. Steroid-5-reductases have highly conserved biochemical functions within the plant and animal kingdoms, enhancing the importance of fully understanding their physiological roles. Even though *srd5alpha* and *srd5beta* do not possess a common ancestor, they do share similar biochemical and biological functions. This chapter helps elucidate the unique and overlapping roles that *srd5alpha* and *srd5beta* play, especially in steroid hormone metabolism. Future studies should investigate if the enhancement of *srd5beta* activity and subsequent alteration of androgen dynamics could help in the treatment of androgen-related diseases such as prostate cancer and benign prostate hyperplasia. Furthermore, since both androgenic and anti-androgenic EDCs are found in the aquatic environment (Jenkins et al. 2003; Urbatzka et al. 2007b) and given the physiological roles of both *srd5alpha* and *srd5beta* in androgen dynamics and sexual development among other functions, it will also be important to characterize the consequences of their disruption (Chapters 5 and 6).

CHAPTER 5

Finasteride exposure modulates sex steroid- and thyroid hormone-related gene expression in *Silurana tropicalis* early larval development

Chapter adapted from **Langlois VS¹**, Duarte-Guterman P², Ing S³, Pauli BD⁴, Cooke GM⁵
and VL Trudeau⁶ (2010a) General and Comparative Endocrinology 166:417-427.

Main contributions of each co-author:

¹Contributed to original ideas, performed animal exposure, real-time RT-PCR assay optimization, aromatase activity assay and 5 β -reductase activity assay optimizations, data analysis and manuscript preparation.

²Performed a parallel study using the cyp19 inhibitor fadrozole for the manuscript.

³Involved in real-time RT-PCR analysis and animal care.

⁴Revised the manuscript using his expertise in amphibian toxicology.

⁵ Revised the manuscript with his expertise on the steroid 5 α -reductase.

⁶Contributed to original ideas and revised the manuscript.

5.1 Introduction

In metamorphic and post-metamorphic frogs, endocrine disrupting chemicals (EDCs) such as pesticides and pharmaceuticals can affect normal gonadal development (Atrazine, Chapter 3; Methoxychlor, Fort et al. 2004; Fadrozole, Olmstead et al. 2009) and alter steroidogenesis (tributyltin, Ahn et al. 2007; paraquat, Quassinti et al. 2009). In vertebrates, sex steroid hormones are detected before gonadal differentiation begins *i.e.* during embryogenesis (in frogs, Bogi et al. 2002; birds, Carere and Balthazart 2007; Goldman-Johnson et al. 2008; mammals, Iwamatsu et al. 2005). Although the functions of sex steroids during embryogenesis remain unclear, it is unknown if EDCs also could affect steroidogenesis during early development.

In vertebrates, steroidogenesis includes the reduction of testosterone to either 5 α -dihydrotestosterone (5 α -DHT) by steroid 5 α -reductases (srd5alpha type 1, 2 and 3) or to 5 β -dihydrotestosterone (5 β -DHT) by steroid 5 β -reductase (srd5beta; Hutchison and Steimer 1984). The steroid 5 α -DHT is a potent androgen most often associated with male sexual development (Russell and Wilson 1994). In humans, disruption of srd5alpha results in pseudohermaphroditism, prostate cancer, polycystic ovarian syndrome and hirsutism (Andersson et al. 1991; Goodarzi et al. 2006; Thomas et al. 2009). Since it has been long believed that 5 β -DHT is biologically inactive (Kokontis and Liao 1999), the reproductive and developmental functions of srd5beta remain largely unexplored; however, several reports suggest that this hormone is biologically active (reviewed in Chapter 4). For example, studies on sexual behaviour indicate important roles of srd5beta, including the regulation of steroid production by testosterone inactivation and/or the direct inhibition of aromatase (cyp19) by 5 β -reduced metabolites of testosterone in the brain of birds

(Hutchison and Steimer 1981; Steimer and Hutchison 1981). Therefore, *srd5alpha* and *srd5beta* are important for vertebrate biological functions and may be potential targets of EDCs.

It is well-established that finasteride is a *srd5alpha2* inhibitor in humans, and it has also been shown to inhibit *srd5alpha1* in humans (Azzolina et al. 1997). Finasteride may also be useful as an inhibitor of *srd5beta* activity since it has been shown to inhibit *srd5beta* activity *in vitro* in human recombinant cells (Drury et al. 2009). This inhibitor was used in this study as a model compound for putative 5α -reductase and 5β -reductase disruptions. However, one of the consequences of finasteride exposure in humans is the accumulation of plasma testosterone (Habib et al. 1997; Roehrborn et al. 2003). Therefore, we also assessed the effects of finasteride on *cyp19* activity since testosterone is also the main steroid substrate for aromatization by *cyp19* into 17β -estradiol.

Thyroid hormones (THs) may also interact with the reproductive axis during amphibian gonadal differentiation since these hormones can regulate the expression of sex steroid-related genes (Duarte-Guterman et al. 2010; Hogan et al. 2007). In *Silurana tropicalis* (Western clawed frog), larvae exposed to triiodothyronine (T3) exhibited a concentration-response increase of *srd5alpha1* and androgen receptor (*ar*) mRNA levels (Duarte-Guterman et al. 2010). Short term exposure of Gosner stage 34 *Lithobates pipiens* (Northern leopard frog) tadpoles to T3 also resulted in an induction of estrogen receptor α (*eralpha*) mRNA and a decrease in *cyp19* mRNA levels (Hogan et al. 2007). Furthermore, chronic inhibition of TH synthesis produces female biased sex ratios in *Xenopus laevis* (African clawed frog; Goleman et al. 2002a). Since some TH-related genes such as TH receptors (*tralpha* and *trbeta*) and deiodinases (*dio1*, *dio2* and *dio3*) are expressed and are functional during amphibian embryogenesis (Havis et al. 2006; Morvan Dubois et al.

2006), an interaction between the TH and reproductive axes may also occur in early amphibian development. For all these reasons, we also measured if a disruption in frog steroidogenesis could alter TH related-gene expression.

We hypothesized that *srd5alpha* and *srd5beta* are present during early anuran development and their inhibition will affect the transcription of sex steroid- and TH-related genes. The objectives of this study were 1) to establish the ontogenic expression of *srd5alpha* (type 1,2 and 3) and *srd5beta* during embryogenesis, 2) to investigate the effects of inhibiting *srd5alpha* and *srd5beta* on sex steroid- and TH-related gene expression; and, 3) to assess the effects of finasteride on *cyp19* activity. The amphibian model *S. tropicalis* was selected because of the ease of studying embryonic development with this species and for its available genomic sequence information (Amaya et al. 1998; Hirsch et al. 2002).

5.2 Materials and Methods

5.2.1. *Breeding and maintenance of Silurana tropicalis*

Frogs were reared in dechlorinated and aerated water from the University of Ottawa Animal Care facility (Ontario, Canada). Fertilized eggs were obtained from eight pairs of frogs by injecting human chorionic gonadotropin hormone (hCG; Sigma) into the dorsal lymph sac of adult *S. tropicalis*. Both males and females received a priming injection of 12.5 IU hCG followed by a boosting injection of 100 IU hCG after 20 h. Staging was determined by following the Nieuwkoop and Faber developmental table (NF; Nieuwkoop and Faber 1994). A 12:12h light:dark cycle was maintained with the light cycle occurring from 7 am to 7 pm at 24 °C ± 1 °C. 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.

5.2.2. *Tissue collection for developmental profiles*

Eggs and larvae were raised in Petri dishes containing modified Ringer's solution (MRS: 0.1 M NaCl, 1.8 mM KCl, 2.0 mM CaCl₂, 1.0 mM MgCl₂, 300 mg/L NaHCO₃; 1:9 v/v) and 0.04 mg/L of the antibiotic gentamycin (Sandoz Canada Inc.). Embryos (NF2 to NF34) and larvae (NF41 and NF46) were pooled to ensure a sufficient amount of RNA could be extracted for gene expression analysis (20 and 25 animals per pool, respectively; n = 6-8 pools) and protein for cyp19 (40 and 50 animals per pool, respectively; n = 7-8 pools) and srd5beta activity analysis (20 and 50 animals per pool, respectively; n = 6 pools). For gene expression profiles, whole embryos and larvae were sampled at NF2, 7, 16, 21, 26-27,

34, 41 and 46 under our husbandry conditions (Fig. 5.1 a). Embryos were frozen immediately on dry ice and stored at -80 °C. Stage NF41 and NF46 larvae were anaesthetized by immersion in 3-aminobenzoic acid ethyl ester (MS-222; 0.01 %; Sigma) and stored at -80 °C. For enzyme activity analyses, whole embryos and larvae were also collected at NF7, 21, 34, and 46 (Fig. 5.1 b). Embryos were frozen immediately on dry ice and stored at -80 °C.

5.2.3. *Finasteride exposure*

Eggs were dejellied at NF6 with 2 % L-cysteine (pH 8.0; Sigma) and washed three times with MRS (1:9). Eggs were placed in Petri dishes in MRS (1:9) containing 0.04 mg/L of gentamycin at a density of 50 eggs per dish. Embryos were exposed from NF12 to NF46 to one of four treatments: 0.05 % ethanol (EtOH; control of the experiment) and 25, 50 and 100 µM finasteride (equivalent to 9, 18 and 36 mg/L; Sigma Canada Ltd, Oakville, ON; Fig. 5.1 c) delivered in 0.05 % ethanol. Throughout the exposure, the medium and antibiotic were refreshed daily. Whole NF46 larvae were sampled from each treatment for gene expression (10 animals per pool; n = 5-8 pools), cyp19 activity (40 animals per pool; n = 5 pools) and srd5beta activity analyses (20 animals per pool; n = 5-6 pools).

5.2.4 *RNA isolation and cDNA synthesis*

Homogenization of samples was achieved using an MM301 Mixer Mill (Retsch, Newton, PA, USA) at 20 Hz for 4 min. For the developmental profile samples, total RNA was obtained using the QIAGEN RNeasy Micro Kit (including the RNase-free DNase treatment) whereas RNA was extracted from the finasteride exposure samples using the QIAGEN RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). Isolated RNA was

resuspended in RNase free water and stored at -80 °C. Concentrations of RNA were determined using the NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc.). Total cDNA was prepared from 2 µg total RNA, 0.2 µg random hexamer primers (Invitrogen) and Superscript II RNase H- reverse transcriptase (Invitrogen).

5.2.5 *Real-time RT-PCR*

Real-time RT-PCR simplex (SYBR Green detection) and multiplex assays (dual labelled fluorescent probes) were used to detect the expression of target genes (details on primer sets can be found in Langlois et al. 2010a). In summary, detection of transcripts for *cyp19* (coding sequence accession no. FJ644565), *srd5alpha1* (accession no. BC076920), *srd5alpha2* (accession no. NM_001017113), *ar* (androgen receptor; expressed sequence tag accession number DT407367), *tralpha* (accession no. AB244213), *trbeta* (accession no. AB244214), *dio1* (expressed sequence tag accession no. DT424550), *dio2* (accession no. EF052283), *dio3* (accession no. NM_001113667) and ribosomal protein L8 (*rpl8*; accession no. BC059744) was performed.

Primers for *srd5alpha3* (accession no. NM_001079071.1) and *srd5beta* (accession no. NM_001030438.1) were designed using Primer 3 (<http://fokker.wi.mit.edu/primer3/input.htm>) and Oligoanalyzer 3.1 (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>) and optimized in simplex. Specificity of every primer set was confirmed by cloning and sequencing the single amplicon obtained (section 5.2.6). For all real-time RT-PCR assays, primer concentrations were optimized to obtain a minimum threshold cycle and a maximum change in fluorescence (Table 5.1).

The simplex reaction and thermocycle program are described in Chapter 3 (section 3.2.5). A multiplex real-time RT-PCR assay was used to measure transcript levels of

estrogen receptors (*eralpha*, *erbeta*), *cyp19* and *rpl8* as described in Langlois et al. (2010a).

The multiplex reaction and thermocycle program used followed the triplex protocol described in Chapter 3 (section 3.2.5).

In every real-time RT-PCR assay, samples were run in duplicate along with two negative controls (no template and no reverse transcriptase controls, respectively). Primer and probe concentrations were optimized to yield an efficiency of $100\% \pm 10\%$ and an $r^2 > 0.985$. The relative standard curve method was used to interpolate relative mRNA abundance of target and reference genes within each sample. For the developmental profiles, the standard curves were generated using a serial dilution of NF46 cDNA mix since all target genes were expressed at that stage. For the finasteride exposure, equal parts cDNA from each treatment were used to produce a standard curve. Several housekeeping genes were tested for the developmental profiles of *S. tropicalis* in our laboratory (e.g., *rpl8*, β -actin, elongation factor 1 α and ornithine decarboxylase) but all tested genes changed significantly during development (for *rpl8* data, refer to Fig. 5.2; for the other gene data, refer to Duarte-Guterman et al. 2010). Therefore, the developmental profile data are normalized to RNA content as recommended by Sindelka et al. (2006). This consists of dividing the gene expression data obtained from the real-time RT-PCR analysis by 2 μ g of RNA used in the cDNA synthesis reaction. Data are presented as fold-change relative to NF2 for comparison purposes. For the finasteride data, the expression of *rpl8* did not change with treatments; therefore it was used as the endogenous control gene. Finasteride exposure data are expressed as fold-change relative to the EtOH control.

5.2.6. *Primer validation*

To validate the specificity of *srd5alpha3* and *srd5beta* primers, their PCR amplicons were cloned and sequenced. The 25 µL-PCR mix contained 1.0X PCR buffer (Invitrogen), 1.5 mM MgCl₂ (Invitrogen), 0.2 mM dNTPs (Invitrogen), 0.4 mM forward and reverse primers (Invitrogen), 0.02U Taq DNA polymerase (Invitrogen) and 1 µL cDNA template. Gene amplification was performed using a Mastercycler gradient Thermal Cycler (Eppendorf, Westbury, NY) with the following thermocycle program: 94 °C (3 min), 40 cycles of 94 °C (45 s), 50 °C (45 s), 72 °C (1 min), and finally 72 °C (10 min) and kept at 4 °C. Samples were run on a 1 % agarose gel, amplification products were excised at the expected sizes (90 bp for *srd5alpha3* and 108 bp for *srd5beta*) and purified using the QIAQuick Gel Extraction kit (Qiagen). Gel extracts were ligated and transformed using the 2.1 TOPO cloning vector kit (Invitrogen). Cells were mixed with ampicillin and X-gal, spread on Luria-Bertani (LB) plates and incubated at 37 °C overnight. Half of each white colonies were used as PCR templates with M13 primers (specific for the 2.1 TOPO cloning vector; Invitrogen) to validate insert size. When validated by PCR, the other half of the positive colonies were added to a mix of LB broth and ampicillin and incubated at 37 °C overnight. Plasmids were purified using the Purelink Quick Plasmid Miniprep kit (Invitrogen) and sequenced by the Centre of Advanced Research in Environmental Genomics (University of Ottawa, ON). Both sets of primers amplified the appropriate genes.

5.2.8. *Enzymatic activity assays*

The activity of cyp19 was measured using the modified radiometric method described in Chapter 3 (section 3.2.6). Briefly, cofactor and ³H-androstendione were

incubated for 30 min at 37 °C. After this pre-incubation, tissue was sonicated in phosphate buffer and incubated for 80 min at 25 °C. Tritiated water was extracted with a charcoal solution and radioactivity was counted. The activity of cyp19 is expressed as fmol $^3\text{H}_2\text{O}/\text{h}\cdot\text{mg}$ protein. Total protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad Co.). Once it was determined that the assay could detect cyp19 activity using *S. tropicalis* tissue, the assay was further validated by incubating *S. tropicalis* brains (pool of 14 brains) with a range of concentrations of fadrozole (a cyp19 inhibitor; 0, 0.5, 1.0, 1.5, 2.0 and 2.5 μM ; Fig. 5.3). The activity of cyp19 was detected in *S. tropicalis* tissue and fadrozole inhibited the enzyme activity in a concentration-dependent manner. Using the validated assay, cyp19 activity was measured at NF46 after finasteride exposure (each pool ranged from 20 to 40 mg; Fig. 5.1 c). Furthermore, different concentrations of finasteride (25, 50 and 100 μM) were also tested *in vitro* on a homogenate of NF46 *S. tropicalis* whole tadpoles (a pool of 240 tadpoles) to determine cyp19 activity.

The activity of srd5beta was measured using the radiometric method optimized in Chapter 3 (section 3.2.7). Briefly, the srd5beta activity was determined by the conversion of testosterone into 5 β -DHT and 5 β -androstane-3 β -17 β -diol (5 β -3 β -diol). Pools of 20 individuals (n = 6 pools per stage or treatment; each pool ranged from 50 to 70 mg) were sonicated in Tris-buffer and incubated at 37 °C with 4- ^{14}C -testosterone. Steroids were extracted by evaporation until dryness and resuspended in chloroform-methanol. Steroid metabolites were extracted using a two-dimensional thin layer chromatography system and analyzed with the Image Quant TL 3.0 software (Amersham Bioscience). The activity of srd5beta is expressed as the sum of 5 β -DHT and 5 β -3 β -diol /h/mg protein. Total protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad Co.). To validate this method in *S. tropicalis* tissue and to determine the concentration to use for the *in vivo*

exposure, an *in vitro* concentration-response assay was performed using 0, 100 and 200 μM of finasteride on NF46 tadpoles (Fig. 5.4). For this, a homogenate of eight NF46 *S. tropicalis* larvae was used to assess srd5beta activity for each finasteride concentration. Results demonstrated that srd5beta activity was detected in *S. tropicalis* tissue and finasteride inhibited > 90 % of srd5beta activity in pool of whole NF46 larvae. Thereafter, the activity of srd5beta was measured in embryos and larvae (from NF7 to NF46; Fig. 5.1 b) to establish the developmental profile and in NF46 livers after the finasteride exposure (Fig. 5.1 c).

5.2.9. Data analysis

Gene expression data analysis was performed as described in Chapter 3 (section 3.2.8). Statistical analyses were performed using S-Plus 8.0 (Insightful Corp. Seattle, Washington, USA). Data were tested for normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test). One-way analysis of variance (one-way ANOVA) was used to analyze the results. If results were significant ($p \leq 0.05$), the Bonferroni post-hoc test was used to evaluate significant differences between groups. When transformed data failed to meet assumptions the nonparametric Kruskal-Wallis test on ranks was used, in which case the Mann-Whitney U test was performed to detect differences between pairs of stages or treatment groups.

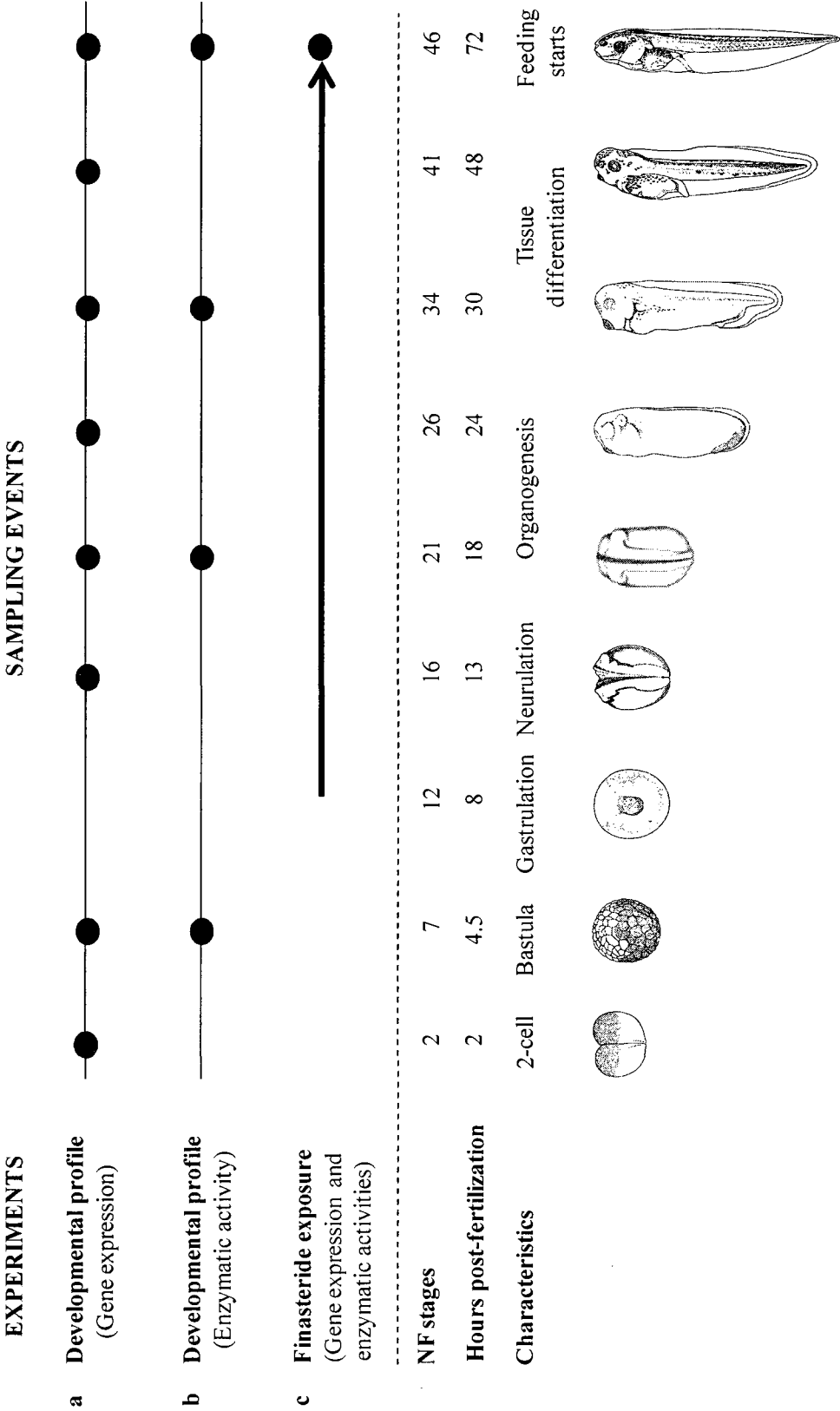


Figure 5.1 Experimental design for developmental profiles and finasteride exposure during early tadpole development. To generate a developmental profile for *Silurana tropicalis* gene expression and enzyme activity, tadpoles were collected at Nieuwkoop-Faber (NF) stages 2, 7, 16, 21, 26, 34, 41 and 46 for gene expression analyses (a) and at NF7, 21, 34 and 46 for enzymatic activity assays (b). Finasteride exposure (0, 25, 50 and 100 µM) began at NF12 until NF46 (c). Each developmental stage is reported with its duration in hours post-fertilization under our husbandry conditions. Main developmental characteristics and anatomical schematics are also presented (schematics are not at scale). Black dots represent tissue sampling events and the arrow represents the duration of the finasteride exposure.

Table 5.1 SYBR-Green real-time RT-PCR primers and assay conditions of genes measured in *Silurana tropicalis*. GenBank accession number, primer sequences (5'-3'), amplicon size (bp) and optimized primer concentration (nM) are presented for both *srd5alpha3* and *srd5beta* target genes.

Target Gene	Accession Number	Primer direction	Sequence (5'- 3')	Annealing temperature (°C)	Amplicon size (bp)	Primer (nM)
<i>srd5alpha3</i>	NM_001079071.1	Forward	GCTGGTCTGAGGAAAAGTGC	58	90	450
		Reverse	AGGGCAGGACACTCTCTCAA			
<i>srd5beta</i>	NM_001030438.1	Forward	GTGGAGTGACCAACCCAGAGT	58	108	450
		Reverse	TTCGGATCAATGAGGAGGA			

srd5alpha3: steroid 5 α -reductase; *srd5beta*: steroid 5 β -reductase.

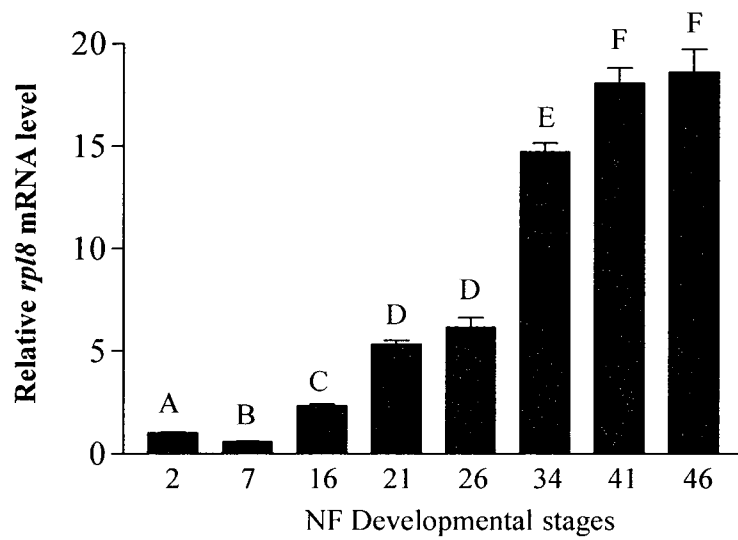


Figure 5.2 Developmental profile of the ribosomal protein L8 during *Silurana tropicalis* embryogenesis and larval development. Gene expression was measured in whole embryos and larvae from NF2 to NF46 (for details, see Fig. 5.1 a). Levels of mRNA levels are expressed relative to NF2. Bars represent the mean + SEM. Different letters indicate statistically significant differences between stages (n = 6-8 pools; $p < 0.05$).

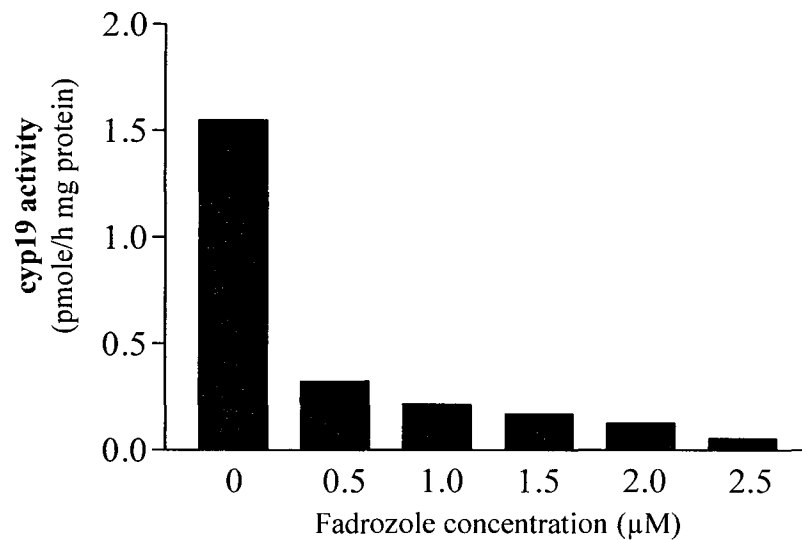


Figure 5.3 Effects of fadrozole on *in vitro* cyp19 activity in the brain of NF66 *Silurana tropicalis* animals. The activity of cyp19 was inhibited in a concentration-dependent manner (0, 0.5, 1.0, 1.5, 2.0 and 2.5 μM) with increasing concentrations of fadrozole in the brain. A brain homogenate of NF66 *S. tropicalis* (pool of 14 brains; n = 1 pool) was used to assess cyp19 activity for each fadrozole concentration. The activity of cyp19 is expressed in pmole/h normalized to total protein content (mg). cyp19: aromatase.

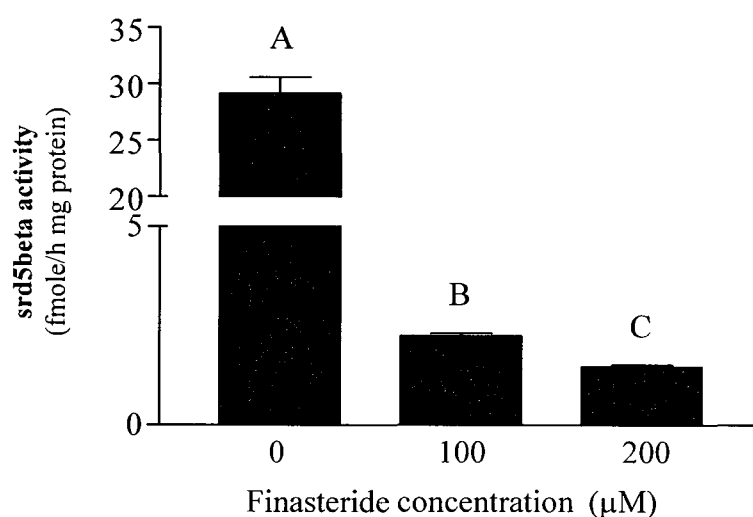


Figure 5.4 Effects of finasteride on *in vitro* srd5beta activity in whole-body NF46 *Silurana tropicalis* larvae. 5 β -reductase activity was inhibited by finasteride (0, 100 and 200 μ M). A pool of eight NF46 *S. tropicalis* larvae was used to assess the srd5beta activity for each finasteride concentration. 5 β -reductase activity is expressed in fmol/h and normalized to total protein content (mg). Bars represent the mean + SEM. Data were analyzed using the non-parametric Kruskal-Wallis rank test ($n = 3$; $p < 0.05$). Different letters indicate statistically significant differences between finasteride concentrations.

5.3 Results

5.3.1 *Steroidogenic enzymes are present during embryogenesis*

Real-time RT-PCR and radiometric assays were used to determine the profiles of all *srd5alpha* and *srd5beta* mRNA levels, and *srd5beta* activity during embryogenesis of *S. tropicalis* (Fig. 5.5 and 5.6). All these enzyme mRNAs were detected at every stages of development studied and show distinct patterns of expression. For *srd5alpha1*, the mRNA levels are low at NF16 and increases until NF41, with a maximum of 2.2-fold change. For *srd5alpha2*, mRNA levels are constant between NF2 and NF21, increase at NF21 and NF34, and stay constant until NF46 (reaching a maximum of 9.9-fold change at NF41). In contrast, *srd5alpha3* shows higher mRNA levels at NF2 and NF7 compared to the other developmental stages examined. The developmental profile of *srd5beta* mRNA decreases by 77 % and 89 % at NF16 and NF21, respectively, followed by a drastic increase of 227-fold change up to NF46. The activity of *srd5beta* was low at NF7 and NF21 (in average, 4.6 fmole/h mg protein) and increased significantly at NF34 (16.5 fmole/h mg protein) and NF46 (732.3 fmole/h mg protein).

5.3.2 *Effects of finasteride on the steroid 5 α -reductase and 5 β -reductase*

The effects of finasteride on *srd5beta* activity and mRNA level in NF46 larvae are shown in Fig. 5.7. Treatment with finasteride significantly inhibited *srd5beta* activity at 25 μ M (38 % inhibition). At higher concentrations (50 and 100 μ M), the activity was reduced by 30 % and 19 %, respectively, although these reductions were not statistically significant. In addition, a 75 % decrease in *srd5beta* and *srd5alpha2* mRNA levels were

observed at the 100 μ M concentration (Fig. 5.7 a and Fig. 5.8 b, respectively). Finasteride did not affect *srd5alpha1* and *srd5alpha3* expression in *S. tropicalis* (Fig. 5.8 a,c).

5.3.3 Effects of finasteride on *cyp19*

The effects of finasteride on *cyp19* were assessed (Fig. 5.9). *In vivo* exposure to finasteride significantly decreased *cyp19* mRNA level by 50 % at 100 μ M whereas *cyp19* activity was significantly reduced at all three concentrations of finasteride (inhibition ranged from 61 % to 80 %; Fig. 5.9 a). Similarly, finasteride inhibited *in vitro* *cyp19* activity at all concentrations tested (Fig. 5.9 b).

5.3.3 Effects of finasteride on the expression of sex receptor, thyroid hormone enzyme and thyroid hormone receptor genes

The effects of finasteride were assessed on gene expression of target genes. The expression of *eralpha* mRNA increased by 4.5-fold at the highest concentration of finasteride (Fig. 5.10 a); however no effects were observed for *erbeta* and *ar* mRNA (Fig. 5.10 b, c). Finasteride treatment resulted in a decrease of more than 50 % in *dio1* and *dio2* mRNA levels at 100 μ M (Fig. 5.11 a, c), but did not affect *dio3* mRNA level. No changes were detected in the expression of either *tr* in any of the treatment groups (Fig. 5.12).

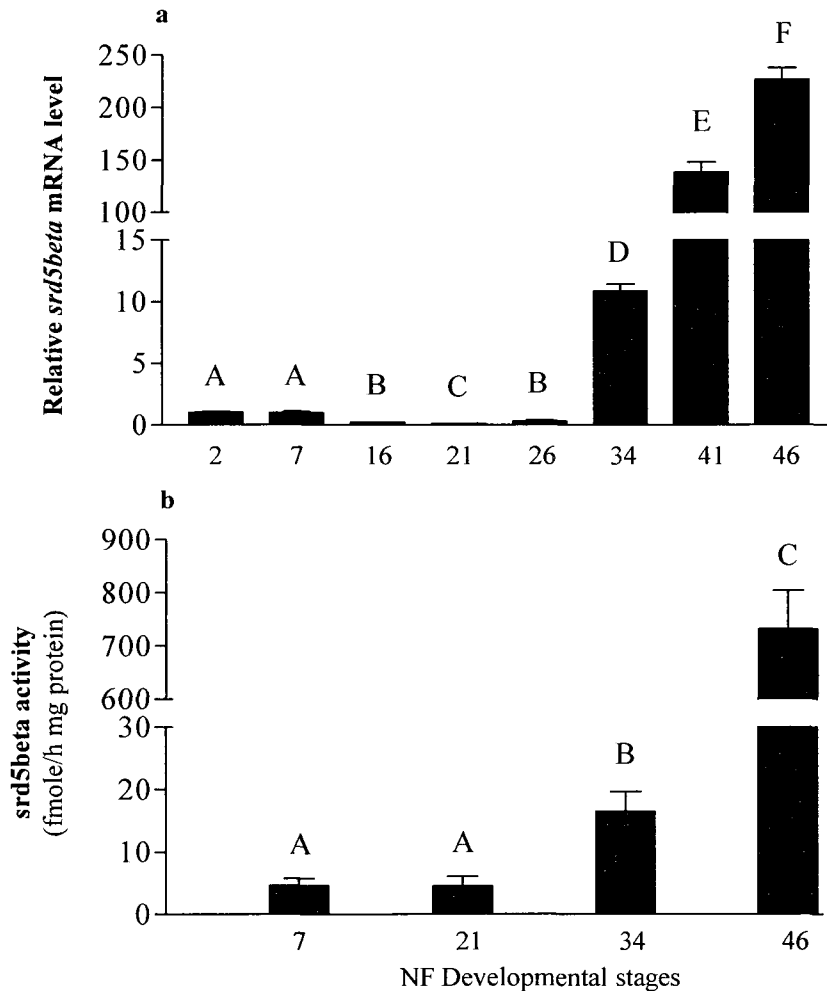


Figure 5.5 Developmental profiles of *srd5beta* expression (a) and activity (b) during *Silurana tropicalis* embryogenesis and early development. Expression and activity of *srd5beta* were measured in whole embryos and larvae from NF2 to NF46 (for details, see Fig. 5.1 a,b). The gene expression profile is expressed relative to NF2 normalized to RNA content. Enzyme activity is expressed in fmole/h normalized to total protein content (mg). Bars represent the mean + SEM. Data were analyzed using the non-parametric Kruskal-Wallis rank test ($n = 6-8$; $p < 0.05$). Different letters indicate statistically significant differences between stages. Note the broken Y-axes. *srd5beta*: 5β -reductase.

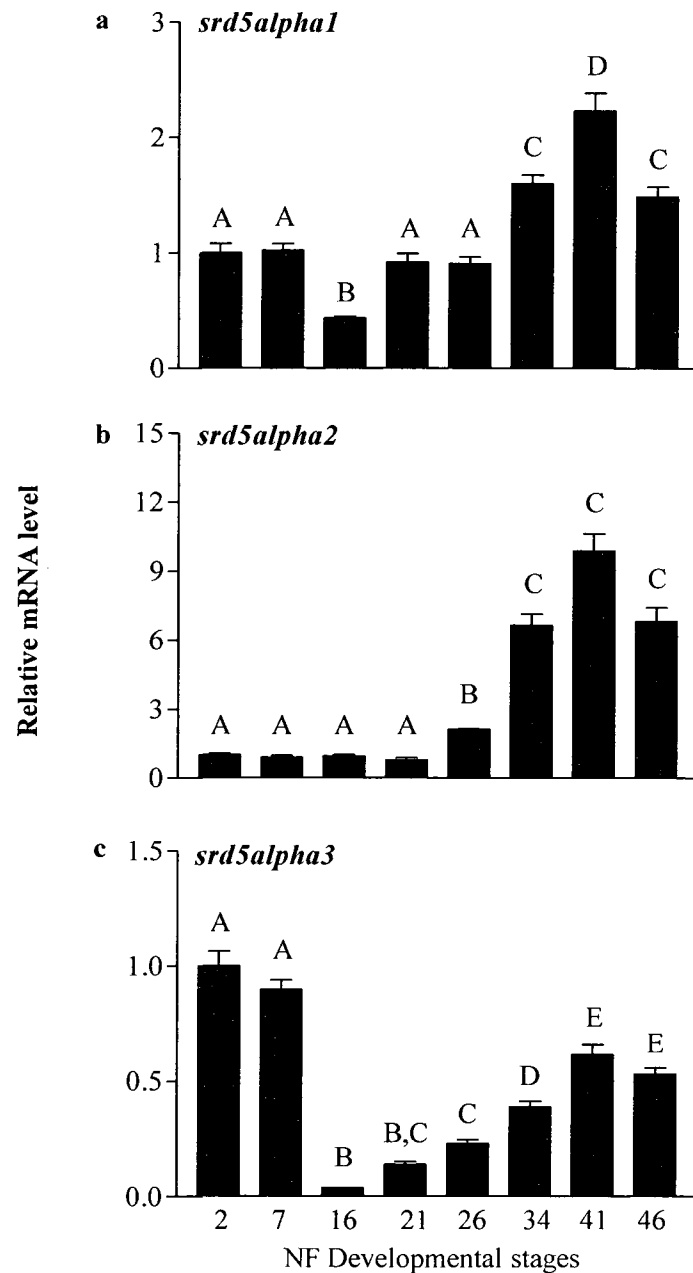


Figure 5.6 Developmental profiles of *srd5alpha1* (a) *srd5alpha2* (b) and *srd5alpha3* (c) during *Silurana tropicalis* embryogenesis and early development. Transcript levels were measured in whole embryos and larvae from NF2 to NF46 (for details, see Fig. 5.1 a). Data are expressed relative to NF2 normalized to RNA content. Bars represent the mean + SEM. Data were analyzed using the non-parametric Kruskal-Wallis rank test ($n = 6-8$; $p < 0.05$). Different letters indicate statistically significant differences between stages. The scales of the Y-axis vary between genes. *srd5alpha*: 5 α -reductase.

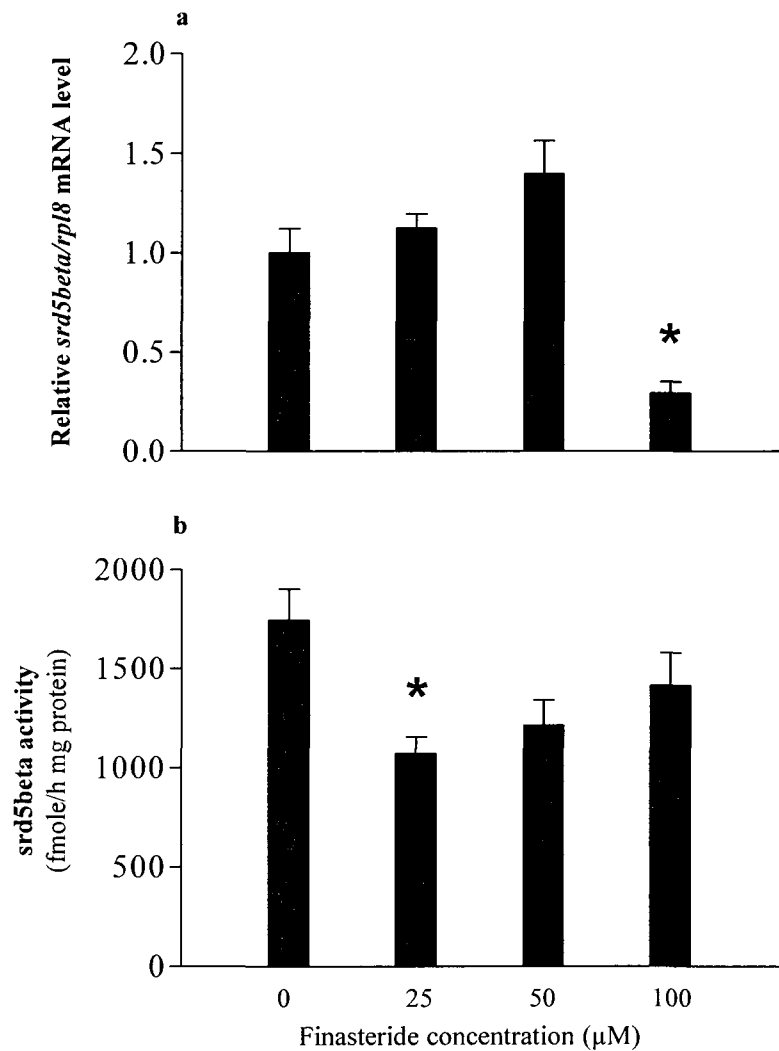


Figure 5.7 Effects of finasteride on gene expression (a) and activity (b) of *srd5beta* in *Silurana tropicalis* larvae. *Silurana tropicalis* tadpoles were exposed to finasteride as described in Fig. 5.1 c. The mRNA levels of *srd5beta* are expressed relative to the control group and are normalized to the expression of *rpl8*. The enzyme activity is expressed in fmole/h normalized to protein content. Bars represent the mean + SEM. Data were analyzed using one-way ANOVA ($n = 5-8$, $p < 0.05$). Asterisks (*) indicate significant differences from the control group. *rpl8*: ribosomal protein L8; *srd5beta*: steroid 5 β -reductase.

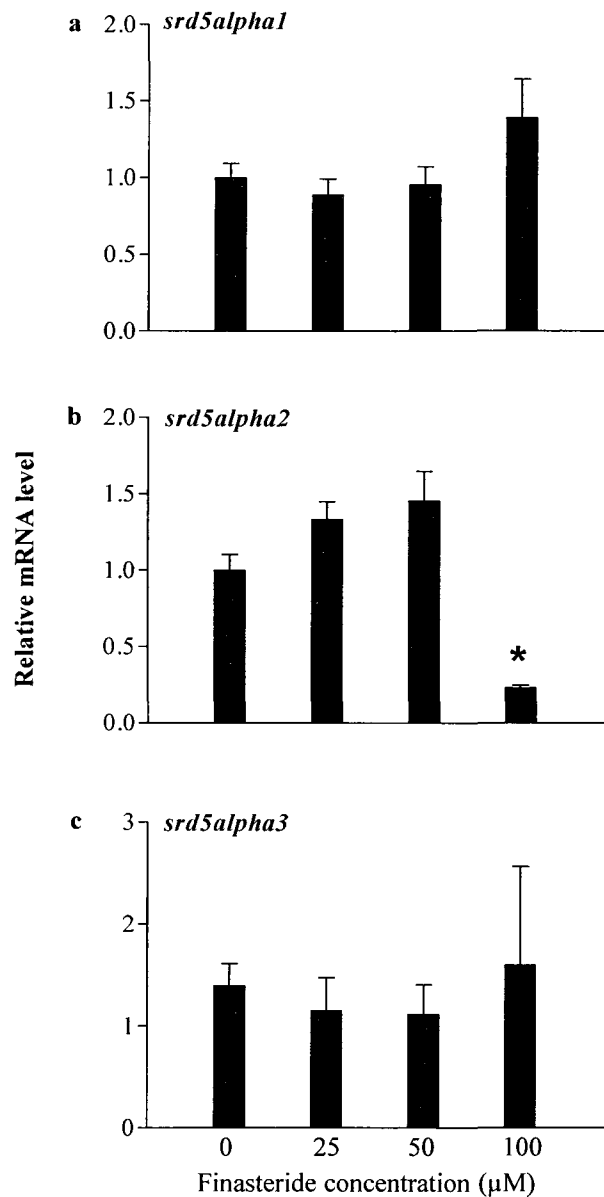


Figure 5.8 Effects of finasteride on gene expression of *srd5alpha1* (a), *srd5alpha2* (b) and *srd5alpha3* (c) in *Silurana tropicalis* larvae. *Silurana tropicalis* tadpoles were exposed to finasteride as described in Fig. 5.1 c. The mRNA levels are expressed relative to the control group and are normalized to the expression of *rpl8*. Bars represent the mean mRNA level + SEM. Data were analyzed using one-way ANOVA ($n = 5-8$, $p < 0.05$). Asterisks (*) indicate significant differences from the control group. The scales of the Y-axis vary between graphs. *rpl8*: ribosomal protein L8; *srd5alpha1*: steroid 5 α -reductase type 1; *srd5alpha2*: steroid 5 α -reductase type 2; *srd5alpha3*: steroid 5 α -reductase type 3.

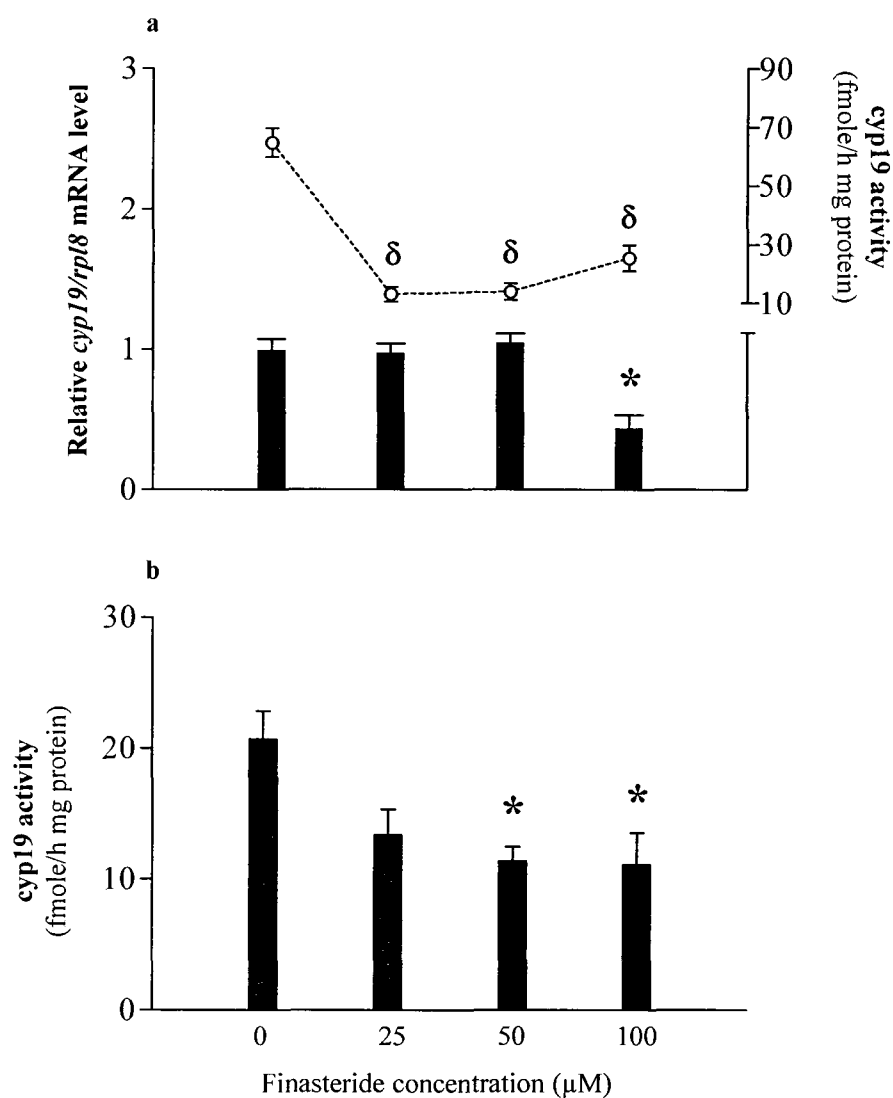


Figure 5.9 *In vivo* (a) and *In vitro* (b) effects of finasteride on cyp19 activity in NF46 *Silurana tropicalis* larvae. (a) *Silurana tropicalis* tadpoles were exposed to finasteride as described in Fig. 5.1 c. The mRNA levels (upper Y-axis on the left) are expressed relative to the control group and are normalized to the expression of *rpl8*. The enzyme activity (upper Y-axis on the right) is expressed in fmole/h normalized to protein content (note the broken Y-axis for a). (b) *In vitro* exposure to four concentrations of finasteride (0, 25, 50 and 100 μM) assessed on a homogenate of NF46 *S. tropicalis* tadpoles (a pool of 240 tadpoles). The enzyme activity is expressed in fmole/h normalized to protein content. Bars represent the mean ± SEM. Data were analyzed using one-way ANOVA (n = 5-8, $p < 0.05$). Asterisks (*) indicate significant differences from the control group for the gene expression data and deltas (δ) report significant differences relative from the control group for enzyme activity. The scales of the Y-axis vary between graphs. cyp19: aromatase, rpl8: ribosomal protein L8.

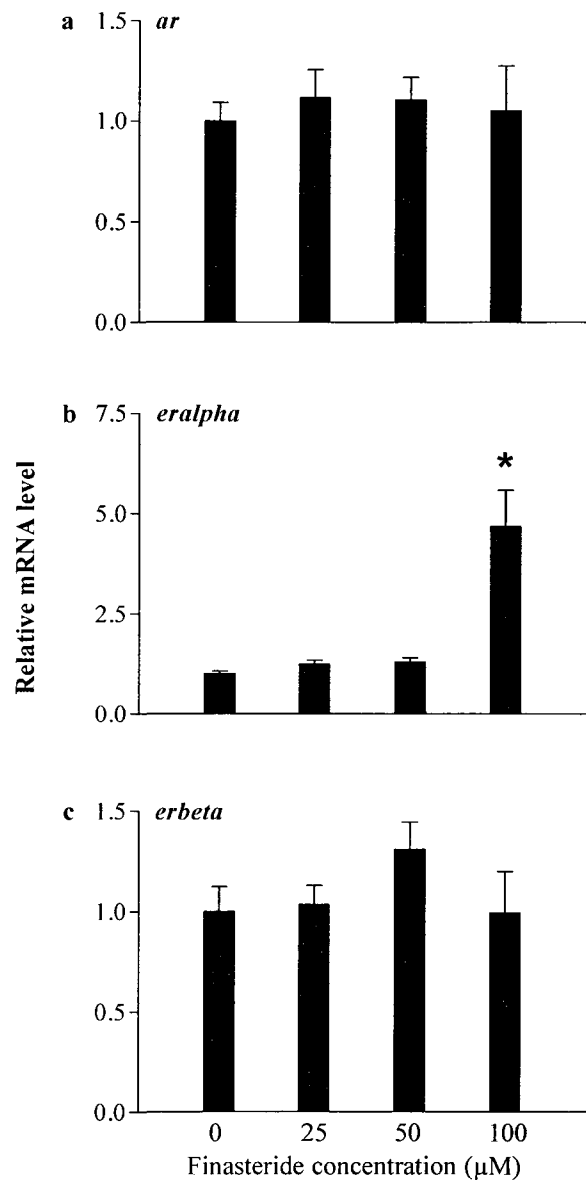


Figure 5.10 Effects of finasteride on gene expression of *ar* (a), *eralpha* (b) and *erbeta* (c) in *Silurana tropicalis* larvae. *Silurana tropicalis* tadpoles were exposed to finasteride as described in Fig. 5.1 c. The mRNA levels are expressed relative to the control group and are normalized to the expression of *rpl8*. Bars represent the mean mRNA level + SEM. Data were analyzed using one-way ANOVA ($n = 5-8$, $p < 0.05$). Asterisks (*) indicate significant differences from the control group. The scales of the Y-axis vary between graphs. *ar*: androgen receptor; *eralpha*: estrogen receptor α ; *erbeta*: estrogen receptor β ; *rpl8*: ribosomal protein L8.

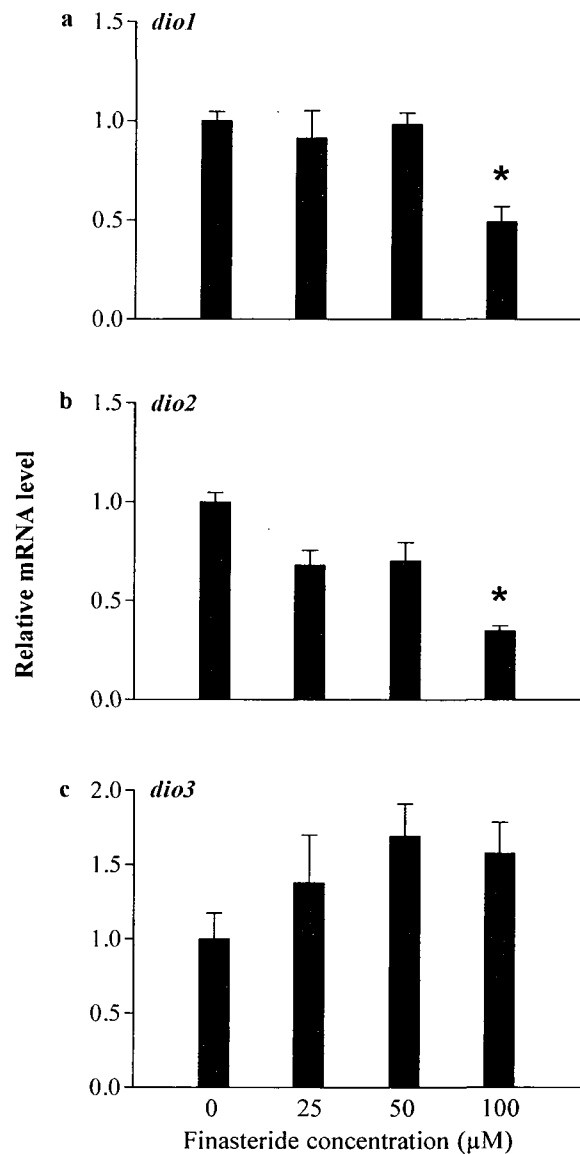


Figure 5.11 Effects of finasteride on gene expression of *dio1* (a), *dio2* (b) and *dio3* (c) in *Silurana tropicalis* larvae. *Silurana tropicalis* tadpoles were exposed to finasteride as described in Fig. 5.1 c. The mRNA levels are expressed relative to the control group and are normalized to the expression of *rpl8*. Bars represent the mean mRNA level + SEM. Data were analyzed using one-way ANOVA ($n = 5-8$, $p < 0.05$). Asterisks (*) indicate significant differences from the control group. The scales of the Y-axis vary between graphs. *dio1*: deiodinase type 1; *dio2*: deiodinase type 2; *dio3*: deiodinase type 3; *rpl8*: ribosomal protein L8.

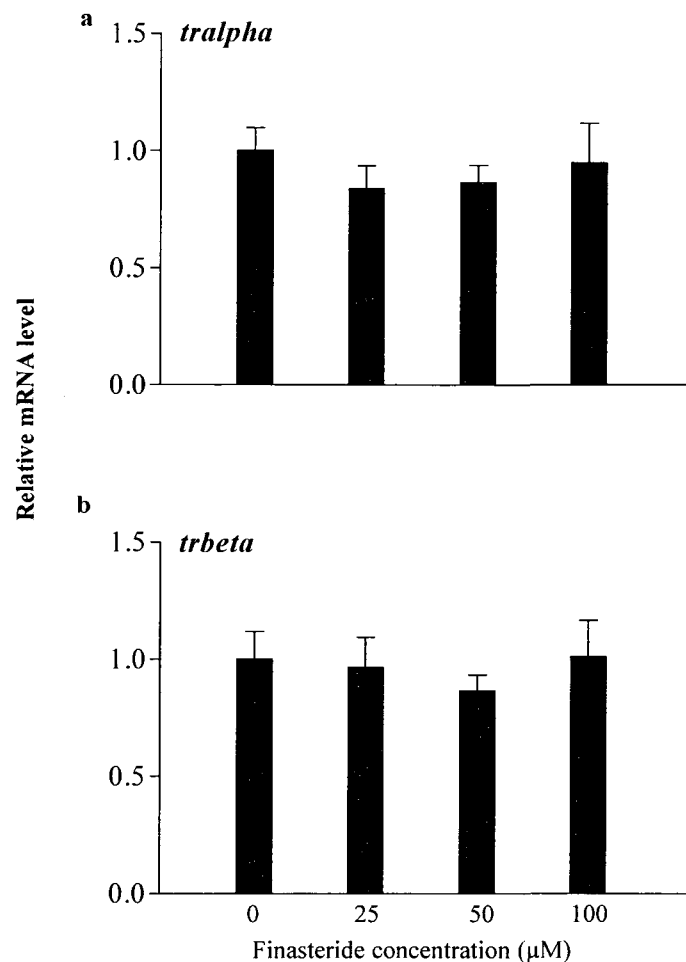


Figure 5.12 Effects of finasteride on gene expression of *tralpha* (a) and *trbeta* (b) *Silurana tropicalis* larvae. *Silurana tropicalis* tadpoles were exposed to finasteride as described in Fig. 5.1 c. The mRNA levels are expressed relative to the control group and are normalized to the expression of *rpl8*. Bars represent the mean mRNA level + SEM. Data were analyzed using one-way ANOVA ($n = 5-8$, $p < 0.05$). *rpl8*: ribosomal protein L8; *tralpha*: thyroid hormone receptor α ; *trbeta*: thyroid hormone receptor β .

5.4 Discussion

Four major findings are reported in this study. First, our results demonstrate that *srd5alpha* (type 1, 2 and 3) and *srd5beta* are all present during amphibian embryogenesis and at the beginning of larval development. This is the first study to report the presence of *srd5beta* and *srd5alpha3* during vertebrate embryogenesis. Second, we demonstrated that *srd5beta* is active in early *S. tropicalis* development. Third, we show that EDCs have the ability to alter steroid pathways since our experiment demonstrated that the sex steroid- and TH-related genes were altered in early development following an exposure to a pharmacological inhibitor used as a model compound. Finally, we showed that finasteride also affected *cyp19* mRNA and activity.

In this study, *srd5beta* mRNA and activity were measured during embryogenesis and results demonstrated that developmentally-regulated increases in *srd5beta* transcription were followed very closely by an increase in enzyme activity. Therefore, we can predict that the time between mRNA induction and synthesis of this functional enzyme during *S. tropicalis* embryogenesis is short since major enzyme activity was detected at the same stage of development than the increase in mRNA observed at NF34.

Detection of *srd5beta* transcripts in NF2 and NF7 embryos indicates maternal transfer of mRNA to the egg. RNA and proteins of maternal origin have been shown to be important in the regulation of the earliest stages of embryonic development (Telford et al. 1990). The presence of *srd5beta* mRNA and activity in embryos suggests that 5 β -DHT is needed before embryonic transcription starts (NF12; gastrulation). Sex steroids have been detected in a wide range of concentrations during embryogenesis in other

vertebrates. For example, estrogens and androgens have been shown to be important for survival, growth and sexual development in fish, bird and mammalian embryos (Adkins-Regan et al. 1995; Carere and Balthazart 2007; Goldman-Johnson et al. 2008; Iwamatsu et al. 2005; von Engelhardt et al. 2006). Sex steroid hormones in eggs can also come directly from maternal transfer (Adkins-Regan et al. 1995); for example, maternal transfer of testosterone has been implicated in offspring begging behaviour in birds (von Engelhardt et al. 2006). The presence of sex steroid hormones in many vertebrate eggs suggests that *srd5alpha1*, *srd5alpha2*, *srd5alpha3* and *srd5beta* could be active and could metabolize testosterone in frog eggs. However, our data only suggests that 5 β -DHT can be synthesised in the frog egg.

While transcripts were also detected at NF2 and NF7, the mRNA levels of *srd5alpha* and *srd5beta* mRNA only began to increase around NF16 to NF21 and this increase continued during organogenesis and tissue differentiation. A similar developmental study in a related species, *X. laevis* also detected *srd5alpha1* and *srd5alpha2* expression in whole embryos (Urbatzka et al. 2007a). However, the overall profiles are different between the two species. For example, *srd5alpha1* decreased from NF12 to NF48 in *X. laevis* embryos whereas we observed the opposite trend in *S. tropicalis*. These differences may be species-related or could reflect that the gene expression data for *X. laevis* in the Urbatzka et al. study were normalized to a housekeeping gene that changed throughout development; we normalized to RNA concentration as it was recently suggested to be more appropriate for developmental studies (Sindelka et al. 2006). In addition, the *X. laevis* study used semi-quantitative PCR, while we used real-time RT-PCR, a more sensitive method. We also analyzed additional

stages of embryonic development to capture important mRNA changes that were perhaps missed in the *X. laevis* study. Nevertheless, *srd5alpha1* and *srd5alpha2* are present in embryogenesis of at least two frog species.

The precise functions of *srd5alpha* and *srd5beta* during embryogenesis and early development remain unclear in vertebrates. Since these developmental periods include the beginning of organogenesis and tissue differentiation, these enzymes could be involved in neurogenesis (Petratos et al. 2000), differentiation of embryonic stem cells into cardiomyocytes (Goldman-Johnson et al. 2008), erythropoiesis (Garavini and Cristofori 1984; Gordon et al. 1970), bile biosynthesis (Kondo et al. 1994), and gonadal development (reviewed in Russell and Wilson 1994). Therefore, the change in transcription and activity of steroidogenic enzymes during embryogenesis can be related to many steroid-sensitive biological processes occurring during organogenesis.

To our knowledge, this is the first study to show that finasteride inhibits *srd5beta* and *cyp19* activities and alters the sex steroid- and TH-related gene expression in an amphibian. Drury et al. (2009) demonstrated that finasteride (designed to be a specific 5α -reductase type 2 inhibitor in humans) has the ability to bind to the *srd5beta* protein and suggested that such inhibition would decrease the hepatic clearance of steroid hormones and reduce the production of 5β -pregnane ligands (natural ligands for the hepatic pregnane-X receptors; Drury et al. 2009). However, there is a clear lack of data on the consequences of *srd5beta* inhibition. In birds, studies have demonstrated that *srd5beta* inactivates testosterone in the brain (Hutchison and Steimer 1984). The regulation of testosterone concentration by *srd5beta* could have important implications for development and reproductive functions. For example, local aromatization of

testosterone to estradiol is known to masculinise the vertebrate brain during development (reviewed in Morris et al. 2004), therefore changes in *srd5beta* activity could affect the available testosterone that needs to be aromatized in the brain. In addition to reduced enzyme activity, our data demonstrated that finasteride decreased *srd5beta* and *srd5alpha2* mRNA levels. Since 5 α -DHT regulates the expression of *srd5alpha2* in mammals (George et al. 1991), our results suggest a similar feed-forward regulation of *srd5beta* mRNA by either or both 5 α -DHT and 5 β -DHT. The analysis of *srd5alpha* and *srd5beta* gene promoters and the design of *srd5alpha* isoform-specific inhibitors will be required to understand the function and regulation of these enzymes.

Unexpectedly, our results demonstrated that finasteride is not a specific inhibitor of *srd5alpha* and *srd5beta* in *S. tropicalis* since it also decreased *cyp19* activity. The reduction of *cyp19* activity without a decrease in *cyp19* mRNA suggests that finasteride can inhibit directly *cyp19* in this species. In order to validate this hypothesis, we tested *in vitro* the effects of finasteride on *cyp19* activity and concluded that finasteride has the ability to directly inhibit *cyp19* in NF46 *S. tropicalis* larvae. In mammalian studies, no effect of finasteride on CYP19 has been reported (Lephart and Husmann 1993; Young et al. 1997). In spite of the effect of finasteride on *cyp19* in *S. tropicalis*, we show here that the effects produced by finasteride are distinct than those resulting from a similar experimental design using the aromatase inhibitor fadrozole (Langlois et al. 2010a; Table 5.2). Despite a possible effect on *cyp19*, chronic exposures to finasteride in amphibians disrupts sexual differentiation in a manner consistent with its intended effects to lower androgen levels rather than what would be expected with aromatase inhibition. A *S. tropicalis* long term exposure to finasteride leads to female-biased sex ratios whereas

exposure to fadrozole leads to a male-biased sex ratio (Chapter 6). Furthermore, finasteride has been reported to disrupt spermatogenesis in *X. laevis*, while the ovaries did not seem to be affected (Urbatzka et al. 2009).

Exposure to finasteride also altered the expression of TH-related genes. There is strong evidence of the importance of *tr* and *dio* in early amphibian embryogenesis (Havis et al. 2006; Morvan Dubois et al. 2006; Tindall et al. 2007). *Xenopus laevis* embryos treated with NH-3 (a *tr* agonist/antagonist) exhibit malformations and alterations in eye development (Havis et al. 2006). *Silurana tropicalis* embryos exposed to methimazole (a thyroid peroxidase inhibitor) showed notochord malformations and an alteration in iodine uptake (Tindall et al. 2007). Treatment of *X. laevis* embryos with iopanoic acid (a *dio1*, *dio2* and *dio3* activity inhibitor) resulted in developmental delays, edema, mobility alteration and deficiencies in eye development (Havis et al. 2006). Therefore, a disruption of TH during early tadpole development can lead to important physiological defects. Data confirm that *dio1* and *dio2* expression can be modulated during frog embryogenesis and further suggest that a compound inhibiting *srd5alpha* and *srd5beta* during that specific developmental period can also alter the TH molecular signalling. These results provide evidence for a crosstalk between the androgen status and TH axis. The physiological consequences of these modulations on further tadpole development remain to be investigated, but we can speculate that decreases in *dio1* and *dio2* mRNA levels can decrease the TH synthesis which in turn, might affect tissue remodelling and metamorphosis later in development. All together, this extends the role of sex steroids beyond sexual development.

Table 5.2 Comparaison of the gene expression changes after fadrozole and finasteride treatments during *Silurana tropicalis* early development.

Genes	Fadrozole ^a (μ M)			Finasteride ^b (μ M)		
	0.5	1.0	2.0	25	50	100
<i>cyp19</i>	-	-	-	-	-	↓
<i>eralpha</i>	-	-	-	-	-	↑
<i>erbeta</i>	-	-	-	-	-	-
<i>ar</i>	-	↑	↑	-	-	-
<i>srd5alpha1</i>	-	-	-	-	-	-
<i>srd5alpha2</i>	-	-	-	-	-	↓
<i>srd5alpha3</i>	-	-	-	-	-	-
<i>srd5beta</i>	-	-	-	-	-	↓
<i>tralpha</i>	-	↑	↑	-	-	-
<i>trbeta</i>	-	↑	↑	-	-	-
<i>dio1</i>	-	-	↓	-	-	↓
<i>dio2</i>	-	-	-	-	-	↓
<i>dio3</i>	-	↑	↑	-	-	-

↑ : increase in fold change

↓ : decrease in fold change

- : no changes

^a data from a collaborative work in Langlois et al. (2010a)

^b data from this chapter

5.5 Conclusions

Taken together, these results indicate a complex interaction between the reproductive and TH axes during amphibian embryogenesis. This is the first study to report that *srd5alpha1*, *srd5alpha2*, *srd5alpha3* and *srd5beta* are present and *srd5beta* is active during early amphibian development and that these enzymes can be disrupted by EDCs. Our results also show that pharmacological inhibition of *srd5alpha* and *srd5beta* affects transcription of sex steroid- and TH-related genes. Although we demonstrated that *srd5beta* is active during amphibian embryogenesis, the precise roles of its key metabolites (e.g., 5 β -DHT) remain to be elucidated. Furthermore, the consequences of a disruption in these steroidogenic enzymes during amphibian sexual development should be assessed (Chapter 6).

CHAPTER 6

Finasteride affects gonadal differentiation and both brain and hepatic gene expression in the frog *Silurana tropicalis*

Chapter adapted from two publications:

Langlois VS¹, Duarte-Guterman P², Hodgkinson K³, Wade MG⁴, Pauli BD⁵, Cooke GM⁶
and VL Trudeau⁷ (2010b) *Sexual Development* 3, 333-341.

Langlois VS¹, Duarte-Guterman P² and VL Trudeau⁷ *Submitted in Aquatic Toxicology*
AQTOX-D-10-00121.

Main contributions of each co-author:

¹ Contributed to original ideas, performed animal exposure, real-time RT-PCR optimization and related-data analysis, aromatase activity assay, 5 β -reductase activity assay optimizations and related-data analysis, histology and prepared the manuscripts.

² Performed a parallel study using the aromatase inhibitor fadrozole for the manuscripts.

³ Participated in animal care, histology and gene expression analysis.

⁴ Revised the manuscript using his expertise in amphibian toxicology.

⁵ Provided histological material and his expertise.

⁶ Revised the manuscript with his expertise on the steroid 5 α -reductase.

⁷ Contributed to original ideas and revised the manuscripts.

6.1 Introduction

Laboratory and mesocosm experiments using frogs have produced skewed sex ratios and/or intersex individuals after exposure to estrogenic (ethinylestradiol; Hogan et al. 2008), anti-estrogenic (fadrozole; Olmstead et al. 2009), androgenic (5 α -dihydrotestosterone [5 α -DHT]; Coady et al. 2004) and anti-androgenic (cyproterone acetate; Hayes et al. 2006b) compounds. These results suggest that one of the mechanisms that contribute to biased sex ratios and the presence of intersex individuals in the environment could be an imbalance between estrogens and androgens after exposure to endocrine disruptor chemicals (EDCs). Furthermore, recent studies have demonstrated that *Lithobates pipiens* (Northern leopard frog) tadpoles exposed chronically to the herbicide atrazine exhibited a skewed sex ratio toward females and an altered activity of the steroid 5 β -reductase (srd5beta; Chapters 2 and 3). These findings suggest that the disruption of srd5beta could also be involved in the sex ratio imbalance. Moreover, finasteride, a human steroid 5 α -reductase type 2 (srd5alpha2) and srd5beta inhibitor, has also been shown to be an effective inhibitor of these two enzymes in frogs (Chapter 5). Exposures to finasteride during larval development altered *srd5alpha2* mRNA and both *srd5beta* mRNA and activity in *Silurana tropicalis* (Western clawed frog; Chapter 5). Therefore, changes in srd5alpha and/or srd5beta after exposure to EDCs could result in imbalanced sex steroid levels and lead to biased sex ratios.

The hypotheses tested in this study were that 1) exposure to a srd5alpha and srd5beta inhibitor during the gonadal differentiation of *S. tropicalis* will affect sex ratio and induce intersex phenotype, and 2) these intersex individuals will express different gene expression profiles when compared to normal males and females. The specific objectives

of this study were to determine the impacts of finasteride on sex ratio and on sex steroid- and thyroid hormone (TH)-related gene expression when exposed during sexual development.

6.2 Materials and methods

6.2.1 *Breeding and maintenance of *Silurana tropicalis**

Siurana tropicalis frogs were reared in dechlorinated and aerated water from the University of Ottawa Animal Care facility (Ontario, Canada). Fertilized eggs were obtained from three pairs of adults by injecting human chorionic gonadotropin hormone (hCG; Sigma) into the dorsal lymph sac (as previously described in Chapter 5). Staging was determined by following the Nieuwkoop and Faber developmental table (NF; Nieuwkoop and Faber 1994). A 12:12h light:dark cycle was maintained where lights came on at 7 am. The water temperature was 25 ± 1 °C. 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.

6.2.2 *Finasteride exposure*

Eggs were dejellied at NF6 with 2 % (w/v) L-cysteine (pH 8.0; Sigma) and washed three times with 10 volumes of modified Ringer's solution (MRS; 0.1 M NaCl, 1.8 mM KCl, 2.0 mM CaCl₂, 1.0 mM MgCl₂, 300 mg/L NaHCO₃). Eggs were placed in Petri dishes at a density of 50 eggs per dish with 10 volumes of MRS containing 0.04 mg/L of the antibiotic gentamycin (Sandoz Canada Inc.). Embryos were exposed from NF12 to NF60 to either 0.05 % ethanol (EtOH; control of the experiment) or 25 µM finasteride (equivalent to 9 mg/L; Sigma Canada Ltd, Oakville, ON) dissolved in 0.05 % EtOH. Finasteride, the antibiotic and the MRS were refreshed daily. At NF46, tadpoles were transferred to 5-L tanks containing activated carbon filtered and aerated water (pH = 7.5 ± 0.5 , dissolved oxygen = 83 ± 2 mg/L, temperature = 25 ± 1 °C, electrical

conductivity = $1000 \pm 50 \mu\text{S}$) and $25 \mu\text{M}$ finasteride. Finasteride and water were refreshed twice a week. Tadpoles were feed with Sera Micron® twice daily. The tadpoles were sampled at NF60, anaesthetized by immersion in 3-aminobenzoic acid ethyl ester (MS-222; 1 %; Sigma), and euthanized by cutting the spinal cord. Wet weight, snout-vent length, tail length and number of days to metamorphose were recorded. Brain and livers were dissected, frozen on dry ice and kept at -80°C . The remainder of the body was fixed and decalcified in Cal-Ex II (Fisher Scientific, Ottawa, ON, Canada) for 48 h and transferred into 70 % EtOH for storage until histological processing.

6.2.3 Histology

Gonad-kidney complexes were trimmed and dissected out from the fixed and decalcified bodies and prepared for histological processing following the methods previously described in Chapter 2. Briefly, gonads were embedded in paraffin and serial $5 \mu\text{m}$ sagital sections were stained with hematoxylin and eosin. Phenotypic sex of each animal was classified as male, female or intersex (if presence of testicular oocytes). Pictures of the sections were taken by a Micro Publisher 3.3 Digital microscope camera (Qimaging Corp.).

6.2.4 RNA isolation and cDNA synthesis

Homogenization of the samples was achieved using an MM301 Mixer Mill (Retsch, Newton, PA, USA) at 20 Hz for 3 min. For the brains, total RNA was obtained using the QIAGEN RNeasy Micro Kit (including the RNase-free DNase treatment) whereas RNA was extracted from the livers using the QIAGEN Mini Plus Kit (Qiagen, Mississauga, ON, Canada). Isolated RNA was resuspended in RNase free water and

stored at -80 °C. Concentrations of RNA were determined using the NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc.). Total cDNA was prepared from 2 µg total RNA and 0.2 µg random hexamer primers using Superscript II reverse transcriptase (Invitrogen).

6.2.5 Real-time RT-PCR analysis

All real-time RT-PCR assays were performed in a MX3000P real-time polymerase chain reaction system (Stratagene, La Jolla, CA, USA) and details regarding specific gene primers, annealing temperatures, real-time RT-PCR conditions and optimization steps are described in Langlois et al. (2010a). The expression of target genes *srd5alpha1*, *srd5alpha2*, *srd5alpha3*, *srd5beta*, androgen receptor (*ar*), TH receptors α and β (*tralpha* and *trbeta*) and deiodinase type 1, 2 and 3 (*dio1*, *dio2* and *dio3*) was analyzed in simplex real-time RT-PCR assays using SYBR Green I Dye (Molecular Probes, Eugene, OR, USA). A multiplex assay was designed to measure the estrogen receptors α and β (*eralpha* and *erbeta*), aromatase (*cyp19*) and ribosomal protein L8 (*rpl8*) gene expression using dual-labelled fluorescent probes (Integrated DNA technologies, Coralville, IA, USA). Primer and probe combinations are described in Langlois et al. (2010a).

Real-time RT-PCR standard curves contained equal parts of each treatment cDNA. Samples were run in duplicate along with a no-template control (RNase-free water was added to the reaction instead of the cDNA template) and a no reverse transcriptase control (RNase-free water was added to the cDNA synthesis reaction instead of the enzyme). All real-time RT-PCR assays achieved an efficiency of 100 % $\pm$ 10 % and an $r^2 > 0.985$. The housekeeping gene *rpl8* was used to normalize the brain data since

its mRNA level did not change significantly between treatments. However, for the hepatic data, the expression of all tested housekeeping genes *rpl8*, *β -actin*, the glyceraldehyde 3-phosphate dehydrogenase and the elongation factor 1 α changed with finasteride treatment (data not shown). Therefore, hepatic gene expression data were normalized to RNA content as recommended by Huggett et al. (2005). Data are presented as fold changes relative to EtOH-exposed control males. The expression of *cyp19* was not performed in the liver since *cyp19* is not detectable in frog liver (for *S. tropicalis*, data not shown; for *L. pipiens*, Hogan 2006).

6.2.6 Enzymatic activities

The activity of *cyp19* was measured using the modified radiometric method described in Chapter 3 (section 3.2.6). Briefly, *cyp19* activity was measured in pools of two brains of same sex animals (NF60; each pool weighed 18.8 to 41.0 mg; n = 6 pools). Cofactor and ^3H -androstendione were first incubated for 30 min at 37 °C. After this pre-incubation, brains were sonicated in phosphate buffer and incubated for 80 min at 25 °C. Tritiated water was extracted with a charcoal solution and radioactivity was counted. The activity of *cyp19* is expressed as fmole of $^3\text{H}_2\text{O}$ /h·mg protein.

The activity of *srd5beta* was also measured using the radiometric method described in Chapter 3 (section 3.3.7). Briefly, the hepatic *srd5beta* activity was determined in individual livers (n = 6 livers per sex and per treatment; liver weights ranged from 6.1 to 34.2 mg) and was assessed by the conversion of testosterone into 5 β -dihydrotestosterone (5 β -DHT) and 5 β -androstan-3 β -17 β -diol (5 β -3 β -diol). Livers were first sonicated in Tris-buffer and incubated at 37 °C with 4- ^{14}C -testosterone. Steroids were extracted by evaporation until dryness and resuspended in chloroform-methanol.

Steroid metabolites were extracted using a two-dimensional thin layer chromatography system and analyzed with the Image Quant TL 3.0 software (Amersham Bioscience). The activity of *srd5beta* is expressed as the sum of 5 β -DHT and 5 β -3 β -diol /h/mg protein. Total protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad Co.).

6.2.7 Data analysis

Gene expression data analysis was performed as described in Chapter 3 (section 3.2.8). Statistical analyses were performed using S-Plus 8.0 (Insightful Corp. Seattle, Washington, USA). Somatic growth, gene expression and enzymatic activity data were tested for normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test) and analyzed using one-way analysis of variance (ANOVA). If results were significant, the Bonferroni post-hoc test was used to evaluate significant differences between groups. When data failed to meet assumptions even after transformation (e.g., Log₁₀), the nonparametric Kruskal-Wallis test on ranks was used. Differences in the proportions of male, female and intersex individuals were analyzed using Fisher's exact test. Statistical significance for all tests was set at $p \leq 0.05$.

6.3 Results

6.3.1 Sex ratio and histological analysis

Treatment with finasteride induced intersex gonads in *S. tropicalis*, while no intersex animals were found in the EtOH control (Fig. 6.1). Finasteride produced 27 % male, 53 % female and 20 % intersex ($p < 0.05$ relative to control). In contrast, the control exhibited 54 % male and 46 % female (Fig. 6.2). Other than the presence of intersex, male and female gonads in finasteride treatments did not show any noticeable morphological differences when compared to controls.

6.3.2 Somatic growth and rate of metamorphosis

The results of the somatic growth and developmental endpoints after chronic exposure to finasteride during *S. tropicalis* development are presented in Table 6.1. The average number of days to metamorphose was 92 in both treatments. At NF60, intersex animals had a 14 % greater body weight compared to treated males and a 4 % greater snout-vent length compared to both treated males and females. Treated females had smaller tails (7 %) compared to control females ($p < 0.05$). No differences in mortality were observed between treated and control animals after the chronic exposure (data not shown).

6.3.3 Effects of finasteride on sex steroid-related gene expression and enzymatic activity

Finasteride decreased hepatic *srd5beta* mRNA levels by approximately 70 % in treated females and intersex animals ($p < 0.05$; Fig. 6.3 a) but did not affect brain

srd5beta mRNA level (Fig. 6.3 b). No effects of finasteride were reported on hepatic *srd5beta* activity in treated females and intersex animals; however a significant 2-fold increase in activity was measured in the treated males (Fig. 6.3 c).

Finasteride affected hepatic *srd5alpha1* and *srd5alpha2* mRNA but did not affect *srd5alpha3* mRNA level. Furthermore, treatment did not affect any of the *srd5alpha* mRNA levels in the brain (Fig. 6.4). The expression of *srd5alpha1* in the livers of intersex animals was more than 40 % lower than in all other groups ($p < 0.05$; Fig. 6.4 a) and finasteride treatment inhibited almost completely the expression of *srd5alpha2* mRNA (96 % decrease; $p < 0.05$; Fig. 6.4 b). Finasteride-induced intersex animals also showed significant changes in the brain, as a 1.7-fold increase in *cyp19* mRNA level (Fig. 6.5 a) was observed compared to control males. No changes in brain *cyp19* activity were found (Fig. 6.5 b).

A sexual dimorphism in hepatic *ar* expression was identified in animals in the control group; expression was 50 % higher in females than in males ($p < 0.05$; Fig. 6.6 a). However, this sex difference disappeared in animals treated with finasteride since treated-females had somewhat lower *ar* mRNA level than treated-males. No such sexual dimorphism was observed on *ar* mRNA level in the brain. However, finasteride increased brain *ar* mRNA levels in treated-males and intersex animals ($p < 0.05$; Fig. 6.6 b). Both treated males and females showed a 4- and 6-fold increase in the expression of hepatic *eralpha*, respectively compared to controls ($p < 0.05$; Fig. 6.6 c); while in the brain only the intersex animals showed an increase in *eralpha* mRNA level ($p < 0.05$; Fig. 6.6 d). A similar significant increase in *erbeta* mRNA was also observed in the brains of intersex animals (Fig. 6.6 f).

6.3.4 Effects of finasteride on thyroid hormone-related gene expression

Finasteride affected brain and hepatic TH-related gene expression in *S. tropicalis* metamorphs. In the liver, *dio2* increased by 4.8-fold (males) and 6-fold (intersex; $p < 0.05$; Fig. 6.7 a), while *dio3* mRNA decreased by 74 % (males) and 92 % (intersex; $p < 0.05$; Fig. 6.7 c). Although no changes were observed for *dio2* mRNA in brain tissue, (Fig. 6.7 b) the expression of *dio3* increased for both treated males (2.5-fold) and intersex animals (4-fold; $p < 0.05$; Fig. 6.7 d). The expression of *trbeta* increased significantly in treated males for both tissues, with a 3-fold increase in the liver and 1.8-fold in the brain; while an increase in expression in intersex animals was only observed in the brain (Fig. 6.8 c,d). No changes in *trbeta* mRNA level were observed for both tissues in females.

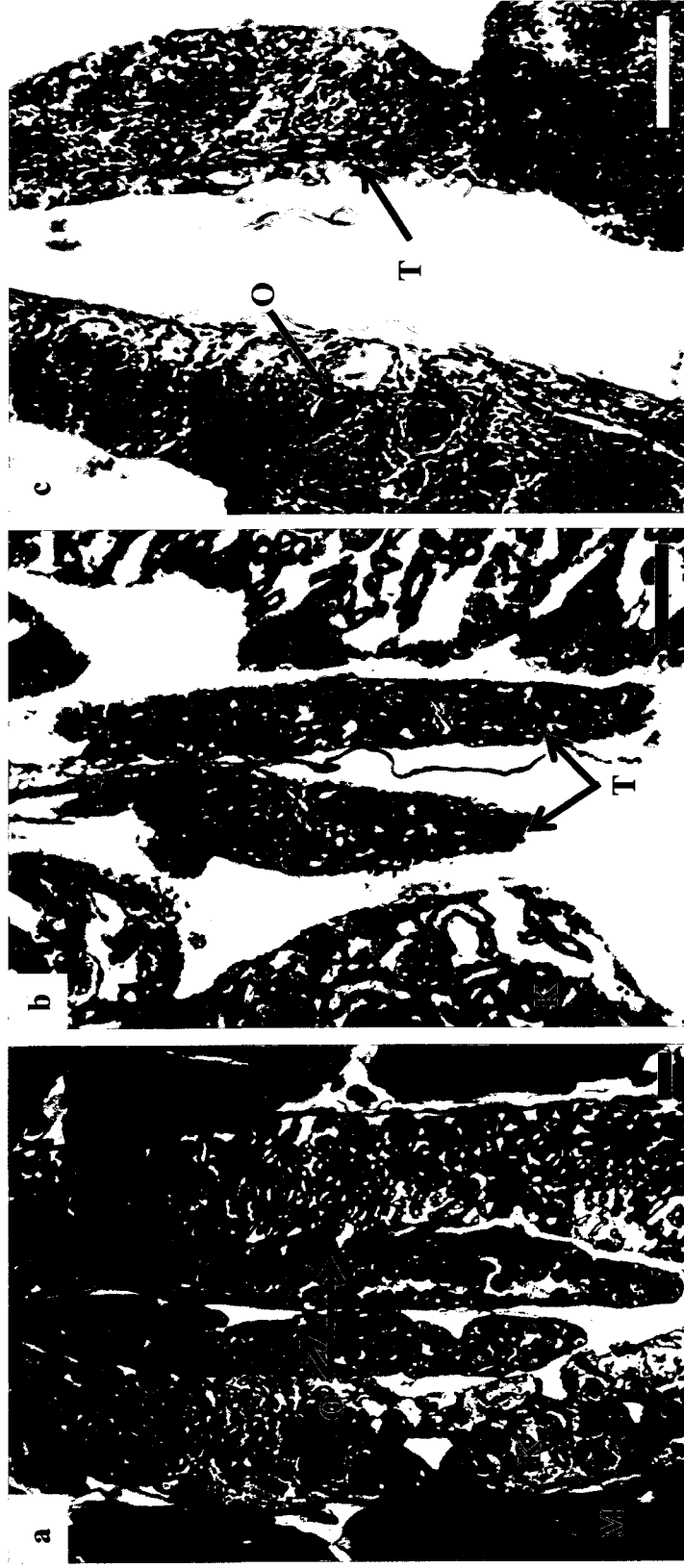


Figure 6.1 Histological sections of NF60 *Silurana tropicalis* gonads for female, male and finasteride-induced intersex individuals. Tadpoles were exposed from NF12 to NF60 to 25 μ M finasteride or to an EtOH control (0.05 %). (a) Normal female, (b) normal male and (c) finasteride-induced intersex. Bar represents 100 μ m. K: kidney; M: muscle tissue; O: ovarian follicle; T: testicular tissue.

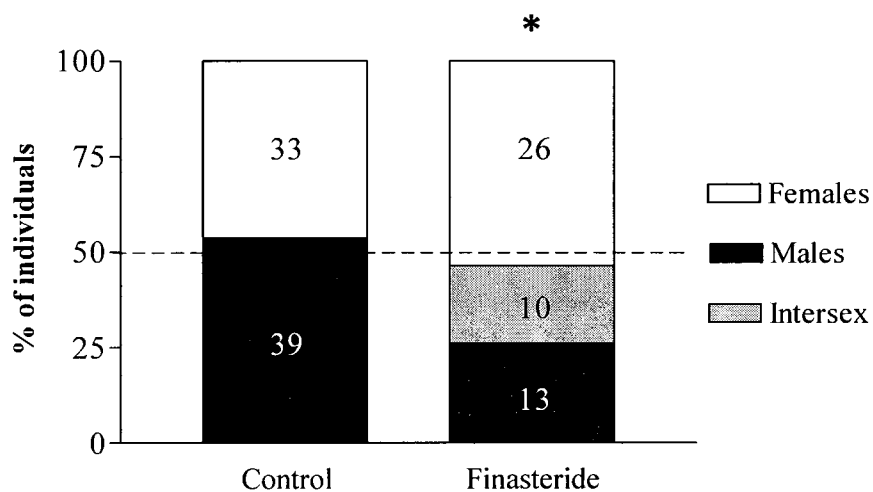


Figure 6.2 Sex ratio of *Silurana tropicalis* after a chronic exposure to finasteride. Tadpoles were exposed to finasteride as described in Fig. 6.1. The numbers on the Y-axis represent the percentage of males, females or intersex individuals per treatment whereas the numbers found inside the bars represent the sample size. Data were analyzed using the Fisher's exact test ($p \leq 0.05$). The asterisk (*) indicates significant differences between the control and finasteride-treated groups.

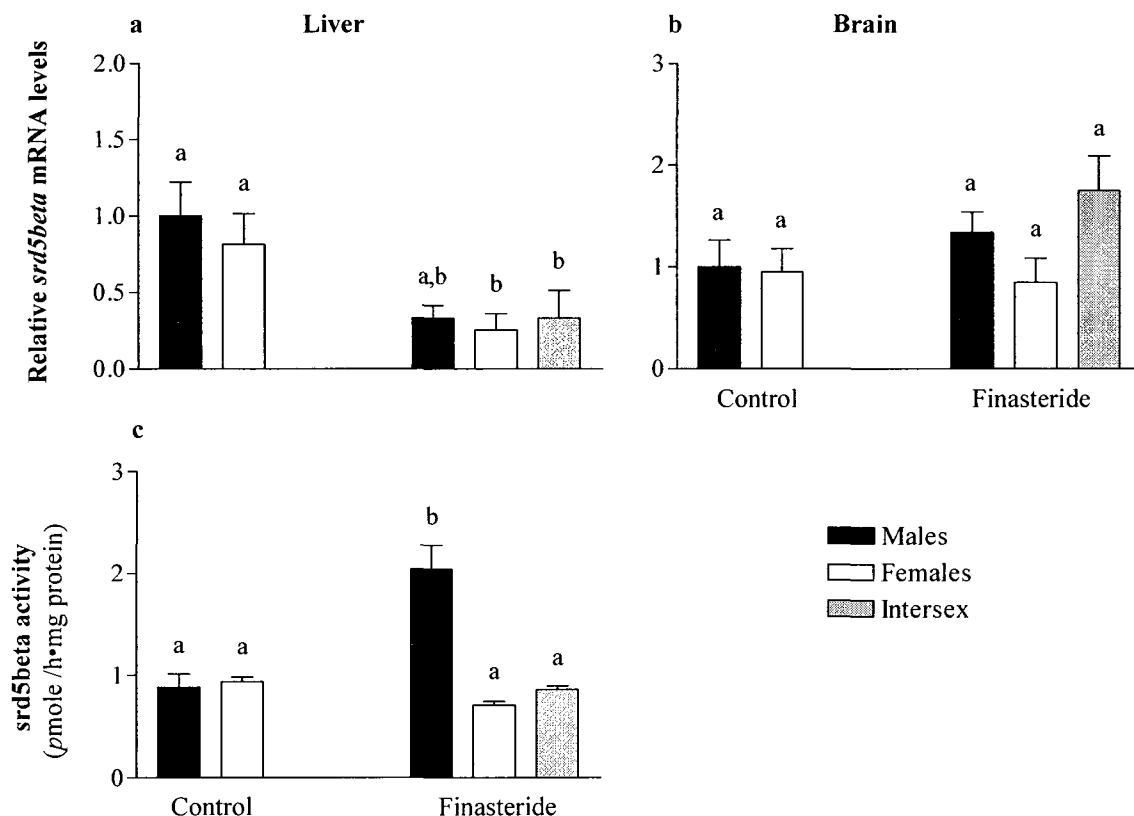


Figure 6.3 Effects of finasteride on gene expression and activity of *srd5beta* in *Silurana tropicalis* tadpoles. Tadpoles were exposed to finasteride as described in Fig. 6.1. Hepatic (a) and brain (b) *srd5beta* mRNA levels are expressed relative to the male control group. Hepatic mRNA levels are normalized to RNA content whereas brain mRNA levels are normalized to the expression of *rpl8*. The enzyme activity of *srd5beta* (c) is expressed in pmole/h normalized to protein content. Bars represent the mean + SEM. Data were analyzed using one-way ANOVA ($n = 6-8$, $p < 0.05$). Different letters indicate statistically significant differences between sex treatments. Note that the scales of the Y-axis vary between graphs. *rpl8*: ribosomal protein L8; *srd5beta*: steroid 5 β -reductase.

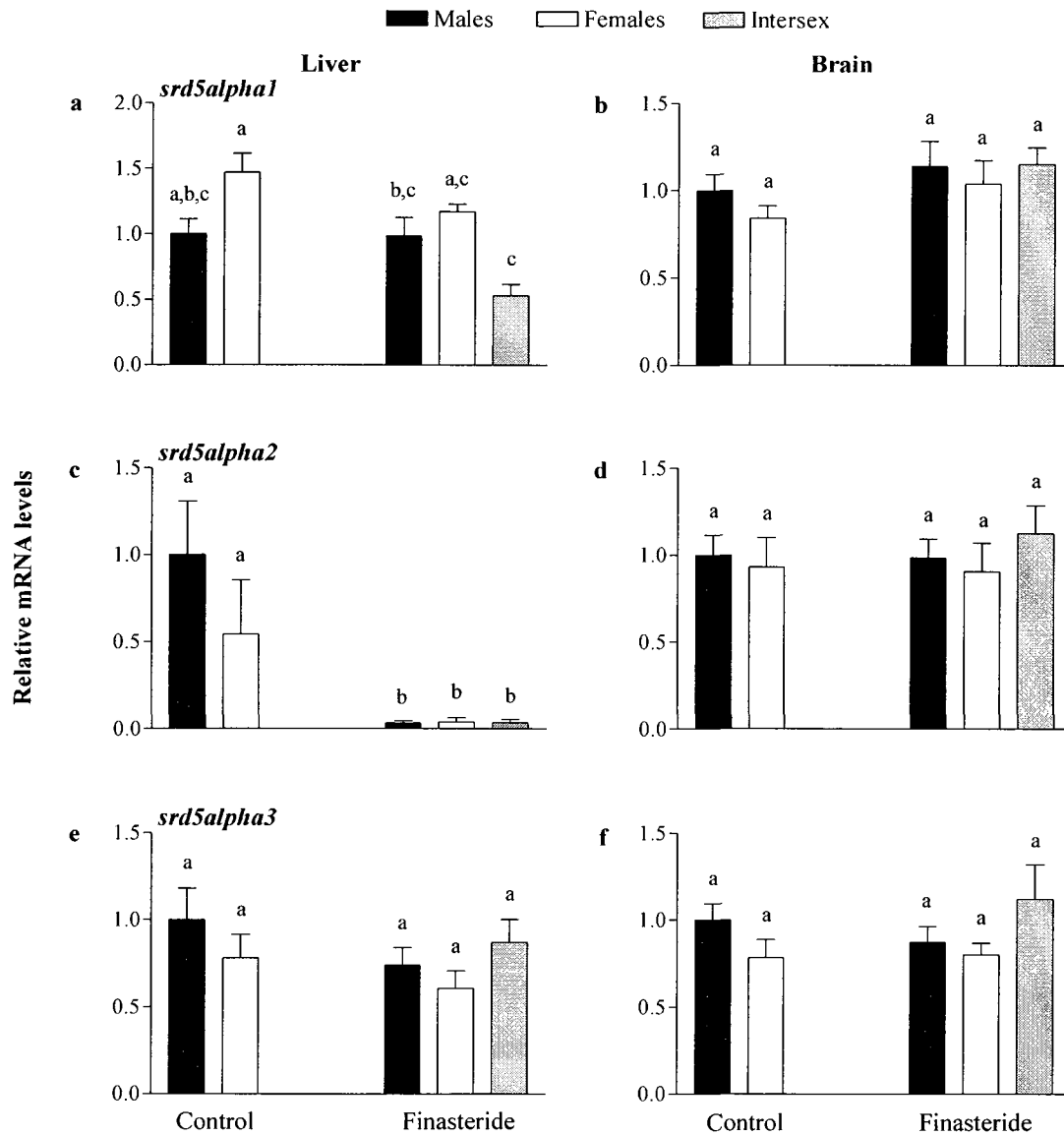


Figure 6.4 Effects of finasteride on gene expression of *srd5alpha1*, *srd5alpha2* and *srd5alpha3* in NF60 *Silurana tropicalis* tadpoles. Tadpoles were exposed to finasteride as described in Fig. 6.1. Hepatic (left panels) and brain (right panels) gene expression data for *srd5alpha1* (a, b), *srd5alpha2* (c, d) and *srd5alpha3* (e, f) are expressed relative to the male control group. Hepatic mRNA levels are normalized to RNA content whereas brain mRNA levels are normalized to the expression of *rpl8*. Bars represent the mean + SEM. Data were analyzed using one-way ANOVA ($n = 8$, $p < 0.05$). Different letters indicate statistically significant differences between sex treatments. Note that the scales of the Y-axis vary between graphs. *rpl8*: ribosomal protein L8; *srd5alpha*: steroid 5 α -reductase.

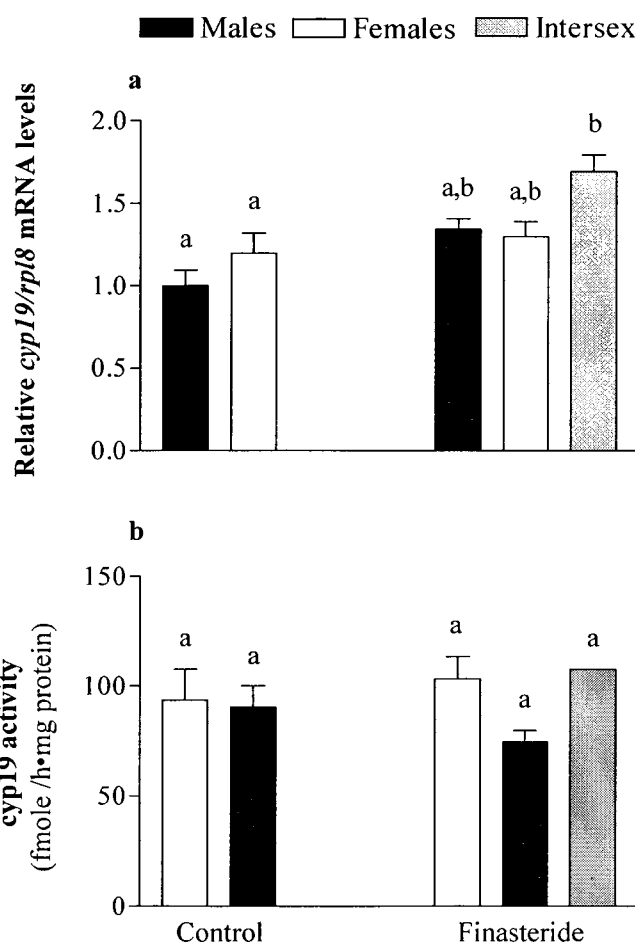


Figure 6.5 Effects of finasteride on *cyp19* mRNA levels (a) and activity (b) in the brain of NF60 *Silurana tropicalis* tadpoles. Tadpoles were exposed to finasteride as described in Fig. 6.1. The mRNA levels of *cyp19* are expressed relative to the male control group and are normalized to the expression of *rpl8* (a). Enzyme activity is expressed in fmole/h normalized to protein content (b). Bars represent the mean + SEM, with the exception of *cyp19* activity of the intersex animal ($n = 1$). Data were analyzed using one-way ANOVA ($n = 6-8$, $p < 0.05$). Different letters indicate statistically significant differences between sex treatments. *cyp19*: aromatase; *rpl8*: ribosomal protein L8.

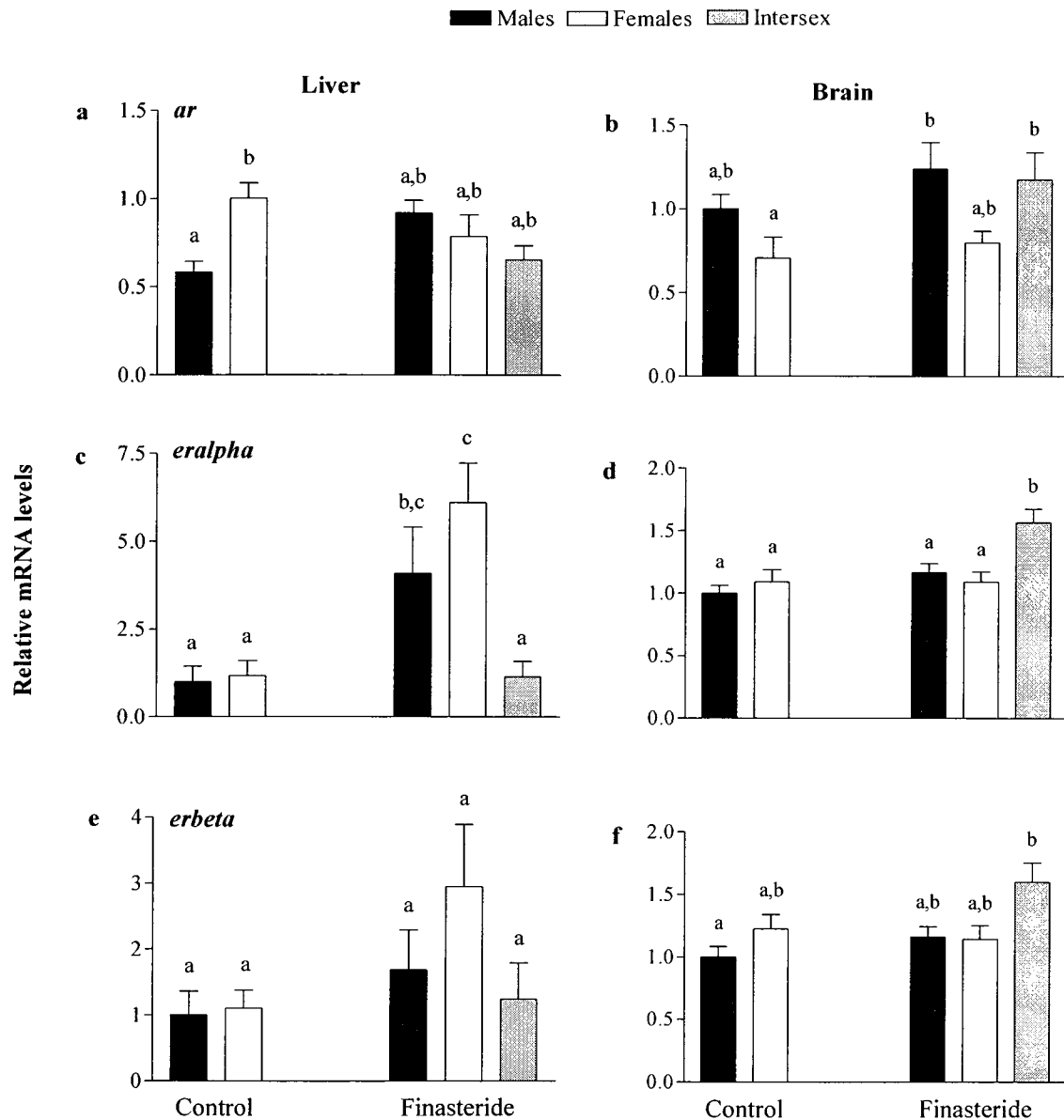


Figure 6.6 Effects of finasteride on gene expression of *ar*, *eralpha*, and *erbeta* in NF60 *Silurana tropicalis* tadpoles. *Silurana tropicalis* tadpoles were exposed to finasteride as described in Fig. 6.1. Hepatic (left panels) and brain (right panels) gene expression data for *ar* (a, b), *eralpha* (c, d) and *erbeta* (e, f) are expressed relative to the male control group. Hepatic mRNA levels are normalized to RNA content; whereas brain mRNA levels are normalized to the expression of *rpl8*. Bars represent the mean + SEM. Data were analyzed using one-way ANOVA ($n = 8$, $p < 0.05$). Different letters indicate statistically significant differences between sex treatments. The scales of the Y-axis vary between graphs. *ar*: androgen receptor; *er*: estrogen receptor; *rpl8*: ribosomal protein L8.

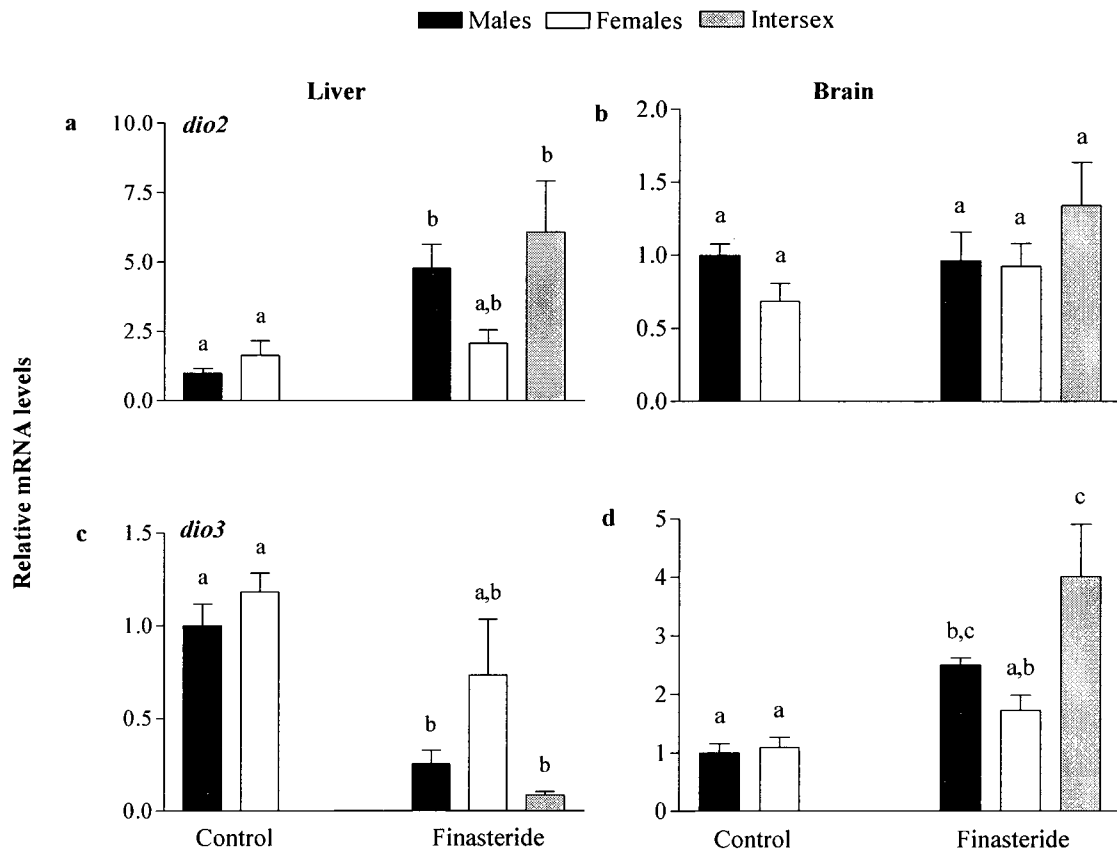


Figure 6.7 Effects of finasteride on gene expression of *dio2* and *dio3* in NF60 *Silurana tropicalis* tadpoles. *Silurana tropicalis* tadpoles were exposed to finasteride as described in Fig. 6.1. Hepatic (left panels) and brain (right panels) gene expression data for *dio2* (a, b) and *dio3* (c, d) are expressed relative to the male control group. Hepatic mRNA levels are normalized to RNA content; whereas brain mRNA levels are normalized to the expression of *rpl8*. Bars represent the mean + SEM. Data were analyzed using one-way ANOVA ($n = 8$, $p < 0.05$). Different letters indicate statistically significant differences between sex treatments. The scales of the Y-axis vary between graphs. *dio*: deiodinase; *rpl8*: ribosomal protein L8.

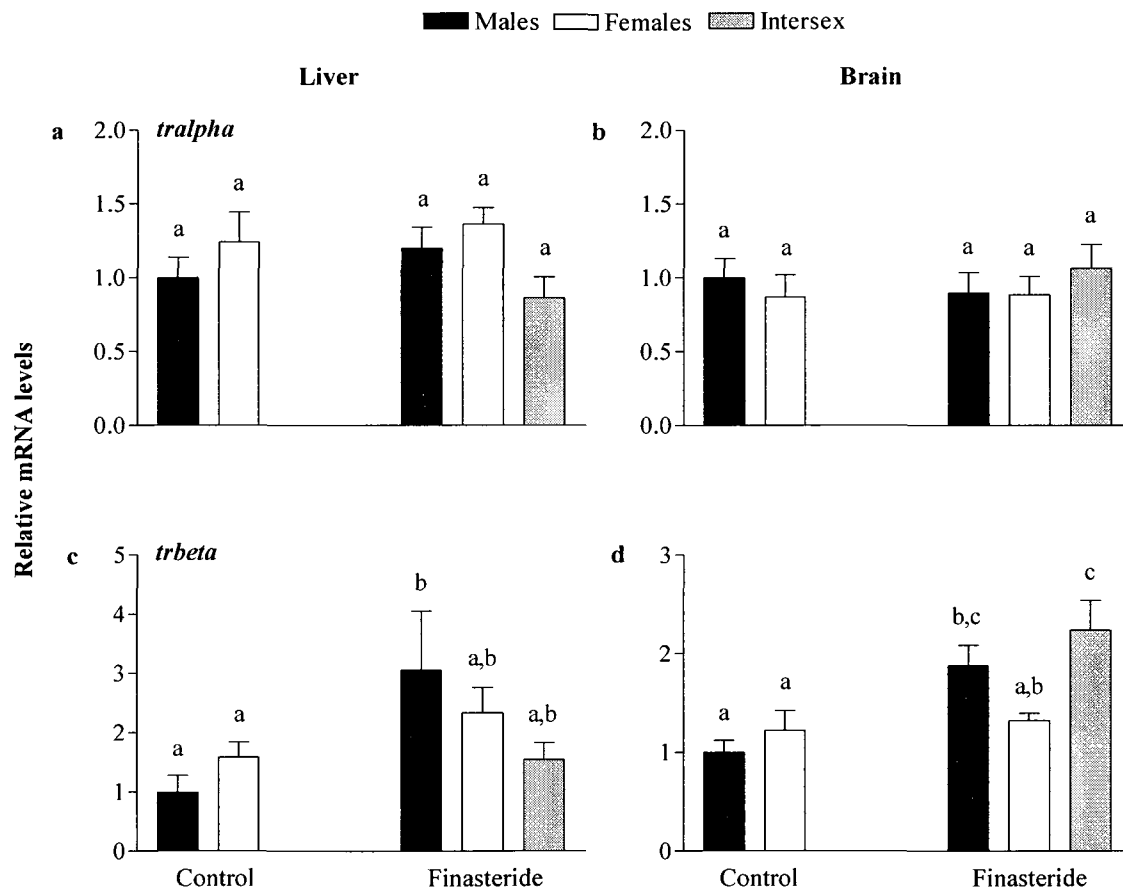


Figure 6.8 Effects of finasteride on gene expression of *tralpha* and *trbeta* in NF60 *Silurana tropicalis* tadpoles. *Silurana tropicalis* tadpoles were exposed to finasteride as described in Fig. 6.1. Hepatic (left panels) and brain (right panels) gene expression data for *tralpha* (a, b) and *trbeta* (c, d) are expressed relative to the male control group. Hepatic mRNA levels are normalized to RNA content; whereas brain mRNA levels are normalized to the expression of *rpl8*. Bars represent the mean + SEM. Data were analyzed using one-way ANOVA (n = 8, $p < 0.05$). Different letters indicate statistically significant differences between sex treatments. The scales of the Y-axis vary between graphs. *rpl8*: ribosomal protein L8; *tr*: thyroid hormone receptor.

6.4 Discussion

Our study demonstrates for the first time that finasteride has the potential of skewing the frog sex ratio by inducing the intersex condition in *S. tropicalis* when exposed chronically during development. We also showed that finasteride exposure altered sex steroid- and TH-related gene expression and demonstrated that brain and liver displayed different gene expression profiles after treatment with finasteride. To our knowledge, this is also the first study to report that intersex frogs express different mRNA patterns than normal males and females, indicating that intersex individuals are physiologically different from the normal animals.

The outcomes of this study revealed that waterborne exposure of tadpoles to a high concentration of finasteride resulted in a decrease in the number of males and an induction of the intersex phenotype. A similar study conducted in *X. laevis* also reported severe disruption of male gonadal development after a chronic exposure to finasteride; however, no effects on sex ratio were observed (Urbatzka et al. 2009). This may be explained by differences between experimental designs (e.g., frog species [*X. laevis*], finasteride concentrations [7 μ M] and developmental stages treated [NF46 to NF66]). In contrast, *Rana clamitans* (green frog) exposed to 100 μ g/L 5 α -DHT had skewed sex ratios toward males (97.7 % of males) and induced the intersex condition (Coady et al. 2004). Although 5 α -DHT levels or *srd5alpha* activity were not measured in this present study, a putative decrease in 5 α -DHT during larval development could explain the mechanism by which finasteride reduces the number of male frogs. However, steroid levels and *srd5alpha* activity need to be assessed.

The ability of finasteride to alter cell differentiation in frog intersex testes may be explained by the lack of 5 α -DHT and/or 5 β -DHT. Real-time RT-PCR analysis revealed that finasteride exposure altered hepatic *srd5alpha2* and *srd5beta* gene expression, which could lead to a decrease in these steroid levels. However, no differences in hepatic *srd5beta* activity between finasteride-induced intersex and normal animals were measured. These results suggest that 5 α -DHT could regulate the expression of these two steroidogenic enzymes in frogs. In rats, 5 α -DHT is known to induce *srd5alpha* mRNA, suggesting that the product of the enzyme positively regulates the expression of the gene (George et al. 1991). A similar positive regulation could also be present for hepatic *srd5alpha2* in frogs and we can speculate that 5 α -DHT could also regulate *srd5beta* gene expression. Furthermore, finasteride treated-male livers expressed 2-fold higher *srd5beta* activity, although this was not accompanied by an increase in *srd5beta* mRNA. This unexpected increase in *srd5beta* activity may be reflective of a compensatory mechanism in males, since this increase was only found in finasteride-treated animals that developed as a male. At the same concentration of finasteride, a decrease of 38 % in *srd5beta* activity was observed in the developing whole *S. tropicalis* larvae (Chapter 5). Therefore, it is possible that finasteride alters *srd5beta* activity in specific tissues such as gonads; future studies should assess gonadal *srd5beta* activity.

Our results also demonstrated that there is a differential response between brain and liver tissues regarding sex steroid-related gene expression (Table 6.2). Of all the genes analyzed only *srd5alpha3* mRNA, which did not change, and *eralpha* mRNA, which increased following finasteride exposure, displayed similar results in both tissues. All other sex steroid-related genes analyzed were altered in only one of the two tissues

analyzed. In the brain, none of the *srd5alpha* and *srd5beta* mRNAs were altered but all the sex steroid receptors (*ar*, *eralpha* and *erbeta*) and *cyp19* mRNAs increased in intersex animals. It has been demonstrated that one of the consequences of finasteride treatment in humans is an increase of plasma testosterone (Habib et al. 1997; Roehrborn et al. 2003). This increase would provide a substrate for *cyp19* to produce estradiol, which in turn may regulate the expression of estrogen-related steroidogenic enzymes and receptors. Although speculative, these increases in estrogen-related gene expression can be explained by a putative increase in testosterone level at the level of the brain.

In another study, reproduction related-gene expression changes were also observed in the brain following chronic exposure to finasteride. Urbatzka and colleagues (2009) demonstrated that treated *X. laevis* males expressed an increase of luteinizing hormone β mRNA and a decrease of the follicle stimulating hormone β mRNA in the brain, confirming the potential of finasteride to disrupt the endocrine signalling related to reproduction in another frog species. Furthermore, in the testes, Urbatzka and colleagues (2009) reported an increase in *srd5alpha2* mRNA level and no changes in *srd5alpha1* and *cyp19* mRNAs. The authors concluded that finasteride alters the testis pathways by increasing steroidogenesis and reducing Sertoli cell proliferation. All together, these results provide the evidence that finasteride alters *srd5alpha* activity in a tissue-specific manner, which consequently affects gene expression of reproduction-related genes.

Interestingly, the expression of *srd5alpha3* mRNA in *S. tropicalis* was never affected by finasteride treatment at early developmental stages (Chapter 5), or later in development in brain and liver (this present study). This newly discovered enzyme seems to be differently affected by finasteride compared to *srd5alpha1* (moderately inhibited)

and *srd5alpha2* (completely inhibited) in humans. This difference in inhibition may be explained by the difference in evolution of these enzymes since *srd5alpha3* is the most ancient form of *srd5alpha*, and for example in humans, only shares approximately 20 and 25 % amino acid similarities with both *srd5alpha1* and *srd5alpha2*, respectively (Chapter 4). To our knowledge, this is the first study assessing the effects of finasteride on *srd5alpha3* in a vertebrate. However, we can speculate that finasteride, a drug designed to reduce 5 α -DHT production, may not be an effective drug against benign prostatic hyperplasia and prostate cancer in humans since *srd5alpha3* can catalyze the conversion of testosterone into 5 α -DHT.

Finasteride also affected TH-related gene expression. Although no effects in metamorphosis were recorded, these changes might have induced alterations in circulating THs or at the tissue level. Therefore, plasma analysis and microscopic examinations of TH-targeted tissue are needed in order to characterize the impacts of these molecular changes in frog morphology. In addition, it has been suggested that there is a cross-regulation between the TH and androgen systems (Chapter 5; Duarte-Guterman et al. 2010; Ram and Waxman 1990). In this present study, finasteride affected *dio* and *tr* expression in the brain and liver in both treated-males and intersex-induced animals. These findings suggest that finasteride treatment results in an increased capacity of hepatic activation of THs (*dio2* gene product converts circulating prehormone thyroxine [T4] into active hormone tri-iodothyronine [T3]); while decreasing the inactivation pathway (*dio3* gene product converts T4 into the inactive reverse T3) in males and intersex. In addition, we measured an increase in *trbeta* mRNA at the brain level, again in both treated-males and intersex. Since *trbeta* expression is autoregulated by THs, this

induction of *trbeta* mRNA supports a putative increase in THs. Based on these data, we can speculate that finasteride-treated males and intersex-induced animals increase their circulating TH concentration to counteract the decrease of *srd5alpha* by finasteride. This is supported by two other studies that demonstrated that TH treatment regulates *srd5alpha* gene expression and activity in vertebrates. Hypothyroid rats exhibited hepatic decreases in *srd5alpha* mRNA level and activity, while T4 addition restored both *srd5alpha* expression and activity (Ram and Waxman 1990). Furthermore, treatment of *S. tropicalis* with T3 increased whole larvae *srd5alpha1* mRNA level in a concentration-dependant manner (Duarte-Guterman et al. 2010). In contrast, during *S. tropicalis* embryogenesis finasteride has been shown to decrease *dio2* mRNA level in whole larvae which would lead to an inactivation of the THs (Chapter 5). This suggests that the developing frog respond differently to finasteride throughout its development. Further studies are required to explore the regulation and the crosstalk between the androgen and TH systems.

In a similar experimental design using developing *S. tropicalis*, treatment with fadrozole (a cyp19 inhibitor) also produces intersex animals, but induced a male-biased sex ratio (Langlois et al. 2010b). Therefore, fadrozole acts as an anti-estrogenic chemical, while finasteride is an anti-androgenic compound. Since both treatments induced intersex animals, we compared their gene expression profiles to establish if the intersex condition refers to only one or to several endocrine pathophysiology (Table 6.3). Finasteride-induced intersex animals have distinct brain and hepatic gene expression profiles compared to the intersex animals produced by fadrozole. Finasteride treatment decreased the expression of *srd5alpha1*, *srd5alpha2* and *srd5beta* and altered TH-related gene

expression, while fadrozole increased the expression of hepatic *srd5alpha1*, *srd5alpha2* and *eralpha* and did not affect TH-related gene transcripts. Hence, we propose that finasteride affects *S. tropicalis* sex ratio by altering male steroidogenesis since it alters androgen-related gene expression. Therefore, the intersex animals were likely originally males. In contrast, fadrozole affects *S. tropicalis* sex ratio by favouring a male-like steroidogenesis pathway since it activates androgen-related gene expression and does not affect the estrogen-related gene expression and therefore, these intersex animals were likely to derive from females (Langlois et al. 2010b). The gene expression results suggest that the intersex condition is a transitional phase during sex reversal from either sex to the other; however the direction of sex reversal (male-to-female or female-to-male) leads to completely different pathophysiologies.

Table 6.2 Summary of brain and hepatic gene expression changes following a chronic exposure to finasteride (25 μ M) during *Silurana tropicalis* development. Statistically significant fold changes are reported along with arrows indicating increase or decrease of fold changes.

Genes	Brain			Liver		
	Male	Female	Intersex	Male	Female	Intersex
<i>ar</i>	1.3 $\uparrow^b$	-	1.2 $\uparrow^b$	-	-	-
<i>srd5alpha1</i>	-	-	-	-	-	1.9 $\downarrow^c$
<i>srd5alpha2</i>	-	-	-	31.8 $\downarrow^a$	25.7 $\downarrow^a$	36.5 $\downarrow^a$
<i>srd5alpha3</i>	-	-	-	-	-	-
<i>srd5beta</i>	-	-	-	-	3.9 $\downarrow^a$	3.3 $\downarrow^a$
<i>eralpha</i>	-	-	1.7 $\uparrow^a$	4.1 $\uparrow^a$	6.1 $\uparrow^a$	-
<i>erbeta</i>	-	-	1.6 $\uparrow^b$	-	-	-
<i>cyp19</i>	-	-	1.7 $\uparrow^a$	<i>nd</i>	<i>nd</i>	<i>nd</i>
<i>tralpha</i>	-	-	-	-	-	-
<i>trbeta</i>	1.8 $\uparrow^a$	-	2.2 $\uparrow^a$	3.1 $\uparrow^a$	-	-
<i>dio2</i>	-	-	-	4.8 $\uparrow^a$	-	6.1 $\uparrow^a$
<i>dio3</i>	2.3 $\uparrow^a$	-	3.8 $\uparrow^a$	3.9 $\downarrow^a$	-	16.1 $\downarrow^a$

nd: not detectable,

$\uparrow$: increase in fold change

$\downarrow$: decrease in fold change

- : no changes

^adifferent than both control males and females ($p < 0.05$)

^bdifferent than control males ($p < 0.05$)

^cdifferent than control females ($p < 0.05$)

Table 6.3 Comparison of hepatic gene expression changes between finasteride-induced and fadrozole-induced *Silurana tropicalis* intersex. Chronic exposures to finasteride (25 µM) or fadrozole (2 µM) during *S. tropicalis* development induced intersex animals. Gene expression data are compared between the two chemically-induced intersex groups. Statistically significant fold changes are reported along with arrows indicating increase or decrease of fold changes.

Genes	Intersex	
	Finasteride ^a	Fadrozole ^b
<i>srd5alpha1</i>	1.9 ↓ ^c	1.8 ↑ ^d
<i>srd5alpha2</i>	36.5 ↓ ^c	3.5 ↑ ^d
<i>srd5alpha3</i>	-	-
<i>srd5beta</i>	3.3 ↓ ^c	-
<i>eralpha</i>	-	4.2 ↑ ^c
<i>erbeta</i>	-	-
<i>cyp19</i>	<i>nd</i>	<i>nd</i>
<i>tralpha</i>	-	-
<i>trbeta</i>	-	-
<i>dio2</i>	6.1 ↑ ^e	-
<i>dio3</i>	16.1 ↓ ^e	-

nd: not detectable

↑ : increase in fold change

↓ : decrease in fold change

- : no changes

^adata reported in this chapter

^bdata from a collaborative work in Langlois et al. (2010b)

^cdifferent than respective control males and females ($p < 0.05$)

^ddifferent than respective control males ($p < 0.05$)

^edifferent than respective control females ($p < 0.05$)

6.5 Conclusions

This study demonstrates that chronic exposure of *S. tropicalis* to finasteride affected gonadal differentiation by altering the sex ratio and inducing intersex animals, which support that *srd5alpha* and *srd5beta* are important enzymes in frog development. Gene expression analyses confirmed that chemically-induced intersex animals display different gene expression profiles when compared to non-exposed animals. Finally, we propose that intersex is a heterogeneous condition with underlying endocrine changes depending on whether the genetic sex is male or female. Therefore, further characterisation at the molecular level of the intersex phenotype is required when studying the effects and the mechanisms of action of EDCs that disrupt sexual development.

CHAPTER 7

General discussion and conclusions

7.1 Thesis summary

The first objective of this doctoral thesis was to determine if the widely used herbicide atrazine (ATZ) alters *Lithobates pipiens* (Northern leopard frog) development using concentrations of ATZ reported in Canadian ecosystems. Waterborne exposures to ATZ were performed in outdoor mesocosms (Chapter 2). The results indicated that 1.8 µg/L ATZ led to a female-biased sex ratio and reduced metamorphic success, which suggests that ATZ could have detrimental effects at the population-level. Modelling of environmental sex reversal scenarios have demonstrated that the consequences of feminization in frog populations are generally negative (e.g., decrease in both population size and persistence of sex chromosomes; Cotton and Wedekind 2009). When comparing our results to those of 38 other peer-reviewed studies on the effects of ATZ in frogs (Chapter 1, Table 1.1, Reference 6), our study is among the 27 studies that have reported an alteration of frog development following exposures to ATZ. Therefore, there was clearly a need to assess the mechanisms by which ATZ is altering both gonadal differentiation and metamorphosis. Real-time RT-PCR and enzymatic activities were assessed in the brain, liver and tail of tadpoles that were exposed and collected at two different developmental stages (Gosner stage 34 and 42) in Chapter 3. The data revealed that ATZ alters the expression of genes in the brain and tail of G34 *L. pipiens* tadpoles and affects the hepatic activity of the steroid 5β-reductase (srd5beta) at metamorphosis. These results confirm that ATZ has an effect at the molecular level in both the reproductive and thyroid axes. These

findings also helped elucidate the mechanisms underlying the disruption of frog development by ATZ and led us to pursue studies on the roles of the *srd5beta* in frogs since, to our knowledge, this was the first report of *srd5beta* disruption in vertebrates by an endocrine disruptor chemical (EDC).

Given that *srd5beta* shares a similar testosterone reducing function as steroid 5 α -reductase (*srd5alpha*), the second objective of this thesis was to characterize the functions of both *srd5alpha* and *srd5beta* in frogs. First, we compared and contrasted the evolution, tissue distribution and biological functions between these enzymes (Chapter 4). The phylogenetic analyses indicated that *srd5alpha* and *srd5beta* represent a convergence in functions in evolution (meaning that they share similar biological functions), but do not have a common ancestor. Furthermore, during this doctoral research work, a third type of *srd5alpha* was identified (Tamura et al. 2007). We therefore established the first phylogenetic analysis of the *srd5alpha* family. Our results indicated that a eukaryotic ancestor likely underwent duplication events to generate three *srd5alpha* subfamilies (*srd5alpha* type 1/2, *srd5alpha*3 and glycoprotein synaptic 2 [*gpsn2*] ancestors). Both *srd5alpha* type 1/2 and *gpsn2* subfamilies may have evolved from ancient duplication events at the early stage of chordate evolution. Another important conclusion from the analyses conducted in Chapter 4 was that data regarding the roles of *srd5alpha* and *srd5beta* in frogs are lacking in the literature. Therefore, we set out to determine if these enzymes were present and functional during frog embryogenesis and undertook studies on the consequences of inhibiting *srd5alpha* and *srd5beta* in frogs.

Developmental expression patterns of *srd5alpha1*, *srd5alpha2*, *srd5alpha3* and *srd5beta* were determined at different developmental stages going from egg to larvae in

Silurana tropicalis (Western clawed frog; Chapter 5). The presence of these enzymes during early frog development indicates that *srd5alpha* and *srd5beta* may have developmental functions during early life. These results were partly supported by the detection of *srd5beta* activity (Chapter 5) and by the work of other studies that identified the presence of the sex steroid hormones in the embryos of vertebrates (Carere and Balthazart 2007; Goldman-Johnson et al. 2008; Iwamatsu et al. 2006). Therefore, to obtain additional evidence that *srd5alpha* and *srd5beta* are functional during early frog development, we inhibited these enzymes to determine whether transcription in larvae would be affected. *Silurana tropicalis* larvae were exposed to finasteride, a known *srd5alpha* and *srd5beta* inhibitor in humans, during embryogenesis and early development (Chapter 5). Results confirmed that finasteride is also an effective inhibitor in *S. tropicalis* and showed that the disruption of *srd5alpha* and *srd5beta*, consequently affected other sex steroid-related and thyroid hormone (TH)-related gene expression and enzymatic activities. All together, this provides strong evidence that *srd5alpha* and *srd5beta* are functional during frog embryogenesis and early development. Furthermore, the ability of finasteride to modulate TH-related gene expression raises questions about the broader roles of *srd5alpha* and *srd5beta* in development.

A chronic exposure to finasteride was carried out from larval to metamorphic stages to determine whether the inhibition of *srd5alpha* and *srd5beta* alters sexual differentiation and development in frogs (Chapter 6). Results demonstrated that finasteride skews the sex ratio by inducing intersex in males. This finding supports the hypothesis that these enzymes are also involved in male sexual development in frogs, as similarly reported in birds and mammals. In addition, gene expression analysis of the brain and liver of these animals confirmed that the inhibition of *srd5alpha* and *srd5beta* caused alterations in the expression of sex steroid-related

genes and provided evidence that 5 α -dihydrotestosterone (5 α -DHT) could regulate the expression of both *srd5alpha2* and *srd5beta* (since mRNA levels were decreased following the treatment despite the increase in *srd5beta* activity in males). Also, data suggest that the putative increase in testosterone level would favour the estrogen production (which could explain the increases in expression of all estrogen-related genes studied in the brain). Furthermore, this chapter also presented data to support the potential cross-regulation between the androgen and thyroid systems.

7.2 Atrazine: what we have discovered and future directions

The contributions of this doctoral thesis to the field of Ecotoxicology regarding the herbicide ATZ are 1) the supporting data that ATZ alters normal development in *L. pipiens* exposed in outside mesocosms, and 2) the investigation of new mechanisms for endocrine disruption caused by this herbicide. Based on the gene expression data presented in this thesis, we propose that completion of metamorphosis is not the best stage of development to measure molecular endpoints. The ideal assessment should occur during the course of sexual development. In other words, we suggest that ATZ exerts its disruption during early larval development via the alteration of the endocrine molecular signalling, which then leads to an imbalance in sex steroid ratios, *i.e.* the balance between androgen and estrogen during sexual differentiation, that consequently affects sex ratio. Therefore, we recommend that future experiments evaluate the molecular actions of ATZ at different periods of the larval development, especially during the sensitive sexual development period.

Another interesting finding that is reported in this thesis is that ATZ eliminates the sexual dimorphism in hepatic *srd5beta* activity in *L. pipiens*. To investigate the importance of this sexual dimorphism, we determined the consequences of inhibiting *srd5beta* activity in frogs. For this study, we used *S. tropicalis* for its wide genomic resources and its ease of manipulation under laboratory settings. Although we found many effects of the inhibition of *srd5beta* on normal sexual development, no hepatic dimorphism in *srd5beta* activity was present between males and females. In fish, a similar differential species pattern exists in *srd5beta* expression where in *Oncorhynchus mykiss* (rainbow trout; Baron et al. 2008) females expressed higher *srd5beta* mRNA levels than males but in *Rhabdosargus sarba* (goldlined seabream; Yeung and Chan 1985), males have higher *srd5beta* levels than females. The reasons for these differences are unknown but could be related to species differences in sexual development or timing of gonadal maturation. Therefore, our *S. tropicalis* data failed to explain the importance of the sexual dimorphism in *srd5beta* activity between *L. pipiens* males and females. Furthermore, the mechanism of inhibition of *srd5beta* by finasteride and ATZ are likely different since different gene expression profiles were observed following exposures to these compounds. For example, ATZ did not affect the expression of *cyp19*, *eralpha*, *tralpha*, *trbeta*, *dio2* and *dio3* in brain and liver at metamorphosis while finasteride did alter the expression of many of these genes. Nevertheless, finasteride data confirmed that a disruption of *srd5beta* activity can have major developmental and physiological effects. Future studies should assess the importance of such a dimorphism in *L. pipiens*, to determine if the action of ATZ on *srd5beta* activity is biologically significant.

The herbicide ATZ has been used since 1960. Over 39 studies have attempted to determine whether or not ATZ alters frog development; however no consensus has yet been established (Chapter 1, Section 1.6.1). The range of different responses that have been reported in the literature may be explained by inter-specific and populational variations. There is evidence that frog populations can respond differently to a contaminant than other populations of the same frog species because of historical exposures to pollutants (Egea-Serrano et al. 2009). Therefore, it is possible that the inter-specific responses of frogs to ATZ can be explained by differences in genetics, maternal transfer of the contaminant or geographic adaptation, all of which could lead, for example, to an increase in the efficiency of metabolic clearance pathways. This range of responses between species and/or populations of frogs has also been reported with other chemicals. For example in frogs, some studies have demonstrated that exposure to nitrogenous compounds reduces the size of larvae (Griffis-Kyle and Ritchie 2007; Shinn et al. 2008), while others have reported no effects of nitrogenous compounds on larval growth (Smith et al. 2004; Vaala et al. 2004). Moreover, differential responses also resulted after exposing tadpoles to ultraviolet B radiation (UVB). In 1926, Higgins and Sheard demonstrated that UVB delayed developmental rate in *L. pipiens* (Higgins and Sheard 1926) whereas scientists remain divided on whether or not UVB alters development rate in *L. pipiens* or in any other species of frogs (reviewed in Croteau et al. 2008). Hence, differences in sublethal responses to a chemical or physical constraint suggest that frog populations and species vary in their ability to respond to a chemical or to a stress; therefore, there is a need to better characterize inter-specific and populational variations (e.g., detoxification pathways) in frogs.

In addition, ATZ does not only affect frog development but has also been shown to alter development in many other vertebrates. In fish, short-term exposure of male *Salmo salar* L. (Atlantic salmon) to 1.0 µg/L ATZ significantly disrupted the olfactory-mediated response to female sex pheromone (prostaglandin F_{2α}). In the same species, exposures to 0.5 and 2.0 µg/L ATZ significantly decreased the levels of milt (Moore and Lower 2001). *Sciaenops ocellatus* (red drum) larvae exposed for four days to 40 and 80 µg/L ATZ exhibited a prolonged larval period and altered swimming behaviour (del Carmen Alvarez and Fuiman 2005). Furthermore, Thibaut and Porte (2004) reported that several EDCs, such as ATZ, organotin, dicofol and phalates, had the capacity to inhibit 5α-reductase activity in *Cyprinus carpio* (common carp) gonads, resulting in decreases in androstenedione and 5α-DHT levels.

Extensive research using the rat model has demonstrated that most of the effects of ATZ are on the reproductive axis. Oral exposures of Long-Evans female rats to ATZ and its metabolite desisopropylatrazine (DIA) induced the hypothalamic-pituitary-adrenal axis response by increasing the plasma concentration of progesterone, corticosterone and adrenocorticotrophic hormones (Fraites et al. 2009). Furthermore, exposures of both Sprague-Dawley and Long-Evans hooded rats to ATZ also demonstrated that ATZ alters the hypothalamic control of luteinizing hormone and prolactin releases by increasing serum levels of these hormones (Cooper et al. 2000). Moreover, the ingestion of ATZ metabolites (DIA, deethylatrazine and diaminochlorotriazine) affected the central nervous system control of the pituitary/gonadal axis in male Wistar rats (Stoker et al. 2002). In addition, ATZ administered during the LH-dependant period of pregnancy in F344 rats caused miscarriage (Narotsky et al. 2001). Concern is rising in the scientific, public and political

communities since ATZ has been shown to have the potential of inducing mammary cancer in adult Sprague-Dawley rats (Birnbaum and Fenton 2003; Ueda et al. 2005).

In humans, Sanderson and colleagues (2002; 2000) studied the effects of ATZ on cyp19 activity and cAMP levels in a human cell line (H295R; adrenocortical carcinoma) and demonstrated that ATZ induced CYP19 activity and increased cAMP levels by approximately 2.5-fold and 1.5-fold above control levels, respectively. Recent epidemiological studies have correlated concentrations of pesticides, including ATZ, to an increase in birth defects in humans in the United States (Winchester et al. 2009). From all the mammalian literature, the World Health Organisation (WHO) has recognized that ATZ is a non-genotoxic chemical capable of inducing mammary tumours in rats by non-genomic mechanisms and has set a guideline for drinking water lower than the guideline established by the U.S. or Canadian governments (WHO 2008; EPA 2009; HC 2008). Therefore, despite the range of study outcomes across vertebrates, we can conclude that ATZ has harmful effects, especially related to sexual development and reproductive function. Finally, it is important to notice that certain governments, such as the European Union completely banned that herbicide from almost its entire territory (EU 2004).

7.3 Steroid-5-reductases: Limitations and future directions

The second objective of this doctoral thesis was to determine the consequences of the inhibition of *srd5alpha* and *srd5beta* in frogs. We provided evidence that *srd5alpha* and *srd5beta* are important enzymes since these enzymes are present in early development and their inhibition resulted in abnormal gonadal development and disrupted endocrine molecular signalling. However, since no specific inhibitor of the different steroid-5-

reductases exists, it remains difficult to conclude which enzyme(s) is(are) responsible for the observed responses to finasteride. *In situ* hybridization and immunohistochemistry could reveal differences in the distribution and abundance of these enzymes after being treated with finasteride which could provide more information about potential functions of *srd5alpha* and *srd5beta*. There is a need to develop specific inhibitors for each of the steroid 5-reductases in order to determine the participation of each of these enzymes.

The roles of 5 β -dihydrotestosterone (5 β -DHT) in vertebrate development remain virtually unexplored. In frogs, studies have demonstrated that 5 α -DHT is involved in the hypothalamic-pituitary-gonadal axis (Tsai and O'Malley 1994) and exposure of *Rana clamitans* (green frog) to this steroid results in quasi-complete female-to-male sex reversal (Coady et al. 2004). Therefore, similar *in vivo* exposures to 5 β -DHT should be attempted to help elucidate the functions of *srd5beta* in the sexual development of frogs. In addition, the regulation of *srd5beta* remains unknown. A recent microarray analysis has identified an induction of *srd5beta* mRNA after treating immortalized human prostate epithelial cells (androgen-responsive and expressing wild-type of androgen receptor) with the synthetic androgen R1881 (Bolton et al. 2007). In fish, treatment of female *Oncorhynchus mykiss* (rainbow trout) treated with an exogenous androgen (11 β -hydroxyandrostenedione) decreased *srd5beta* transcription in gonads (Baron et al. 2008). In birds, exogenous exposure to testosterone and 17 β -estradiol decreased *srd5beta* activity in the preoptic area of the brain of castrated male *Streptopelia risoria* (ring dove, Steimer and Hutchison 1981). Furthermore, this doctoral research provides data suggesting that 5 α -DHT could also be involved in the regulation of *srd5beta* (Chapters 5 and 6). Despite differences in the response of *srd5beta* to androgens, there is evidence that sex steroids do indeed regulate

srd5beta transcription in vertebrates. Clearly, a more systematic comparison of species differences and tissue responses of srd5beta to hormones are required. Also, critically missing are studies analyzing the promoter region of the srd5beta gene (e.g., for the presence of the androgen-, estrogen- and TH response elements), which are needed since this would allow a better understanding of the regulation between the androgen-, estrogen- and TH systems.

Cross-regulation between the reproductive and TH systems has previously been recognized between the estrogen and TH axes in frogs (Hayes 1997; Hogan et al. 2007); however data from this doctoral thesis provides evidence that the TH axis could also be regulated by androgens (Chapters 5 and 6). Alterations of *trbeta*, *dio1*, *dio2* and *dio3* expression following the inhibition of srd5alpha and srd5beta suggest a complex relationship between the TH-responsive genes and the androgen status in early frog development and at metamorphosis. *In vivo* somatic gene transfer approaches could help determine if 5 α -DHT and/or 5 β -DHT can directly modulate TRE-driven reporter genes, as has been performed previously in frogs (Fini et al. 2007; Trudeau et al. 2005). There is also a report that the expression of androgen-responsive genes (e.g., *srd5alpha1* and *ar*) was altered following treatments to THs (Duarte-Guterman et al. 2010). Therefore, there is also evidence for a cross-regulation between the androgen and TH axes in frogs. Co-localization studies using *in situ* hybridization and immunohistochemistry could confirm if srd5alpha, srd5beta, *ar*, *dio1*, *dio2*, *dio3* and *trbeta* are present in same cells and provide support for a crosstalk between these two endocrine systems.

Finally, despite the limitations of this doctoral thesis, we provide evidence that srd5alpha and srd5beta are important in frog development and that there is a potential for

cross-regulation between the androgen and TH axes in frogs. Furthermore, a disruption of these enzymes during larval development results in the alteration of normal endocrine molecular signalling, which can lead to a change in the sex ratios of frog populations.

7.4 Concluding remarks

Chemically-induced changes in sex ratios and disruptions of metamorphosis are important physiological consequences that could potentially alter amphibian population fitness and contribute to worldwide amphibian population declines. Mesocosm exposures were useful to deliver ATZ, and our findings confirm that this herbicide is harmful for *L. pipiens* endocrine molecular signalling, gonadal development and metamorphosis at concentrations reported in Canadian waters. Furthermore, disruption of *srd5alpha* and *srd5beta* activity by finasteride led to many physiological defects (e.g., alteration of gonadal development, targeted gene expression and enzymatic activities). This doctoral research contributes to the fields of classic endocrinology and ecotoxicology by demonstrating that *srd5alpha* and *srd5beta* are important enzymes during frog development and are ‘new’ targets for EDCs.

Annexe 1. Other contributions to research and development

a. Other articles published in refereed journals

3. Duarte-Guterman P, **Langlois VS**, Pauli BP and VL Trudeau. 2010. Expression and T3 regulation of thyroid- and sex steroid-related genes during *Silurana (Xenopus) tropicalis* early development. *Gen Comp Endocrinol.* 166, 428-435.
2. Zhang D, Duarte-Guterman P, **Langlois VS** and VL Trudeau. 2009. Expression profiles of piRNA pathway component proteins during *Silurana (Xenopus) tropicalis* embryogenesis. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. In press (MS17630)*
1. Croteau MC, Renner VE, Archibald F, **Langlois VS**, Cahn J, Ridal J, Trudeau VL, DRS Lean. 2007. Investigation of Pathogenic *Escherichia coli* and Microbial Pathogens in Pulp and Paper Mill Biosolids, *Water Environ Res* 79:9, 1050-6.

b. Conference proceedings (^aOral presentation, ^bPoster presentation)

14. **Langlois VS^a**, Duarte-Guterman P, Ing, S, Hodgkinson KM, Pauli BD, Wade MG, Cooke GM and VL Trudeau. The Effects of the Herbicide Atrazine and the Prostate Drug Finasteride on 5-beta-reductase in Two Species of Frogs. 36th Aquatic Toxicity Workshop (La Malbaie, Qc), September 27-30th. *Recipient of an honorable mention for best presentation.*
13. **Langlois VS^a**, Duarte-Guterman P, Hodgkinson K, Wade MG, Pauli BD, Cooke GM and VL Trudeau (2009) The Aromatase Inhibitor Fadrozole and the 5-Reductase Inhibitor Finasteride affect Gonadal Differentiation and Gene Expression in the Frog *Silurana tropicalis*. 16th International Congress of Comparative Endocrinology (Hong Kong, SAR), June 22-26th. *Recipient of the International Travel Award.*
12. **Langlois VS^b**, Duarte P, Ing S, Pauli BD, Wade, MG, Cooke G and VL Trudeau (2008) Consequences of Steroid 5 α - and 5 β -reductase Inhibition during Amphibian Embryogenesis 13th International Congress on Hormonal Steroids and Hormones & Cancer (Québec), September 27-30th.
11. Duarte P^b, **Langlois VS**, Ing S, Pauli BD, Wade MG, Cooke GM and VL Trudeau (2008) The Aromatase Inhibitor Fadrozole affects Thyroid- and Androgen-related Gene Expression and Sexual Differentiation in the Frog *Xenopus tropicalis*. 13th International Congress on Hormonal Steroids and Hormones & Cancer (Québec), September 27-30th.

10. Trudeau VL, Hogan, NS^a, **Langlois VS**, Davidson M, Duarte P, Croteau MC, Pauli BD, Wade MG and DRS. Lean (2008) Disruption of Sexual Development and Metamorphosis by Pollutants and UV-B Radiation: Can this Contribute to Amphibian Decline? 5th Society of Environmental Toxicology and Chemistry (SETAC) World Congress, Sydney (Australia), August 3-7th.
9. Ing S, Duarte P, **Langlois VS**^b and VL Trudeau (2008) The Effect of Inhibiting Sex Steroid Synthesis on Gene Expression and on Sexual Differentiation in Early *Xenopus tropicalis* Development, Annual Ottawa Reproductive Biology Workshop (ON), May 14th. *Recipient of the 2nd prize for Best Ph.D. Poster Presentation.*
8. **Langlois VS**^a, Carew AC, Pauli BD, Wade M, Cooke G and VL Trudeau (2007) Environmentally Relevant Levels of Atrazine in Outdoor Microcosms disrupt Metamorphosis and Sexual Development in *Rana pipiens*, SETAC North American, 28th Annual Meeting, Milwaukee (WI), November 11-15th.
7. **Langlois VS**^b, Carew AC, Pauli BD and VL Trudeau (2007) The Effects of the Herbicide Atrazine on Metamorphosis in *Rana pipiens* Canadian Society of Zoology Annual Meeting Montréal (QC), May 21-25th.
6. **Langlois VS**^b, Carew AC, Pauli BD and VL Trudeau (2007) A Microcosm Approach for Atrazine Aquatic Toxicology Experiments. Canadian Water Network Annual Meeting Montréal (QC), November 20-23th.
5. **Langlois VS**^b, Duarte P, Hogan NS, Pauli, BD and VL Trudeau (2006) The Effects of the Herbicide Atrazine on Metamorphosis in *Rana pipiens*. SETAC North America, 27th Annual Meeting, Montréal (QC), November 6-9th.
4. **Langlois VS**^a, Duarte P, Hogan NS, Pauli BD and VL Trudeau (2006) Effects of the Herbicide Atrazine on Sexual Development and Metamorphosis of the Northern Leopard Frog from the Raisin River, Cornwall (ON) Source Water Protection, 13th Annual International Conference on the St. Lawrence River/Great Lake Ecosystems, Cornwall (ON) May 16-18th.
3. **Langlois VS**^a, Duarte P, Hogan NS, Pauli BD and VL Trudeau (2006) Effects of the Herbicide Atrazine on Sexual Development and Metamorphosis of the Northern Leopard Frog from the Raisin River, Cornwall (ON), 9th Annual GSAÉD Interdisciplinary Conference – The Politization of Research, February 8 – 10th.
2. **Langlois VS**^a, Duarte P, Hogan NS, Pauli BD and VL Trudeau (2006) Effects of the Herbicide Atrazine on Sexual Development and Metamorphosis of the Northern Leopard Frog from the Raisin River, Cornwall (ON), Comparative Physiology and Biochemistry Workshop, Rice Lake (ON), February 3 – 5th.
1. **Langlois VS**^b, Croteau MC, Renner V, Cahn JN, Ridal J, Lean DRS and VL Trudeau (2005) Development of a Polymerase Chain Reaction Assay for the Detection of Pathogenic *Escherichia coli* in Pulp and Paper Biosolids, AMERICANA,

Montréal (QC), May 21-26th. *Recipient of the ECO Canada's Student Award for Environmental Excellence.*

c. Other creative scientific contributions

1. Media appearances

- University of Ottawa (Article) - Press release about my ATZ article published in Environ Health Perspect , F2009, http://www.medias.uottawa.ca/salledesmedias/nouvelles-details_1818.html
 - CBC (Canadian Broadcasting Corporation), Canada (TV)
<http://www.cbc.ca/technology/story/2009/12/03/ottawa-frogs-sex-change-atrazine.html#socialcomments>
 - Washington Post, Washington, D.C., USA (Article)
http://www.washingtonpost.com/wp-dyn/content/article/2010/01/11/AR2010011103384_pf.html
 - InSciencesOrganisation, Switzerland (Article)
http://insciences.org/article.php?article_id=7813
 - ScienceDaily, Washington, D.C., USA (Article)
<http://www.sciencedaily.com/releases/2009/12/091203225038.htm>
 - Yubanet, California, USA (Article)
<http://yubanet.com/scitech/University-of-Ottawa-researchers-discover-popular-herbicide-affects-sexual-development-in-frogs.php>
 - Reuters, USA (Article)
<http://www.reuters.com/article/idUSTRE5B24AM20091203>
 - La Nouvelle, Eastern ON, Canada (Article)
<http://www.journallanouvelle.ca/article-408324-Un-herbicide-dommageable-pour-les-grenouilles-disent-des-chercheurs-de-lUniversite-dOttawa.html>
 - TagRadio, Ottawa, Canada (Radio) www.tagradiox.com (16:00-16:10 in French)
- Radio-Canada, (TV) - Interviewed for a report on Amphibian Decline, S2006

2. Outreach teaching

- Organized and taught the *Envirothon Environmental Workshop* to high school students (S2008; Matawa, ON, 50h)
- Organized and taught the *Biology Mini-Course's* week to high school students (S2008; Ottawa, ON, 60h)
- Teacher for *Let's Talk Sciences* to elementary and high school students (F2005-W2006)

3. Conference organization

5th Annual Symposium and Research Day of the Ottawa-Carleton Institute of Biology, University of Ottawa, ON, May 28-29, 2008.

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