INFLUENCE OF TESTOSTERONE METABOLIC PATHWAYS ON DEVELOPMENT AND REPRODUCTION IN AMPHIBIANS

LE ROLE DU METABOLISME DE LA TESTOSTERONE SUR LE DEVELOPPEMENT ET LA REPRODUCTION DES AMPHIBIENS

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Abstract

Amphibian populations are declining worldwide partly due to environmental pollution. Certain chemicals (e.g., pesticides, pharmaceuticals, and plasticizers) present in our ecosystem have been shown to disrupt the endocrine system in vertebrates by interfering with normal steroidogenesis. Steroidogenesis includes the synthesis of androgens, which are essential hormones involved in vertebrate development and reproduction. Androgens are synthesized from the precursor testosterone (T) by specific enzymes (e.g., steroid 5-reductases) and need to be present in a balanced ratio for an organism to function normally. Three types of 5α-reductases (Srd5α) and one type of 5β-reductase (Srd5β) are involved in the conversion of T into 5α-dihydrotestosterone (5α-DHT) and 5β-dihydrotestosterone (5β-DHT), respectively. 5α-DHT is a potent androgen regulating male fertility, whereas 5β-DHT and other 5β-metabolites synthesized by Srd5β have been shown to be involved in bile acid synthesis, initiation of hormone clearance, drug metabolism, erythropoiesis, vasorelaxation, parturition, and synthesis of neuroactive steroids. Given the vital functions of steroid metabolites produced by Srd5α and Srd5β, it is not surprising that a reduced synthesis and functionality of these enzymes can cause substantial health issues. In amphibians, the inhibition of the activity of Srd5α2 leads to feminization and decreased spermatogenesis. However, little is known about the biological functions and regulation of Srd5 in this taxon and how endocrine disrupting chemicals affect these enzymes. Hence, the main objective of this thesis was to further our understanding on Srd5 in developing and adult *Silurana tropicalis* frogs. First, I confirmed that endocrine disrupting chemicals (i.e., phthalates) modify *srd5* mRNA levels during embryogenesis in *S. tropicalis*. Then, to gain a better understanding of the tissue distribution of *srd5* (*srd5α1, srd5α2, srd5α3,* and *srd5β*) during embryogenesis, whole mount *in situ* hybridization was performed and showed a unique expression pattern for all *srd5*. Data suggested that metabolites synthesized by Srd5 are required in the central nervous, sensory, cardiac, respiratory, and detoxifying systems aside from reproduction in early anuran development. Complementary hormonal and specific DNA methylation assays were conducted during anuran embryogenesis and adulthood. Experiments demonstrated that specific DNA methylation of *srd5α1* and *srd5α3* is involved in regulation of the gene expression during embryogenesis as well as in mature gonads. In addition, exposure to T modified *srd5* mRNA levels in gonads in a sex-specific manner demonstrating that androgens also regulate *srd5* in frog gonads. Taken together, my thesis confirms the importance of Srd5 in biological functions related to anuran reproduction, but also provides evidence that this enzyme family is crucial in other biological functions for proper development.

Résumé

Les populations d'amphibiens sont en déclin dans le monde, et ce, en partie en raison de la pollution environnementale. Certains produits chimiques (par exemple, les pesticides, les produits pharmaceutiques, et des plastifiants) présents dans notre écosystème ont été perturbent le système endocrinien des vertébrés en interférant avec la stéroïdogenèse normale. La stéroïdogenèse comprend la synthèse des androgènes, qui sont des hormones essentielles impliquées dans le développement et la reproduction des vertébrés. Les androgènes sont synthétisés à partir de la testostérone (T) par des enzymes spécifiques (i.e., 5-réductases stéroidiennes) et doivent être présents dans un rapport équilibré afin qu'un organisme puisse fonctionner normalement. Trois types de 5α-réductases (srd5α) et un type de 5β-réductase (srd5β) sont impliqués dans la conversion de la T en 5α-dihydrotestostérone (5α-DHT) et en 5β-dihydrotestostérone (5β-DHT), respectivement. La 5α-DHT est un androgène puissant qui régule la fertilité masculine, alors que la 5β-DHT et d'autres 5ß-métabolites synthétisées par la Srd5β sont impliquées dans la synthèse des acides biliaires, l'initiation de la clairance des hormones, le métabolisme des médicaments, l'érythropoïèse, la vasorelaxation, la parturition et la synthèse des stéroïdes neuroactifs. Compte tenu des fonctions vitales de métabolites des stéroïdes produites par Srd5α et Srd5β, il est pas surprenant qu'une synthèse réduite de ces enzymes peut causer des problèmes de santé importants. Chez les amphibiens, l'inhibition de l'activité de la Srd5α2 conduit à la féminisation des individus et à la diminution de la spermatogenèse. Cependant, les fonctions biologiques et la régulation des Srd5 ainsi que les effets des perturbateurs endocriniens qui affectent ces enzymes sont peu connus chez ce taxon. Par conséquent, l'objectif principal de cette thèse était d'étudier les fonctions et la régulation des Srd5 lors du développement et à l'âge adulte de la grenouille *Silurana tropicalis*. Tout d'abord, j'ai confirmé que des perturbateurs endocriniens (i.e., les phtalates) modifient les niveaux d'ARNm *srd5* lors de l'embryogenèse chez *S. tropicalis*. Ensuite, afin d'obtenir une meilleure compréhension de la distribution tissulaire des *srd5* (*srd5α1, srd5α2, srd5α3* et *srd5β*) au cours de l'embryogenèse, l'hybridation *in situ* a été réalisée et a montré un profil d'expression unique, et ce, pour tous les *srd5*. De plus, mes données suggèrent que les métabolites synthétisés par les Srd5 seraient nécessaires dans le système nerveux central, sensorielle, cardiaque, respiratoire et du système de détoxification durant le développement précoce des grenouilles. Des essais hormonaux et de methylation spécifique de l'ADN complémentaires ont été effectués au cours de l'embryogenèse et à l'âge adulte des amphibiens. Ces expériences ont démontré que la méthylation de l'ADN spécifique de *srd5α1* et *srd5α3* est impliquée dans la régulation de l'expression de ces gènes au cours de l'embryogenèse ainsi que dans les gonades matures. De plus, l'exposition à la T a modifié les taux d'ARNm des *srd5* dans les gonades d'une manière spécifique au sexe. Finalement, ma thèse confirme l'importance des Srd5 dans les fonctions biologiques liés à la reproduction, mais également que cette famille d'enzymes est cruciale pour d'autres fonctions biologiques essentielles au bon développement des amphibiens.

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List of Abbreviations

aa	Amino acid
ANOVA	Analysis of variance
AR	Androgen receptor
ARE	Androgen responsive element
BPA	Bisphenol A
BzBP	Benzylbutyl phthalate
CA	Chenodeoxycholic acid
CNS	Central nervous system
CpG	Cytosine guanidine dinucleotide
Cyp19	Aromatase
DBP	Dibutyl phthalate
DCHP	Dicyclohexyl phthalate
DEHP	Diethyl-hexyl phthalate
DEP	Diethyl phthalate
5α-DHT	5α-dihydrotestosterone
5β-DHT	5β-dihydrotestosterone
DMP	Dimethyl phthalate
DPeP	Di-n-pentyl phthalate
E2	Estradiol
EE2	Ethinylestradiol
EDC	Endocrine disrupting chemical
EEF1α1	Eukaryotic elongation factor 1α1
ER	Estrogen receptor
FIN	Finasteride
FDR	False discovery rate
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
HPG	Hypothalamus-pituitary gonadal axis
HSD	Hydroxysteroid dehydrogenase
LH	Luteinizing hormone
MBP	Mono-butyl phthalate
MEHP	Mono(2-ethylhexyl) phthalate
MEP	Mono-ethyl phthalate
MnOP	Mono-n-octyl phthalate
MMP	Monomethyl phthalate
MPeP	Mono-n-pentenyl phthalate
MS-222	Ethyl 3-aminobenzoate methanesulfonate
MSRE	Methyl sensitive restriction enzyme
MT	17α-methyltestosterone

NF	Nieuwkoop and Faber
PAGE	Parametric analysis of gene set enrichment
RIN	RNA integrity number
SD	Standard deviation
SEM	Standard error of the mean
SNEA	Sub-network enrichment analysis
Srd5α	Steroid 5α-reductase
Srd5α1	Steroid 5α-reductase Type 1
Srd5α2	Steroid 5α-reductase Type 2
Srd5α3	Steroid 5α-reductase Type 3
Srd5β	Steroid 5β-reductase
Star	Steroidogenic acute regulatory protein
T	Testosterone
UA	Ursodeoxycholic acid

CHAPTER 1: GENERAL INTRODUCTION

1.1 PROBLEM IDENTIFICATION

Amphibian populations are experiencing a worldwide decline in diversity and number (Wake and Vredenburg, 2008). Many factors, including habitat destruction, climate change, emerging diseases, and environmental pollution have been shown to affect the biodiversity (Wake, 2012). Amphibians live in both, terrestrial and aquatic ecosystems, which increases their risk of exposure to contaminants present in the environment at the embryo and adult life stage (Boyer and Grue, 1995). The permeable skin of amphibians allows penetration of toxic substances present in the environment, resulting in a disruption of normal growth and sexual development (Reeder et al., 2004). The interaction of chemical compounds with the sexual development can ultimately result in a decreased amphibian population (Reeder et al., 2004). Therefore, the sexual development of amphibian species is often assessed in studies investigating the effects of chemical compounds present in the environment. Most commonly, these compounds interfere with the endocrine system and are known as endocrine disrupting chemicals (EDCs) that have been shown to interfere with the reproductive systems in vertebrates (Milnes et al., 2006). However, the effects of EDCs on amphibians are currently not as well understood as compared to mammalian species (Milnes et al., 2006).

The purpose of this thesis was to investigate how EDCs interfere with steroidogenic enzymes involved in the testosterone (T) metabolism in amphibians, in particular in the frog *Silurana tropicalis*. This first chapter introduces the concepts of EDCs, androgens, and steroidogenic enzymes involved in T metabolism, and also provides an overview of all the objectives and hypotheses of this thesis.

1.2 ENDOCRINE DISRUPTING CHEMICALS

EDCs have gained increasing attention due to their proven adverse effects on the environment, wildlife, and humans. Sources of EDCs include plasticizers, detergents, pharmaceuticals, cosmetics, flame retardants, herbicides, and pesticides that are released from households, hospitals, and industries; resulting in widespread detection of these compounds in rivers and lakes (Bolong et al., 2009; Metcalfe, 2003). Thus, aquatic wildlife such as amphibians are at great risk in developing adverse effects due to their exposure to contaminated water in particular during tadpole development (Beck and Slack, 2001). EDCs interfere with the development and have been shown to affect the endocrine, immune, and reproductive systems (Colborn et al., 1993). A broad range of EDCs has been shown to negatively impact the reproduction system in wild frog populations and laboratory exposed animals (reviewed in both Egea-Serrano et al., 2012 and Orton and Tyler, 2015). For example, gestagens and substances with estrogenic, anti-estrogenic, androgenic, and anti-androgenic properties are compounds known to result in adverse effects on the amphibian life cycle (gestagens: Orlando and Ellestad, 2014; estrogens and androgens: Fort et al., 2004; Gyllenhammar et al., 2009; Levy et al., 2004). For example, changes in male fertility, including a biased sex ratio and the occurrence of intersex animals has been observed in anurans (Gyllenhammar et al., 2009; Langlois et al., 2010a; Milnes

et al., 2006). Intersex animals are characterized by the occurrence of oocytes in the seminiferous tubules or spermatogenic nests in oocytes and were observed following exposure to certain EDCs including ethinylestradiol (EE2), atrazine, tamoxifen, finasteride, and methyldihydrotestosterone (Cevasco et al., 2008; Duarte-Guterman et al., 2009; Langlois et al., 2010a). Moreover, laboratory studies have demonstrated that exposure to compounds, including ammonium perchlorate, EE2, 4-nonylphenol, bisphenol A (BPA), butylhydroxyanisol, octylphenol, and dibutyl phthalate (DBP) during frog metamorphosis resulted in feminization (Duarte-Guterman et al., 2009; Goleman et al., 2002; Kloas et al., 1999; Ohtani et al., 2000). Thus, EDCs have been shown to result in adverse effects in the reproductive system of anurans.

EDCs mediate their actions through different pathways. Currently, EDCs are known to 1) bind to receptors, 2) inhibit steroidogenic enzymes, and 3) disturb the function or production of nuclear receptors. Chemicals with a similar structure to steroid hormones such as synthetic estrogens or androgens can interfere with receptors and therefore mimic the presence of a certain hormone (Lintelmann et al., 2003). For example, EE2 binds to the estrogen receptor and mimics the presence of estradiol (E2; Bai et al., 2011). This mechanism of action has been shown to lead to feminization (Cevasco et al., 2008). Moreover, herbicides and pharmaceuticals are known to bind to androgen and estrogen receptors (AR, ER) and subsequently modify steroid levels in amphibians (Orton et al., 2009; Urbatzka et al., 2007; Wyk et al., 2003). A change in steroid level can ultimately lead to an unbalanced steroid ratio, which can also have detrimental effects on the reproductive system. Additionally, EDCs can also interfere with enzymes involved in the steroid synthesis (Lintelmann et al., 2003). For example, exposure to the antifouling chemical tributyltin lead to a decreased level of aromatase (Cyp19) expression (enzyme converting T to E2); which ultimately resulted in intersex animals in snails (Oehlmann et al., 1996). Lastly, EDCs can also disturb the function or production of nuclear receptors (Goksøyr, 2006). Since the production of certain steroid hormone receptors is under autoregulatory control, some hormone mimics may activate the receptor gene itself through their agonistic or antagonistic effect (Rotchell and Ostrander, 2003). These findings provide evidence that EDCs interact with the vertebrate reproductive system in various ways. In particular, males are dependent on a proper androgen level to successfully develop and maintain a working reproductive system.

1.3 ANDROGENS

Androgens are essential hormones required for developing male sexual characteristics and to maintain fertility (Davison and Bell, 2006). However, these male sex hormones also have other vital biological functions, including immune response, growth, osmoregulation, apoptosis, transport and oxidation of lipids, synthesis and transport of hormones, protein metabolism, cell proliferation, and in the central nervous system (CNS; Águila et al., 2013; Lieberman et al., 2001; Martyniuk and Alvarez, 2013; Sangiao-Alvarellos et al., 2006; Sparks et al., 2003). For example, the level of androgens is crucial in the regulation of neurogenesis in the brain in both males and females and contribute to the sexually different structure of the brain (reviewed in Mahmoud et al., 2016). In the liver, androgens have a crucial role in fat metabolism (Vehmas et al., 2016). Androgens are also the precursor for estrogens, which bind to the ER to regulate gene expression (Chang, 2012). Estradiol is the dominant estrogen involved in sexual differentiation and during the reproductive cycle in females. Both androgens and estrogens are synthesized by a series of enzymes known as steroidogenic enzymes. Steroidogenesis is a complex biological

process where cholesterol is converted into many sequential steroids. Five major classes of steroids exist and are all structurally related: progestagens, glucocorticoids, mineralcorticoids, androgens, and estrogens (Norris and Carr, 2013). Cholesterol is first converted to progestagens by cholesterol side-chain cleavage enzyme and 17α-hydroxylase and then further transformed into T by 17, 20 lyase, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-HSD. Testosterone is then converted into the more potent androgen 5α-dihydrotestosterone (5α-DHT) by steroid 5α-reductase (Srd5α). Testosterone can also be converted to 5β-DHT by steroid 5β-reductase (Srd5β) or to E2 by aromatase. Figure 1 shows a simplified overview of the compounds that are synthesized from T.

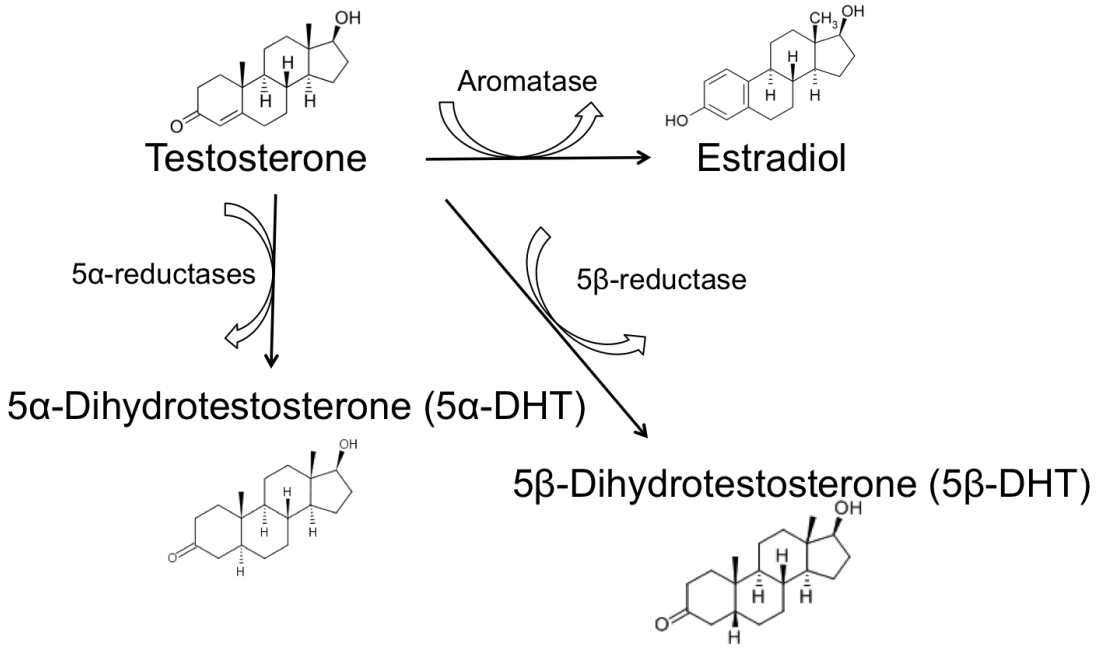

Figure 1 Synthesis of androgens and estrogens from testosterone by steroidogenic enzymes steroid 5α-reductase, steroid 5β-reductase, and aromatase.

1.4 STEROID 5α-REDUCTASE

Srd5α enzymes convert the Δ-4,5 bond of C-19 and C-21 steroids to 5α-stereoisomers. The best-known reaction is the synthesis of 5α-DHT from T due to its strong androgenic properties and its necessity in male sexual differentiation (Davison and Bell, 2006). According to their reaction catalysis, Srd5α enzymes belong to the enzyme superfamily oxidoreductase. There are three isoforms of Srd5α: Srd5α1, Srd5α2, and Srd5α3 (reviewed in Azzouni et al., 2012). Even though the three isoforms are able to catalyze the same reaction, the protein sequence homology is only partly identical. A comprehensive analysis of the three isoforms in different species was previously performed (Langlois et al., 2010b). In *Homo sapiens*, the three isoforms share only 46%, 25%, and 20% sequence homology between Srd5α1 and Srd5α2, Srd5α2 and Srd5α3, and Srd5α1 and Srd5α3, respectively. Throughout different species, the protein sequence homology for Srd5α1, Srd5α2, and Srd5α3 is 53, 65, and 61% alike. The protein sequence for *Homo sapiens* consists of 259 amino acids (aa) for Srd5α1, 254 aa for Srd5α2, and 318 aa for Srd5α3. In contrast, protein sequences for the frog *Silurana tropicalis* for Srd5α1, Srd5α2, and Srd5α3 are 257, 239, and 308 aa, respectively. The genes for the three isoforms are located on different chromosomes and have different biochemical properties (reviewed in Azzouni et al., 2012; Table 1).

Table 1 Chromosomal location and pH optimum for human steroid 5α-reductase type 1, 2, and 3.

Isoform	Chromosomal location	pH optimum
Srd5α1	5p15	6 - 8.5
Srd5α2	2p23	5 - 5.5
Srd5α3	4q12	6.5 - 6.9

While Srd5α1 has a lower affinity for T it has a higher turnover rate compared to Srd5α2, which has a higher affinity for T but a lower turnover number. However, biochemical data for Srd5α3 is scarce. Some *in vitro* studies have suggested that human and hamster Srd5α3 have no catalytic capability to convert T and other 3-keto-4-ene steroids (Chávez et al., 2015); while other research shows the ability for Srd5α3 to reduce T and other steroids (Titus et al., 2014). Thus, data for the characterization of Srd5α3 is currently lacking.

1.4.1 BIOLOGICAL FUNCTIONS OF SRD5α

Srd5α are known to be involved in several crucial biological functions, including reproduction. As such, the best recognized role of Srd5α is the synthesis of 5α-DHT. 5α-DHT is a very potent androgen in most vertebrates, including amphibians and is involved in the development and maintenance of male secondary characteristics (Russell and Wilson, 1994). Exposure to 5α-DHT during amphibian embryogenesis caused a biased sex ratio, producing 98% males (Coady et al., 2005). In contrast, inhibition of Srd5α results in a female biased sex ratio (Duarte-Guterman et al., 2009). Moreover, human Srd5α2 deficiency also results in diseases including pseudohermaphroditism, prostate cancer, polycystic ovarian syndrome and hirsutism (reviewed in Langlois et al., 2010b). In addition to being essential in functions related to male sexual development, 5α-metabolites synthesized by Srd5α are also involved in erythropoiesis (5α-androstanes) and bile acid synthesis (7α,12α-dihydroxy-5α-cholestan-3-one; Azzouni et al., 2012). Recent studies have also provided evidence that Srd5α can be used as a potential biomarker for hormone dependent tumor malignancy. For example, *Srd5α3* expression was demonstrated to be useful as a biomarker of lung, breast, papillary thyroid, and testicular cancer biopsies (Godoy et al., 2011; Rui et al., 2008). Additionally, mRNA level of *SRD5a3* is also induced in the cells of hormone refractory prostate cancer (Uemura et al., 2008). Moreover, patients with metastatic prostate carcinomas had an increased level of *SRD5α1* and *SRD5α3*, but a decreased level of *SRD5α2* in the prostate (Mitsiades et al., 2012).

Srd5α is also expressed in both neuronal and glial cells in the rat brain (Morita et al., 2004). Neuroactive 5α-reduced steroids were shown to promote glial cell differentiation (Morita et al., 2014). Srd5α1 is known to be the rate limiting step in the biosynthesis of the neurosteroid allopregnanolone, as *SRD5a1* mRNA levels are decreased in the prefrontal cortex in depressed patients (Agís-Balboa et al., 2007). Previous studies correlated Srd5α inhibition to depression suggesting that Srd5α enzymes have also a crucial role in brain function (Traish et al., 2011).

The most recently discovered isoform Srd5α3 has a crucial role in the N-glycosylation of proteins (Cantagrel et al., 2010). Mutations of the human *SRD5α3* led to congenital disorders of glycosylation in children (Kara et al., 2014). The ability of Srd5α3 to synthesize 5α-DHT is debated. However, with the important function of N-glycosylation of proteins, all three enzyme isoforms are vital. But little research has focused on the involvement of Srd5α3 in biological functions related to development and reproduction. In particular, research is lacking for non-mammalian species. In addition, no literature is currently available for functions during embryogenesis.

1.4.2 REGULATION OF SRD5α

The regulation of Srd5α is complex and not completely clear. However, it is known that the most potent androgen 5α-DHT synthesized by Srd5α is tightly regulated by the hypothalamus-pituitary-gonadal (HPG) axis (Chang, 2012). Gonadotropin releasing hormone is emitted from the hypothalamus and the signal triggers the anterior pituitary to produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH then travel via blood to androgen responsive tissues such as the gonad and stimulate the production of androgens (Norris and Carr, 2013). Androgens, including T and 5α-DHT, bind to AR; however, 5α-DHT has a much higher affinity than T for AR. After binding to AR, the receptor complex binds in a homodimer within a

zinc finger complex to androgen responsive elements (AREs) to activate/regulate gene expression. AREs are specific DNA sequences and are known to be present in the promoter region of *srd5α* (Matsui et al., 2002). *In silico* analysis of the promoter region of *srd5α1*, *srd5α2*, and *srd5α3* in the species *Mus musculus*, *Silurana tropicalis*, and *Oryzias latipes* demonstrated that all three isoforms contain AREs, suggesting that all three isoforms are regulated by androgens (Flood et al., 2013). Indeed, exposure to 5α-DHT in adult rat hepatocytes induced the *Srd5α1* mRNA level (El-Awady et al., 2004). However, other experiments demonstrated that androgens do not regulate *srd5α* in all developmental stages and tissues. For example, perinatal expression of *Srd5α1* in the mouse hypothalamus was not controlled by androgens (Karolczak et al., 1998). Likewise, other researchers demonstrated that the transcription of *SRD5α2* is not directly regulated by T or 5α-DHT in human genital skin fibroblasts (Hellwinkel et al., 2000). In contrast, the expression of *SRD5α1* in cultured human lymphoid cells was induced by 5α-DHT, but not T (Zhou and Speiser, 1999). This discrepancy could be partly explained by differing DNA methylation patterns at various developmental stages and tissues. Additionally, other hormones are involved in the regulation of *srd5α.* For example, in the rat liver, thyroid hormones act at the pretranslational level to regulate *Srd5α1* mRNA levels (Ram and Waxman, 1990). Moreover, the mRNA levels of *SRD5α1* in cultured human lymphoid cells (Zhou and Speiser, 1999) and *Srd5α2* in the mouse brain (Matsui et al., 2002) were induced by progesterone. The induction by progesterone can be explained by a progesterone response element in the promoter of *srd5α2* (Matsui et al., 2002). These findings suggest that Srd5α are strongly regulated by hormones, including androgens. However, research on the regulation of Srd5α3 is lacking and the role of androgen regulation of Srd5α in frogs is currently not clear either.

The regulation of *srd5α* in eukaryotic cells is also dependent on a series of transcription factors. For example, Sp1and Sp3 (zinc finger transcription factors), GATA4, sterol regulatory element binding proteins, and members of the E26 transformation-specific transcription factors are involved in regulating *Srd5α* (Blanchard et al., 2007; Her et al., 2010; Schrade et al., 2015; Seenundun and Robaire, 2005; Seo et al., 2009; Yang et al., 2006). Literature suggests that regulation of *Srd5α* can also be achieved by suppressing the degradation of *Srd5α* mRNA by polyamines (Morita et al., 2014). Activation of the cAMP/protein kinase A-mediated intracellular signaling pathway was suggested as another pathway to induce the *Srd5α* mRNA and protein levels in rat C6 glioma cells after stimulation of the β-adrenergic receptors (Morita et al., 2004). However, the regulation of *srd5α* is isoform- and tissue-specific. For example, in the brain, nerve growth factor has been suggested to be involved in the regulation of *Srd5α1* (Her et al., 2004). *Srd5α1* also contains sequence-specific DNA binding for the transcription factor Su2 (Gaston and Fried, 1992). In the liver, the early growth response gene 1 is able to transactivate the transcription of *Srd5α1* (Rui et al., 2008). Prostatic fibroblast cells secrete a diffusible factor, which was suggested to be responsible for the transcription of *Srd5α2* in primary prostatic epithelial cells (Bayne et al., 2003). These findings suggest that regulation of *srd5α* is complex and tissue-specific. However, research is only available for mammalian species and is scarce for lower vertebrates.

1.4.2.1 METHYLATION OF SRD5α

DNA methylation is an epigenetic regulation mechanism crucial for tissue-specific gene expression (Chen and Riggs, 2005). Methyl groups attached to the cytosine in a cytosine guanidine dinucleotide (CpG) are often associated with gene silencing. This methylation process can suppress gene expression by either eliminating access of transcription factors to the promoter site or by forming a tight chromatin structure that creates an inactive region (Casati et al., 2015). DNA methylation has been associated with transcriptional repression in mammals leading to low mRNA levels of highly methylated genes (Chen and Riggs, 2005). A lot of research is dedicated to understand epigenetic regulation of gene expression, in particular at the DNA methylation level. Steroidogenic enzymes, including *Cyp19* and steroidogenic acute regulatory protein (*Star*), have been shown to be regulated via DNA methylation (reviewed in Martinez-Arguelles and Papadopoulos, 2010). However, only a few studies have previously investigated the epigenetic regulation of *srd5α*. For example, differential DNA methylation of *Srd5α1* and *Srd5α2* in rat testis and liver was shown to control their expression level (Reyes et al., 1997). In particular, the cytosine DNA methylation was lower in testis than liver for both *Srd5α1* and *Srd5α2* and correlated with their expression level. Moreover, the DNA methylation pattern of *SRD5α2* in human prostate has been observed to increase with age and subsequently results in decreased protein expression of SRD5α2 in older men (Bechis et al., 2015; Isaacs, 2015). Likewise, a recent study demonstrated that absent SRD5α2 is correlated to hypermethylation in the promoter of *SRD5α2* in adult human prostate (Ge et al., 2015).

Interestingly, the DNA methylation pattern of *srd5α* has also been used as a biomarker in cancer tissue, which makes *srd5α* isoforms important candidates for cancer screening purposes. For example, in hepatocellular carcinoma, DNA methylation patterns of *Srd5α2* is increased (Tsunedomi et al., 2010). Hypermethylation in the promoter region of *SRD5α2* has been previously correlated to absent or decreased SRD5α2 (Ge et al., 2015; Horning et al., 2015). These findings suggest that the DNA methylation pattern of *srd5α* is crucial for regulating their expression level and also provides a potential method for early detection of cancer. Thus, it is important to first establish a baseline for DNA methylation pattern in different organs as well as throughout development. Literature suggests that DNA methylation is crucial in vertebrate development by regulating genes throughout the vertebrate life cycle (Ehrlich, 2003; Meehan, 2003). In *Xenopus laevis* embryos, DNA methylation is typically associated to gene silencing prior to transcriptional initiation (Stancheva and Meehan, 2000). After onset of acquiring the ability of gene transcription at the mid-blastula transition stage, global DNA methylation level decreases, possibly leading to changes in DNA methylation of individual gene promoters (Stancheva et al., 2002). These findings suggest that DNA methylation could also be involved in regulating *srd5α* in amphibians. However, no previous studies have examined the DNA methylation of *srd5α* in non-mammalian animals.

1.5 STEROID 5β-REDUCTASE

The enzyme Srd5β converts the Δ 4,5 bond of C-19 and C-21 steroids to 5β-stereoisomers and is present in a wide range of vertebrates, including mammals, birds, reptiles, amphibians, and fish (reviewed in Langlois et al., 2010b). Srd5β belongs to the aldo-keto reductase (AKR) superfamily, which contains more than 100 other proteins and is categorized in family 1 as member D (AKR1D1; Barski et al., 2008). The human *AKR1D1* gene expressing SRD5β is located on chromosome 7q32-33 (Barski et al., 2008). The resulting protein is composed of 326 amino acids that is encoded by a 2.7 kb mRNA (Charbonneau and The, 2001). The protein sequence is highly conserved along different species (Table 2).

Table 2 Comparative analysis of protein sequence of Srd5β of different species. The % homology is related to the *homo sapiens* protein sequence (XP_001148679.1). Accession numbers were obtained from NCBI.

Species	**Protein similarity (%)**	**NCBI Accession #**
Pongo abelii (Monkey)	99	XP_002818550.1
Rattus norvegicus (Rat)	89	NP_620239.1
Mus musculus (Mouse)	90	NP_663339.1
Felis catus (Cat)	95	XP_003983144.1
Ailuropoda melanoleuca (Panda)	96	XP_002914246.1
Bos taurus (Cow)	91	NP_001179287.1
Gallus gallus (Chicken)	94	XP_416341.3
Silurana tropicalis (Frog)	91	NP_001025609.1
Anoplopoma fimbria (Fish)	88	ACQ58910.1

The protein is a soluble, cytosolic monomeric enzyme with a molecular weight of 37 kDa and requires the cofactor nicotinamide adenine dinucleotide phosphate for substrate conversion (Chen and Zhang, 2012; Okuda and Okuda, 1984). Srd5β has enzymatic activity to many substrates, including steroid hormones T, cortisol, cortisone, progesterone, androstenedione, aldosterone, cholestenone, epitestosterone, corticosterone, 7 α-hydroxy-4-cholesten-3-one, 4-estren-17β-ol-3-one, 1,4-androstadien-17β-ol-3-one, 11-deoxycorticosterone, and 7α,2α-dihydroxy-4-cholesten-3-one (Chen et al., 2011; Okuda and Okuda, 1984). The optimal pH value for Srd5β varies among species. For example, chicken liver tissue showed optimal enzyme activity at around pH 5-6.5 (Sugimoto, 1990); while rat and human SRD5β have an optimal at pH 7.4 (Chen et al., 2011; Onishi et al., 1991). A physiological pH provides evidence that only one isoform is necessary for the catalysis of the Δ 4,5 bond of C-19 and C-21 steroids to 5β-stereoisomers.

1.5.1 BIOLOGICAL FUNCTIONS OF SRD5β

5β-metabolites synthesized by SRD5β are crucial in a variety of biological functions. The major function of Srd5β is bile biosynthesis. Creation of bile acids in the liver is important to maintain the cholesterol homeostasis and is required for lipid absorption and secretion, and vitamin uptake. Bile acid synthesis is the main cholesterol catabolism and elimination process in mammals (Lee et al., 2009), birds (Hagey et al., 2011), reptiles (Hofmann et al., 2010), amphibians (Hagey et al., 2010; Une et al., 1980), and fish (Hagey et al. 2010). Depending on the species, C_{27}-bile alcohols, C_{27}-bile acids or C_{24}-bile acids are found as bile salt variations, while C_{27}-bile alcohols are likely to be the oldest evolutionary form (Hofmann et al., 2010). To synthesize the two main bile acids, cholic acid and chenodeoxycholic acid (CA), 15 different enzymatic steps are required (Chiang, 2009). SRD5β is involved in the reduction of 7α,12α-dihydroxy-4-cholesten-3-one to 5β-cholestane-3α,7α,12α-triol and 7α-hydroxy-4-cholesten-3-one to 5β-cholestane-3α,7α-diol, two intermediates of cholic acid and CA, respectively (Lee et al., 2009; Lefebvre et al., 2009). These two bile acids also act as natural inhibitors for Srd5β and thus regulate the expression level of *srd5β* (Chen et al., 2011). Srd5β is the only enzyme capable of transforming compounds into 5β-stereospecific compounds necessary for bile acid synthesis in all vertebrates, indicating that all species creating 5β-C_{27} or C_{24} bile acids require this enzyme (Barski et al., 2008). A mutation in the human *AKR1D1* gene led to a SRD5β deficit, which resulted in the inability to synthesize the two primary bile acids, cholic acid and CA (Clayton et al., 1996). A lack of Srd5β ultimately causes diseases, including neonatal cholestasis, hepatitis, and liver failure (Barski et al., 2008; Lemonde et al., 2003; Setchell et al., 1988).

Furthermore, Srd5β has also been shown to have an important role in erythropoiesis. 5β-androstane and 5β-pregnane steroid metabolites, synthesized by Srd5β, induce the formation of porphyrins in chick embryo liver cells (Granick and Kappas, 1967) and stimulate a more rapid synthesis of haemoglobin in the erythroblasts of chick blastoderm (Levere et al., 1967). 5β-metabolites in rat and human blood vessels stimulate vasorelaxation (Perusquia et al., 1996; Perusquía et al., 2007). Patients with a SRD5β deficiency were prone to essential hypertension, obesity, atherosclerosis, coronary artery disease and high serum cholesterol, and alcohol liver injury (Charbonneau and The 2001). Additionally, there is a link between overweight and Srd5β regulation as obese rodents showed an increased mRNA level of *Srd5β* (Barat et al., 2007;

Livingstone et al., 2009). These findings demonstrate a strong involvement of 5β-metabolites in the cardiovascular system.

Although Srd5β is known for its role in the conversion of many steroid metabolites (Chen and Penning, 2014), research on the involvement of Srd5β and 5β-DHT on the reproductive cycle is lacking. Historically, it was believed that 5β-DHT has no androgenic effects as 5β-DHT did not compete for the androgen binding site in mammalian prostate (Kokontis and Liao, 1999; Mainwaring, 1977). Furthermore, it was also shown that β-androstanes did not exhibit a negative feedback on LH and FSH in bird plasma (Davies et al., 1980). In contrast, methyltrienolone (synthetic androgen R1881) exposure was shown to induce SRD5β in human prostate epithelial cells (Bolton et al., 2007). This could suggest that Srd5β is regulated by androgens.

Srd5β plays an important function in the synthesis and inactivation of steroids (Penning et al., 2001). 5β-DHT has been shown to be important in the regulation of T availability in the blood as a protective mechanism to prevent masculinisation of female zebra finch during sexual differentiation (Balthazart et al., 1986). Hutchison and Steimer (1981) suggested that T inactivation is achieved by synthesizing 5β-DHT in dove brain through hormonal changes to androgen-sensitive brain areas, and therefore, was suggested to be involved in the regulation of brain sensitivity to androgens in birds. Moreover, the high Srd5β activity in the brain of female zebra finch during sexual differentiation was associated to a protective mechanism to prevent masculinisation (Balthazart et al., 1986). Indeed, the level of Srd5β was found to be different in male and female rat liver (Mode and Rafter, 1985), fish gonads (Baron et al., 2008), and bird brains (Schumacher et al., 1983). In birds, it was also found that Srd5β protein expression is not only dependent on sex, but also on age and season (Balthazart et al., 1986; Hutchison and Steimer, 1981; Silverin and Deviche, 1991). Another study suggested that the different amount of Srd5β activity in male and female Japanese quail brains could be attributed to behavioural dimorphism (Schumacher et al., 1983). According to these findings it may be possible that 5β-DHT does have an important role in sexual development.

1.5.2 REGULATION OF SRD5β

The transcriptional regulation of *srd5β* is not well characterized and less is known than for *srd5α*. Research has suggested that similar to *srd5α*, the transcription of *srd5β* is also controlled by several hormones. Previous data reported that the human *AKR1D1* gene contains an estrogen response element (Chen and Zhang, 2012). Indeed, exposure to E2 decreased Srd5β activity in the dove brain (Steimer and Hutchison, 1981). Furthermore, *in silico* analysis of the promoter region of *srd5β* indicated the presence of AREs (Flood et al., 2013) suggesting that androgens could also modulate *srd5β* expression. Indeed, androgen exposure increased *SRD5β* mRNA level in human prostate epithelial cells and decreased *srd5β* expression in fish gonads and the bird brain (Baron et al., 2008; Bolton et al., 2007; Steimer and Hutchison, 1981). These results suggest that Srd5β could have biological functions in reproduction as sex steroids are regulating it throughout vertebrates. Further research on how androgens regulate Srd5β will help to understand the roles of Srd5β in androgen responsive tissues and may reveal new roles of 5β-metabolites currently not known.

1.6 PHTHALATES

Phthalates belong to one group of EDCs that has been shown to substantially trigger adverse effects on the vertebrate endocrine system (Mathieu-Denoncourt et al., 2015a). Phthalates are additives that are used worldwide to increase the flexibility of plastic compounds (Daniels, 2009). Due to the non-covalent bounds of phthalates to polymers, leaching into the environment is possible. Phthalates are commonly detected in soil, water bodies, and organism tissues (Bauer and Herrmann, 1997; Blair et al., 2009). For example, diethyl phthalate (DEP) was found at a concentrations of ~100 ng/L in the False Creek in Vancouver, BC, Canada (Blair et al., 2009). Other studies discovered that the Tama River in Tokyo contained amounts of dibutyl phthalate (DBP) and DEP of 88−540 ng/L and 4-310 ng/L, respectively (Suzuki et al., 2001). In contrast, river water in China contained 20 ng/mL of DBP (X. Liu et al., 2015). Environmentally relevant concentrations of phthalates have been shown to adversely affect development and reproduction of female and male vertebrates (Kay et al., 2013; Latini et al., 2006). Most of the research on the effects of phthalates has been performed in mammalian species. However, a growing body of literature provides evidence that amphibians also experience adverse effects from exposure to these chemicals (reviewed in Mathieu-Denoncourt et al., 2015a). For example, the frog sex ratio was altered following phthalate exposure. High concentrations of mono-methyl phthalate (MMP) (128.7 mg/L) resulted in a male biased sex ratio (Mathieu-Denoncourt et al., 2015b). In contrary, exposure to DBP to genetically male *Rana rugosa* tadpoles during sexual differentiation resulted in intersex animals (Ohtani et al., 2000). Moreover, spermatogenesis in frogs was severely affected by DBP exposure, which was demonstrated by a lower seminiferous tubule diameter, decreased number of germ cell nests per tubule, and an increase in tubules containing no germ cells (Lee and Veeramachaneni, 2005). These findings show that the male reproductive system of frogs is altered by phthalate exposure.

Phthalates mediate their action through different pathways (reviewed in Mathieu-Denoncourt et al., 2015a). Studies have demonstrated that phthalates exert their action through interfering with hormone synthesis via the modulation of the expression of sex steroid related genes (Lehmann et al., 2004; Thompson et al., 2004; Wong and Gill, 2002). In particular, phthalates have been shown to alter cholesterol transport, which is the steroid precursor to all steroids. mRNA level of the *StAR* was decreased in rat testes after diethyl-hexyl phthalate (DEHP) exposure and increased after dicyclohexyl phthalate (DCHP) exposure during embryogenesis in *S. tropicalis* (Borch et al., 2006; Mathieu-Denoncourt et al., 2016). StAR is necessary to transport cholesterol to the inner mitochondria and a modified protein level may lead to a change in the synthesis of downstream production of androgens, including T. Several studies demonstrated that plasticizers like bisphenol A (BPA), DEP, mono-ethyl phthalate (MEP), DBP, mono-butyl phthalate (MBP), di-n-pentyl phthalate (DPeP), mono-n-pentenyl phthalate (MPeP), benzylbutyl phthalate (BzBP), mono-n-octyl phthalate (MnOP), DEHP, and mono(2-ethylhexyl) phthalate (MEHP) altered T levels (Mathieu-Denoncourt et al., 2015a). This suggests, that the decrease of T due to a decreased StAR activity is a possible mechanism for the disruption of the male reproductive system due to the presence of a lower than normal level of androgens (Borch et al., 2006). Likewise, a recent study demonstrated that DBP exposure of prenatal male rats resulted in decreased T levels accompanied by a decreased expression of *Srd5α2* in testicular tissues (Jiang et al., 2016). This study showed evidence that Srd5 are potential targets for phthalates. Likewise, other researchers have found correlations between phthalate exposure and Srd5α2 activity. For example, a decrease in Srd5α2 activity was also

reported following DBP *in vitro* treatment in gonad microsomal homogenates isolated from the common carp (Thibaut and Porte, 2004). An experiment exposing primary cultures of rat Leydig cells to MEHP decreased Srd5α activity in immature, but not in adult Leydig cells (Svechnikov et al., 2008). Moreover, prenatal exposure to DBP in rats significantly decreased Srd5α2 in the proximal penis (Kim et al., 2010). In contrast, DEHP exposure resulted in increased SRD5α activity in testis of pubertal rats (Kim et al., 2003). In addition to alteration of Srd5α activity; MMP, dimethyl phthalate (DMP), and DCHP did not alter *srd5α2* mRNA level in *S. tropicalis* (Mathieu-Denoncourt et al., 2016). The exact mechanism of action of how plasticizers interfere with Srd5α is not clear. A dysfunction of Srd5α is known to be associated with human diseases, including acne, hirsutism, male pattern baldness, benign prostate hyperplasia, prostate cancer, and congenital glycosylation disorder (Azzouni et al., 2012; see sections 1.4.1 and 1.5.1 for details). Thus, disruption of Srd5α by phthalates can have detrimental health effects. Furthermore, no studies have investigated the effects of phthalates on Srd5β. Consequently, it is important to first understand the functions and regulation of Srd5 in order to understand how disruption by EDCs can affect an organism.

1.7 MODEL SPECIES

In this doctoral thesis, the frog *S. tropicalis* was chosen as a model species. Frogs, in particular of the *Xenopus* genus, are often used as surrogate organisms to answer developmental and reproductive-related research questions (Beck and Slack, 2001; Grainger, 2012). The genome of *S. tropicalis* has been sequenced and embryos are large in size (0.6 mm), abundant (between 1,000-3,000 eggs per breeding), and develop externally, which facilitates investigations of molecular and cellular functions during embryogenesis (Beck and Slack, 2001; Hellsten et al., 2010). Furthermore, their endocrine system resembles the one from higher mammals, including humans, making it a perfect species to investigate fundamental endocrine functions and serve as a model to identify the mode of actions of EDCs (Burggren and Warburton, 2007; Kloas and Lutz, 2006). Thus, *S. tropicalis* is a well-suited model organism to examine the expression, functions and regulation of Srd5 during development and adulthood.

1.8 AIM, HYPOTHESES, AND RESEARCH OBJECTIVES

The overall aim of this thesis was to gain a better understanding of the enzymes Srd5α and Srd5β in relation to their molecular regulation and biological functions during development and reproduction in amphibians. The general hypothesis was that *srd5α* and *srd5β* are expressed in various tissues throughout frog embryogenesis and are regulated by androgens and DNA methylation. In conjunction with investigating the expression and regulation of *srd5* in amphibians, the hypothesis that EDCs (phthalates) target the transcription of *srd5* was tested. The specific hypotheses of this thesis are summarized in Table 3.

This doctoral research is of particular significance considering that the enzymes Srd5α and Srd5β have important biological functions and have been shown to be disrupted by EDCs in other species. The fundamental understanding of possible modes of action of how EDCs interfere with the amphibian reproductive system may help understand the worldwide amphibian decline.

1.9 THESIS PRESENTATION

This doctoral thesis is based on four manuscripts (two published, one submitted, and one in preparation). Chapter 2 is currently in preparation to be submitted to *Reproductive Toxicology*. Chapter 3 has been published in *General and Comparative Endocrinology* and was an invited publication for a special edition of the 3rd North American Society for Comparative Endocrinology conference, which was held on June 21-25, 2015 in Ottawa, ON, Canada. Chapter 4 was submitted to *Mechanisms of Development* on April 5, 2016. Chapter 5 was published in *General and Comparative Endocrinology* in 2014.

To simplify the reading of this thesis, the organization of the thesis is presented in Table 3. This table shows the title, the hypotheses, the main outcomes, and the next research questions for each chapter to help the reader understand the linkages between the chapters.

Table 3 Outline of the overall progression of the doctoral thesis.

CHAPTER 1: General Introduction

↓

CHAPTER 2: Phthalates modulate mRNA levels of *steroid 5-reductases* in *Silurana tropicalis*

Hypotheses

1. Acute exposure to phthalates during embryogenesis induces changes at the transcriptional level of *srd5* in *S. tropicalis*.
2. Exposure of mature frog testes to phthalates alters the transcriptional level of *srd5*.

Main outcomes

Exposure of embryos to phthalates altered the mRNA levels of *srd5* in frogs.

Further questions

1. Given that EDCs can alter *srd5* transcription, does a change in *srd5* mRNA levels lead to modified steroid levels? (Chapter 3)
2. What are the biological functions of Srd5 in frogs? (Chapter 3)

↓

CHAPTER 3: Transcriptomic profiling in *Silurana tropicalis* testes exposed to finasteride

Hypotheses

1. Steroid levels (T, 5α-DHT, E2) are modified after inhibition of Srd5.
2. Srd5 are involved in synthesizing steroid metabolites for biological functions associated and not associated to sexual development and reproduction.

Main outcomes

Inhibition of Srd5 led to significantly increased T levels (build up) in the media of exposed liver and testis and disrupted the expression of genes related to reproduction, oxysterol synthesis, apoptosis, and epigenetic regulation.

Table 3 Continued

Further questions

1. Are *srd5* also functional during early frog development? (Chapter 4)
2. What are the roles of steroid metabolites synthesized by *srd5* during embryogenesis? (Chapter 4)
3. Are the *srd5* isoforms differentially regulated throughout development? (Chapter 4)
4. Are *srd5* regulated in the same way during early development and adulthood? (Chapter 4 and 5)

CHAPTER 4: Steroid 5-reductases are functional during early frog development and are regulated via DNA methylation

Hypotheses

1. Srd5 are expressed and functional in different tissues during frog embryogenesis.
2. The expression pattern of *srd5* varies among the isoforms.
3. Gene expression of *srd5* during embryogenesis is regulated by DNA methylation. A high percentage of methylation is leading to a lower expression level compared to a low methylation percentage that results in higher gene expression.

Main outcomes

Srd5 are functional and expressed in the central nervous, sensory, cardiac, respiratory, and detoxifying systems in early anuran development. The three different *srd5α* isoforms showed different localization pattern and DNA methylation profiles throughout embryogenesis.

Further questions

1. Does the mRNA levels of *srd5α1*, *srd5α2*, *srd5α3*, and *srd5β* vary among different organs in adult frog tissues? (Chapter 5)
2. Do *srd5* isoforms respond differently to androgens between tissues? (Chapter 5)

Table 3 Continued

CHAPTER 5: Androgens modulate gene expression and specific DNA methylation pattern of *steroid 5α-reductases* in the frog *Silurana tropicalis*

Hypotheses

1. Gene expression of *srd5* is different between male and female organs and is tissue-specific.
2. Androgen regulation of *srd5* varies among isoforms.
3. Androgens modify DNA methylation pattern of *srd5α.*
4. The metabolite 5β-DHT has androgenic properties and is involved in steroid clearance in frogs.

Main outcomes

The mRNA levels of *srd5α1* and *srd5α3* were modulated after exposure to T, 5α-DHT, and 5β-DHT in a sex-and tissue-specific manner; while the DNA methylation pattern of *srd5α2* was enhanced after 5α-DHT exposure.

CHAPTER 6: General discussion and conclusions

CHAPTER 2: PHTHALATES MODULATE MRNA LEVELS OF *STEROID 5-REDUCTASES* IN *SILURANA TROPICALIS*

Chapter adapted from **Sonja Bissegger** [1] and Valerie S. Langlois [2]

Will be submitted to *Reproductive Toxicology*

Main contributions of each co-author :

[1] Contributed to original ideas, optimized *ex vivo* assay, performed animal exposure, real-time RT PCR, DNA methylation analysis, ELISA analysis, data analysis, and manuscript preparation.

[2] Contributed to original ideas and revised manuscript.

2.1 INTRODUCTION

Phthalates are used worldwide in the manufacturing of plastic compounds (Daniels, 2009). Leakage into our ecosystems has been observed due to the non-covalent bounds of phthalates to polymers. Moreover, phthalates are also used as additives in cosmetic products, personal care products, pharmaceuticals, medical devices, and paints (reviewed in Magdouli et al., 2013). Thus, phthalates are commonly detected in soil, surface water, and organism tissues (Bauer and Herrmann, 1997; Blair et al., 2009). DEHP is one of the most widely used plasticizers and thus occurs readily in our ecosystems (reviewed in Magdouli et al., 2013). For example, DEHP was detected at concentrations ranging between 0.01 and 25 μg/L in rivers in Japan (Suzuki et al., 2001; Yuwatini et al., 2006). Wastewater influent at a wastewater treatment plant in France identified concentrations of DEHP up to 44 μg/L (Dargnat et al., 2009). DEP and DBP are two other compounds that have been widely detected in waters. The False Creek in Vancouver, BC, Canada showed ~ 0.1 μg/L DEP (Blair et al., 2009). These phthalate esters were also detected in the Tama River in Japan at concentrations ranging from 0.004 to 0.31 μg/L DEP and 0.088 to 0.54 μg/L DBP (Suzuki et al., 2001). Research has shown that phthalates have androgenic and/or anti-androgenic properties and adversely affect development and reproduction of male vertebrates (Kay et al., 2014; Latini et al., 2006). For example, feminization of gonads by exposure to DBP was found in juvenile Murray rainbowfish (Bhatia et al., 2015). In addition, disrupted spermatogenesis was observed in the frog *Xenopus laevis* after DBP exposure (Lee and Veeramachaneni, 2005). The mechanism of action by which phthalates mediate their action is still not completely understood (Mathieu-Denoncourt et al., 2015a). Previous research suggested that phthalates interfering with hormone synthesis by modulating the expression of sex steroid-related genes (Lehmann et al., 2004; Thompson et al., 2004; Wong and Gill, 2002). For example, decreased mRNA and protein levels of StAR have been observed in rat testis after DEHP exposure (Borch et al., 2006). StAR is responsible to transport cholesterol to the inner mitochondria in order to synthesize steroids, including androgens. Decreased expression levels of StAR have been correlated with reduced levels of the androgen T (Borch et al., 2006) suggesting that T metabolism can be affected by phthalate exposure.

Testosterone is converted to 5α-DHT by Srd5α and to 5β-DHT by Srd5β. Few research studies have previously investigated if one of the mechanisms of action of phthalates is modulating mRNA and/ or protein levels of Srd5α and Srd5β. Furthermore, all prior experiments have been carried out in mammals. For example, in rodents, BPA was found to increase the expression of *Srd5α3* and decrease *Srd5α1* and *Srd5α2* transcript levels and protein activity in the rat prostate (Castro et al., 2013). Exposure of MEHP decreased SRD5α protein levels in a primary cell culture of immature rat Leydig cells (Svechnikov et al., 2008). BPA, but not DBP exposure, to androgen receptor (AR) knockout mice resulted in a decreased SRD5α2 protein level (D. Liu et al., 2015). Moreover, prenatal exposure to DBP in rats significantly decreased SRD5α2 protein in the proximal penis (Kim et al., 2010). In contrast, a concentration dependent correlation was found to increase SRD5α activity in testis after DEHP exposure of pubertal rat (Kim et al., 2003). Similarly, in females, prenatal exposure of BPA to Suffolk ewes resulted in increased *SRD5α1* mRNA levels in ovaries at gestational day 65 (Veiga-Lopez et al., 2013). These studies show that phthalates can modulate the gene and protein expression of Srd5α. No literature is currently available that investigated if Srd5β expression can be modified by phthalates.

Srd5 are involved in vital biological functions (reviewed in Langlois et al., 2010b) and their dysregulation leads to a variety of diseases in humans, in particular in the male reproductive system and liver (reviewed in Azzouni et al., 2012). Thus, there is a need to determine how phthalates or other EDCs with androgenic and/or anti-androgenic properties affect Srd5α and Srd5β, in particular in lower vertebrates such as amphibians.

Amphibians are at risk to be adversely affected by environmental contamination due to their external egg development in aquatic habitats (Hayes et al., 2006). To improve our understanding of how EDCs with androgenic and/or anti-androgenic properties (e.g., phthalates) interfere with *srd5* transcription in amphibians, we exposed *S. tropicalis* to DEHP, DBP, and DEP during the critical period of early development. In addition, mature frog testes were exposed *ex vivo* to DBP in order to analyze the androgen levels secreted by the organ. Thus, this study presents novel insights in regards to interactions between EDCs and *srd5* in amphibians during embryogenesis and reproduction in adulthood.

2.2 MATERIALS AND METHODS

2.2.1 EXPERIMENTAL ANIMALS

Maintenance of male and female *S. tropicalis* occurred in dechlorinated and aerated water at the Queen's University Animal Care facilities (Kingston, ON, Canada) in accordance with guidelines of the Institution's animal care protocols and the Canadian Council on Animal Care. Animals were kept in a 12:12 h light:dark cycle with a water temperature of 26 ± 1 °C. Breeding was induced by injecting human chorionic gonadotropin (hCG; Fisher scientific, Ottawa, ON, Canada). Both male and female frogs were injected with 12.5 U hCG 24 h before the mating and with 200 U hCG directly prior to the start of the breeding. The male and female frogs were kept in a tank with dechlorinated and aerated water at a pH of 6 and were left in a dark and quiet room for mating. Eggs were collected and kept in Frog Embryo Teratogenesis Assay-Xenopus (FETAX) solution consisting of 625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4 \cdot 2H_2O$, 30 mg KCl, 15 mg $CaCl_2$/L, plus 0.04 ppm of the antifungal agent gentamycin sulphate. At Nieuwkoop and Faber (NF; Nieuwkoop and Faber, 1994) stage 8, the fertilized eggs were dejellied using 2% (w/v) L-cysteine (Fisher scientific, Ottawa, ON, Canada).

2.2.2 ACUTE EXPOSURE TO PHTHALATES

Embryos at NF 11 were exposed to DEHP (Sigma, Oakville, ON, Canada), DBP (Sigma, Oakville, ON, Canada), or DEP (Sigma, Oakville, ON, Canada) for 72 h until the larvae reached NF 46. Nominal concentrations of 0.1, 1, and 10 µM were chosen for each phthalate ester and corresponds to 39, 390, and 3900 µg/L for DEHP, 28, 280, and 2800 µg/L for DBP, and 22, 220, 2200 µg/L for DEP. NF 11 embryos were also exposed to finasteride (a human SRD5α2 inhibitor) as a positive control for *srd5α2* mRNA inhibition in frogs (Langlois et al., 2010c). The FETAX solution was kept at 26 °C and changed every 24 h. Mortality was recorded everyday. Embryos were sampled in pools (n = 10) and flash frozen on dry ice until RNA isolation.

2.2.3 GONADAL *EX VIVO* EXPOSURE TO PHTHALATES

As DMP is widely used and detected in our ecosystem, it was chosen to pursue the gonadal *ex vivo* experiment. The *ex vivo* assay was adapted from A Marca Pereira et al. (2011) and was optimized for the first time in frog tissue. Six male adult frogs were anesthetized in 0.1% MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma, Oakville, ON, Canada). Testes were carefully dissected from each animal. From each frog, one testis was used as a control sample and the other testis was exposed to DBP (n = 6 per treatment). Each testis was weighed (to correct for steroid production). Testes were placed in a separate 1.5 mL centrifuge tube filled with 500 µL ice cold Lebovitz (L-15 media, Sigma, Oakville, ON, Canada) containing 10 mM HEPES, 50 µg/mL gentamicin (Fisher Scientific, Ottawa, ON, Canada) and 2% synthetic serum replacement (Sigma, Oakville, ON, Canada) at pH 7.4. Before the exposure was started, the testes were transferred into designated wells in a 24-well plate containing 500 µL ice cold L-15 media. Prior to the start of the incubation, the media in each well of the 24-well plate was replaced by 500 µL of L-15 media containing 0.05% ethanol (control samples) or L-15 media containing 10 µM of DBP. The 24-well plates were incubated for 6 h at 26 °C using an orbital shaker. This time point was

selected according to a time dependent experiment (2–10 h) that showed that RNA extracted from frog testes began to degrade with an incubation time longer than 6 h. After 6 h, the organs were snap-frozen on dry ice and stored at -80 °C for subsequent RNA isolation. In addition, the media of each sample was also collected and stored at -80 °C for steroid analysis.

2.2.4 MALFORMATION ANALYSIS

A subset (n = 46 – 103, due to different starting numbers and mortality rates of each treatment) of randomly collected animals at NF 46 was fixed in 10% formalin for each treatment in order to conduct morphological analysis. Malformation analysis was performed based on the Atlas of Abnormalities (Bantle et al., 1998). A Nikon SMZ18 microscope (Nikon, Mississauga, ON, Canada) was used to observe malformations in eyes (reduction in size, asymmetric formation, incomplete separation from the brain and cyclops), tails (shortening and flexure), hearts (failure to coil in an 'S' shape), guts (failure to coil), gills (shredded appearance), and head and face (reduction in size and unusual shape).

2.2.5 RNA ISOLATION AND cDNA SYNTHESIS

Total RNA from embryo (NF 46) samples was performed with the E.Z.N.A Total RNA kit II (VWR, Mississauga, ON, Canada). Sample homogenization and disruption was carried out by a Mixer Mill MM400 (Retsch, Newtown, PA, USA) at 20 MHz for 1 min. In contrast, total RNA from testes was isolated using the Trizol reagent (Life Technologies Inc., Burlington, ON, Canada). Sample homogenization and disruption was done using a sonicator (Fisher Scientific, Toronto, ON, Canada). RNA was resuspended in 20 µL nuclease free water for both embryo and testis samples. RNA concentration and quality was assessed using the NanoDrop-2000 spectrophotometer (Fisher Scientific, Toronto, ON, Canada). Residual genomic DNA was eliminated by the TURBO DNA-free kit (Life Technologies Inc., Burlington, ON, Canada). Total cDNA from embryo samples was obtained from 1 µg RNA and 0.5 µg random primers utilizing the GoScript reverse transcriptase (Fisher Scientific, Toronto, ON, Canada), while cDNA from testis samples was obtained from 0.5 µg RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Toronto, ON, Canada).

2.2.6 REAL-TIME RT-PCR

The expression levels of *srd5α1*, *srd5α2*, *srd5α3*, *srd5β*, *star*, *cyp19*, *AR*, and *ERα* was determined relative to the reference gene *ornithine decarboxylase* (*odc*) by real-time RT-PCR using the SYBR Green detection system. Specific primers were used for amplification and quantification of cDNA from embryo and testis samples (Table 4).

Each sample was diluted 1:80 (for the embryos) or 1:40 (for the testes) and analyzed in duplicate using the GoTaq qPCR Master Mix (Fisher Scientific, Toronto, ON, Canada) with the optimized concentration of forward and reverse primer (0.1 to 0.65 µM) on a CFX96 Touch™ real-time RT-PCR machine (Bio Rad, Mississauga, ON, Canada). The program used to run all samples included an enzyme activation step at 95 °C for 2 min followed by 40 cycles with 95 °C for 15 sec and 58 or 62 °C (depending on target gene) for 1 min. After the amplification phase, a

dissociation curve was established in order to ensure the presence of a single amplicon. Reaction efficiencies were 100 ± 10 % with an $R^2 > 0.990$ and calculated by the CFX Manager Software (Bio Rad, Mississauga, ON, Canada).

In each assay, a standard curve (0.048 to 50 ng), a no template control, and a no reverse transcription control (to ensure the absence of genomic DNA in the samples) were run with the samples. The standard curve was generated by pooling equal amounts of the treated and control samples and was then serially diluted 1:4 to obtain concentrations from 50 to 0.048 ng. The standard curve was then used to interpolate and calculate the mRNA level of target and reference gene in each sample. The mRNA level of each target gene was calculated relative to the reference gene *odc*.

Table 4 qPCR primers and assay conditions of genes for *S. tropicalis*. Complete list of target genes, primer sequences (5′-3′), annealing temperature (°C), and primer concentration (micromolar).

Target gene	Primer direction	Sequence (5' - 3')	Annealing temperature (°C)	Primer (nM)
odc	Forward	TGAATGATGGCGTGTATGGA	62	150
	Reverse	GTCCCCAAATGCTGCTTG		
srd5α1	Forward	GTTGAATGGTCTGGCTTTGC	62	350
	Reverse	CTGTTGTGCCCTGGAAGTC		
srd5α2	Forward	ACCAGAAGGGAAGCACACAA	62	650
	Reverse	CCATAAGCAGCAGGATAAGTGA		
srd5α3	Forward	GCTGGTCTGAGGAAAAGTGC	58	450
	Reverse	AGGGCAGGACACTCTCTCAA		
srd5β	Forward	GTGGAGTGACCACCCAGAGT	58	450
	Reverse	TTCGGATCAATGAGGAGGA		
ar	Forward	TGACAACAACCAACCAGACA	62	600
	Reverse	GCCTTTGCCCACTTTACAAC		
erα	Forward	CCCAACATTTTACAGGTCAAGTTC	62	200
	Reverse	GGCCCTTATCATTAGCTGATGTC		
cyp19	Forward	GAATCCCGTGCAGTATAACAGC	62	110
	Reverse	ACAGGTCTCCTCTTGATTCCATAG		
star	Forward	GAGCAGAAAGGCACAAACCC	58	350
	Reverse	TTCCAGCCACTAAGCCTCTC		

2.2.7 SEX STEROID MEASUREMENT

Concentration of T and 5α-DHT excreted from the testes into the media was measured using commercially available enzyme-linked immunosorbent assays (T: Cedarlane, Burlington, ON, Canada; 5α-DHT: Diagnostics Biochem Canada, Dorchester, ON, Canada). Media samples were thawed on ice and diluted in the immunoassay buffer. The immunoassay protocol was followed as described by the manufacturer. All samples were measured in duplicate. The absorbance of samples after the designated incubation time of 2 h for T and 1 h for 5α-DHT were measured using a TECAN Infinit M1000 PRO microplate absorbance reader (TECAN, Männedorf, Switzerland) at 415 nm for T and 450 nm for 5α-DHT. The limit of detection according to the manufacturer was 6 pg/mL for T and 5α-DHT. Hormone concentrations were normalized to tissue weight.

2.2.8 STATISTICAL ANALYSIS

All statistical analysis was performed using the software GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Gene expression results of the embryo samples were analyzed using one-way analysis of variance (ANOVA) with a subsequent post-hoc test (Dunnett's) to determine significant differences between treatments. Gene expression and hormone concentration results of the testes exposed to DBP *ex vivo* were analyzed using an unpaired t-test. Significant differences were reported when $p < 0.05$.

2.3 RESULTS

2.3.1 DEHP, DBP, AND DEP INDUCE MALFORMATIONS IN FROG LARVAE

Survival rates of both, the water and solvent controls, were above 90% as recommended by the American Society for Testing and Materials (2004; Table 5). None of the phthalate treatments significantly increased the mortality rate (Table 5). However, phthalate exposure increased the occurrence of malformations. Exposure to DEHP, DBP, and DEP increased both, incomplete gut coiling and eye malformations. Moreover, DEHP and DEP increased tail abnormalities, and DEP increased the occurrence of edemas.

Table 5 Effects of di(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DBP), and diethyl phthalate (DEP) spiked water on mortality and malformation of *S. tropicalis* larvae at exposure completion (Nieuwkoop and Faber stage 46). The mortality data are expressed as percent mean ± SD (%) and the malformation results are expressed as a percentage (%) of malformed animals to the total number of animals analyzed. Asterisks indicate statistically significant differences between treatments (DEHP, DBP, and DEP) and solvent (0.05% ethanol) control.

Treatments (µg/L)	Mortality (%)	Average malformation (%)	Malformations observed (%)				
			Tail	Edema	Eye	Heart	Gut
Water Ctrl	4.5 ± 2.1	9.5 ± 5.1	12.8	2.3	12.8	0.0	11.6
Solvent Ctrl	5.0 ± 4.0	7.0 ± 2.2	8.7	6.8	4.9	0.0	9.7
39 DEHP	4.4 ± 3.1	16.5 ± 6.4*	21.2*	7.7	17.3*	0.0	21.2*
390 DEHP	3.8 ± 3.2	8.2 ± 4.5	14.3	4.1	6.1	0.0	10.2
3,900 DEHP	5.6 ± 2.4	12.3 ± 9.6	21.3	2.1	21.3*	0.0	8.5
28 DBP	3.8 ±3.2	14.5 ± 4.0*	14.9	2.1	19.1*	0.0	25.5*
280 DBP	8.8 ± 6.0	5.2 ± 4.4	10.0	0.0	8.0	0.0	8.0
2,800 DBP	5 ± 2.0	6.1 ± 3.2	4.3	0.0	8.7	0.0	15.2
22 DEP	8.1 ± 4.3	33.8 ± 8.1*	44.8*	13.8*	51.7*	0.0	31.0*
220 DEP	4.4 ±2.4	18.6 ± 5.5*	30.5*	8.5	28.8*	1.7	10.2
2,200 DEP	8.1 ±3.8	12.3 ± 8.2	21.2*	1.9	13.5	0.0	7.7

2.3.2 PHTHALATE ESTERS MODULATE GENE EXPRESSION OF *SRD5* IN FROG LARVAE

Exposure to phthalate esters modulated androgen related mRNA levels. Exposure to 1 μM DEHP increased the expression levels of *srd5β* and *AR* (*srd5β*: 1.4-fold increase, p = 0.0057; *AR*: 1.4-fold increase, p = 0.0228; Figure 2D, Figure 3A). At a concentration 10 x higher (10 μM), the frog larvae responded with increased mRNA levels for all four *srd5* genes (*srd5α1*: 1.4-fold increase, p = 0.0001; *srd5α2*: 1.7-fold increase, p = 0.0003; *srd5α3*: 1.5-fold increase, p = 0.0010; and *srd5β*: 1.8-fold increase, p = 0.0001; Figure 2A-D). DBP treatment induced significant changes at the lowest (0.1 μM) and highest (10 μM) concentration only. Low DBP stimulated the mRNA level of *srd5β (*1.4-fold increase, p = 0.0302); while higher concentration of DBP increased the transcript level of *srd5α1, srd5α2, srd5α3, srd5β,* and *AR* (*srd5α1*: 1.3-fold increase, p = 0.0046; *srd5α2*: 1.6-fold increase, p = 0.0029; *srd5α3*: 1.4-fold increase, p = 0.0178; *srd5β*: 1.6-fold increase, p = 0.0001; and *AR*: 1.4-fold increase, p = 0.0287). Exposure to DEP increased the expression of *srd5α1* (10 μM: 1.3-fold, p = 0.0094) and *srd5β* (1 μM: 1.4-fold, p = 0.0042). None of the phthalates affected the expression of *cyp19*, *ERα,* or *star* (Figure 3B-D). The positive control for Srd5α2 inhibition, finasteride, significantly decreased *srd5α2* mRNA level (2-fold decrease, p = 0.01; Figure 2B) validating the experimental design.

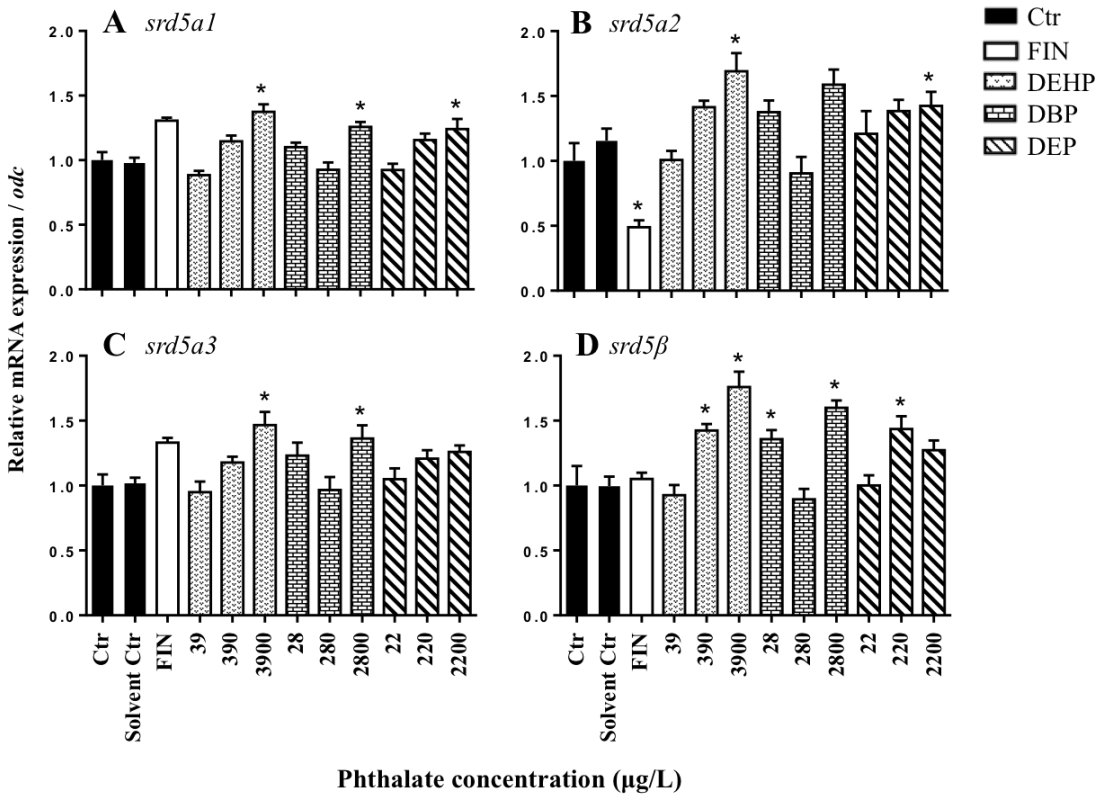

Figure 2 mRNA levels of A) *srd5α1*, B) *srd5α2*, C) *srd5α3*, and D) *srd5β* in frog embryos following exposure to FIN, DEHP, DBP, and DEP. Data are expressed relative to the reference gene *odc*. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance (n = 4-8; $p < 0.05$). Legend: DBP, dibutyl phthalate; di-2-ethylhexyl phthalate DEHP, diethyl hexyl phthalate; DEP, diethyl phthalate; FIN, finasteride; *, significant different from control at $p < 0.05$.

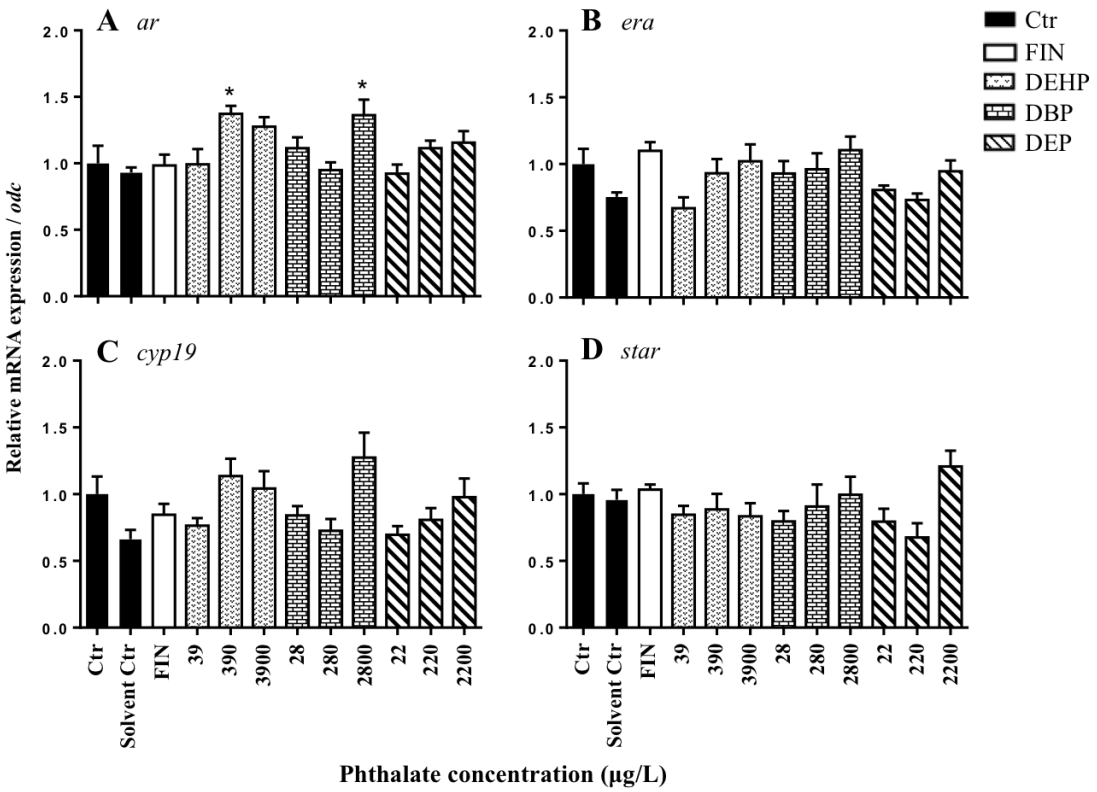

Figure 3 mRNA levels of A) *ar*, B) *erα*, C) *cyp19*, and D) *star* in frog embryos following exposure to FIN, DEHP, DBP, and DEP. Data are expressed relative to the reference gene *odc*. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance (n = 4-8; $p < 0.05$). Legend: DBP, dibutyl phthalate; di-2-ethylhexyl phthalate DEHP, diethyl hexyl phthalate; DEP, diethyl phthalate; FIN, finasteride; *, significant different from control at $p < 0.05$..

2.3.3 STEROID LEVELS AND *SRD5* EXPRESSION IN FROG TESTES WERE NOT MODIFIED BY DBP EXPOSURE

As *srd5* were altered by DBP at the larval developmental stage, androgen levels and gene expression analysis were conducted on the DBP-exposed testes. The total amount of androgens was measured in the media of control and DBP-exposed testes tissues, but DBP treatment did not significantly altered T and 5α-DHT levels ($p > 0.05$; Figure 4). In addition, the expression of *srd5α1, srd5α2, srd5α3,* and *srd5β* was also not significantly modified after DBP exposure (Figure 5).

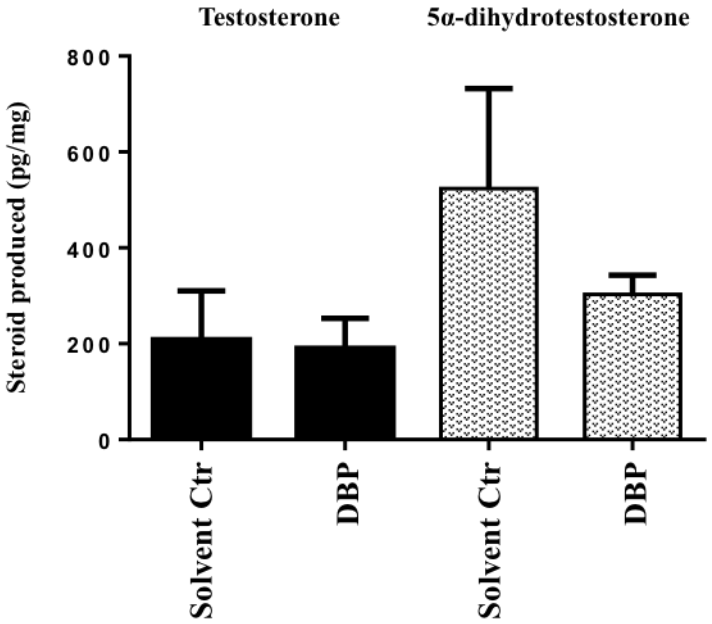

Figure 4 Concentration of testosterone and 5α-dihydrotestosterone in the media after ex vivo exposure of testes to dibutyl phthalate (DBP). Bars represent the mean + STD. Data were analyzed using a two tailed t-test (n = 6).

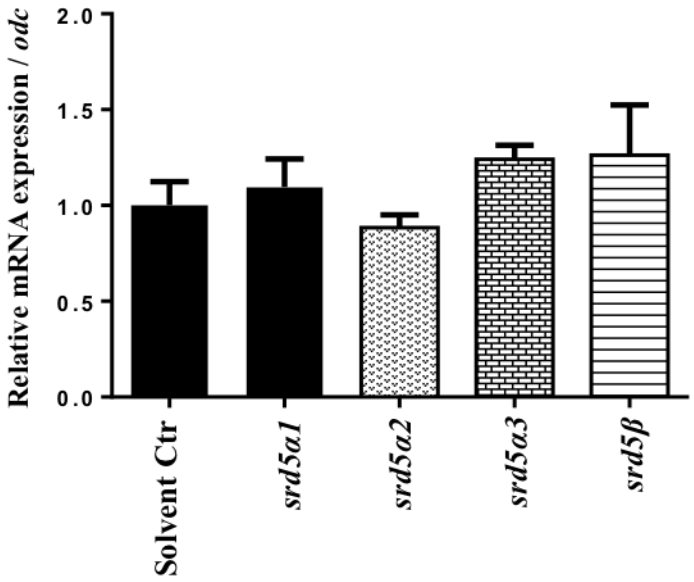

Figure 5 mRNA levels of *srd5α1, srd5α2, srd5α3,* and *srd5β* in frog testes after ex vivo exposure to 10 µM dibutyl phthalate (DBP). Data are expressed relative to the reference gene odc. Bars represent the mean + SEM. Data were analyzed using a two tailed t-test (n = 4).

2.4 DISCUSSION

Phthalates have been shown to induce endocrine disruption in wildlife. Amphibians are at a high risk due to their external egg development and permeable skin, which easily allows chemical penetration. Thus, the objective of this study was to investigate if phthalates interfere with amphibian development and reproductive system. As a first step, we established that the phthalate concentrations chosen did not affect the survival rate. Similarly, previous studies have shown that exposure to 1,000 µg/L DBP did not result in decreased mortality of fathead minnow embryos (Mankidy et al., 2013), while exposure to 5,000, 10,000, and 15,000 µg/L DBP significantly decreased the survivability of *Xenopus laevis* larvae (Lee et al., 2005). Likewise, higher DEP concentrations as the ones used in the present study (e.g., 10,000 µg/L and above), decreased survival rate of fathead minnow and zebrafish embryos (Kim et al., 2015; Mankidy et al., 2013). Japanese medaka exposed to 10 µg/L DEHP did not result in significantly decreased mortality (Chikae et al., 2004) which correlates with our finding.

In the present study, exposure to DEHP, DBP, and DEP augmented the occurrence of malformations in *S. tropicalis*, especially the presence of incomplete gut coiling and eye malformations. Similarly, previous studies have also observed that phthalate exposure yielded malformed animals. For example, exposure to DBP and DEP at concentrations exceeding 0.5 mg/L, resulted in significantly higher malformations, including abnormal gut coiling, cardiac abnormalities, and malformed faces, eyes, and brains in *Xenopus laevis* (Bantle et al., 1999; Lee et al., 2005; Gardner et al., 2016). Furthermore, *S. tropicalis* exposed to DMP and DCHP also resulted in malformations, such as edemas, improperly developed hearts, tail abnormalities, improperly coiled guts, and/or absent gills (Mathieu-Denoncourt et al., 2016). Altogether, these findings propose that several phthalate esters induce developmental abnormalities when in contact with the developing frog embryo.

In addition to inducing malformations, phthalates are known to alter the endocrine system in vertebrates. Previous studies have suggested that phthalates can interfere with steroidogenesis and affect both the female and male reproductive systems. Multiple regulating pathways involved in the maintenance of steroid homeostasis have been shown to be affected by phthalates (reviewed in Mathieu-Denoncourt et al., 2015a). In order to examine if this is the case in amphibians, a series of endocrine related targets were analyzed. First, the expression of a gene involved in cholesterol transport (e.g., *StAR*) was analyzed due to previous findings that demonstrated that phthalates interfere with this critical step in steroid synthesis. StAR is the protein that transports cholesterol to the inner mitochondrial membrane. In the present study, none of the phthalates of interest modulated *star* transcription, suggesting that each phthalate has unique molecular mechanisms of action. Prior studies have shown that DEHP decreased *StAR* in rat testis (Borch et al., 2006; Thompson et al., 2004; Vo et al., 2009). In contrary, treatment with DCHP during Western clawed frog embryogenesis increased the *star* mRNA level (Mathieu-Denoncourt et al., 2016), proposing that the chemical properties of the different phthalate esters differently affect StAR expression.

Previous literature has also demonstrated that the synthesis and signaling of the female sex steroids were affected by phthalate treatments. For example, mRNA levels of *Cyp19*, the enzyme responsible to aromatize T to E2, decreased in rodent cell lines when treated with MEHP and DEHP (Gupta et al., 2010; Lovekamp and Davis, 2001). In contrast in amphibians, exposure to DCHP during embryogenesis in the Western Clawed frog increased *cyp19* mRNA level;

however, DMP and its metabolite, MMP did not alter *cyp19* expression in the same species (Mathieu-Denoncourt et al., 2016). The later is similar to the data found in this study as none of DEHP, DBP, or DEP modulated the expression of estrogen-related genes, such as *cyp19,* and *ERα,* which furthermore supports the hypothesis that various phthalate esters act on different molecular pathways.

Androgen synthesis is also known to be altered by phthalate exposure in mammalian species. Generally, it is recognized that phthalates do not exert their action through the AR (reviewed in Rouiller-Fabre et al., 2015). As an example, juvenile and adult liver tissues exposed to MMP did not alter transcript levels of *AR* (Mathieu-Denoncourt et al., 2015b). Similarly, *AR* expression was not changed following exposure to 10 mg/L DEP and 1 mg/L DBP in fathead minnow embryos (Mankidy et al., 2013). In contrast, our data revealed that exposure to 10 μM DEHP and DBP increased *AR* transcription. In addition to the measured increase in *AR* mRNA level, *srd5* expression was also augmented in the frog larvae. DEHP, DBP, and DEP increased the mRNA level of *srd5α1*. In addition, *srd5α2* and *srd5α3* expression were increased by DEHP and DBP exposures. Similarly in mammalian species, DEHP also amplified the activity of SRD5α in the pubertal rat testes (Kim et al., 2003). An increase of *srd5α* could lead to a higher than normal conversion of T to 5α-DHT. However, other studies demonstrated that phthalate exposure decreases SRD5α activity. For example, a decrease in Srd5α2 activity was demonstrated after DBP exposure *in vitro* in gonad microsomal homogenates isolated from the common carp (Thibaut and Porte, 2004). Moreover, prenatal exposure to DBP in rats significantly decreased Srd5α2 protein expression in the proximal penis (Kim et al., 2010). In contrast, MMP, DMP, and DCHP did not alter *srd5α2* mRNA level in *S. tropicalis* (Mathieu-Denoncourt et al., 2016). These results suggest that the chemical nature of the phthalates as well as the developmental stage or tissue is important as to how the specific compound interferes with certain genes/ proteins.

Interestingly, not only *srd5α* isoforms, but also *srd5β* transcription was increased by DEHP, DBP, and DEP. Srd5β is known to be involved in clearing excess steroids in bird brains (Steimer and Hutchison, 1981). The observation that *srd5β* increased after phthalate treatment may suggest that EDCs can induce clearing of steroids, which may disturb the normal balance of sex steroids. This may lead to a change in the ratio between estrogens and androgens and adversely affect biological functions. However, experimental designs that investigated a change in *srd5β* are atypical. Limited studies examined the expression of *srd5β* after EDC treatments. For example, methyltrienolone and atrazine have been shown to alter *SRD5β* levels in human prostate cells and frog liver, respectively (Bolton et al., 2007; Langlois et al., 2010a). However, to our knowledge, no studies currently exist on the effect of phthalates on *srd5β.* Our results provide evidence that phthalates can interfere with *srd5β* and perhaps result in adverse reproductive effects. Since *srd5β* is involved in many other biological functions, including bile acid synthesis and erythropoiesis, other adverse effects may be observed. To test this hypothesis, exposure of phthalates to animals throughout development and sexual differentiation would be required. Taken together, these present findings suggest that phthalates exert their action through different mechanisms depending on species and tissues and affect androgen synthesis in various ways.

As androgen-related genes were altered by DBP during frog early development, further investigations were pursued in DBP-exposed testes of adult males due to a high androgen synthesis in gonads. DBP *ex vivo* exposure did not alter the T or 5α-DHT steroid levels nor did it alter *srd5α* expression in frog testes. Previous studies demonstrated that phthalates, including DEHP, DBP, DEP, MEP, MBP, DPeP, MPeP, BzBP, MnOP, and MEHP decreased T levels in

mammalian species (reviewed in Mathieu-Denoncourt et al., 2015a). As an example, a recent study demonstrated that DBP exposure of prenatal male rats resulted in decreased T levels, which was accompanied by a decreased expression of *Srd5α2* in testicular tissues (Jiang et al., 2016). Similarly, an experiment exposing primary cultures of rat Leydig cells to MEHP decreased Srd5α activity in immature, but not in adult Leydig cells (Svechnikov et al., 2008). Taken together, our data suggest that early frog developmental stages are more sensitive to phthalate disruption than adulthood.

2.5 CONCLUSION

This is the first study that demonstrated that DEHP, DEP, and DBP interfere with gene expression of *srd5* in amphibians. However, mRNA levels of *srd5* only changed after phthalate exposure during embryogenesis and not at the testicular maturation in frogs. This finding suggests that the early anuran development is critical and readily disrupted by chemicals present in the environment. This study provides evidence that phthalates and other EDCs with androgenic or anti-androgenic properties can disrupt *srd5* and place amphibian species at risk for endocrine disruption, in particular with regards to reproductive dysfunction.

ACKNOWLEDGEMENTS

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CHAPTER 3: TRANSCRIPTOMIC PROFILING IN *SILURANA TROPICALIS* TESTES EXPOSED TO FINASTERIDE

Chapter adapted from **Sonja Bissegger** [1], Christopher J. Martyniuk [2] and Valerie S. Langlois [3] (2014) General and Comparative Endocrinology 203:137-145.

Main contributions of each co-author:

[1] Contributed to original ideas, optimized ex vivo assay, performed animal exposure, ELISA analysis, data analysis, and manuscript preparation.

[2] Performed microarray analysis and revised manuscript with his microarray expertise.

[3] Contributed to original ideas and revised manuscript.

3.1 INTRODUCTION

During the last two decades, research has become increasingly focused on investigating the biological effects of EDCs that are present in the aquatic ecosystems. Compounds exerting effects through both androgenic and estrogenic signalling cascades have been shown to adversely affect the endocrine system in vertebrates by interfering with reproduction, development and sexual behaviour (reviewed in Colborn et al., 1993; Sanderson, 2006; Söffker and Tyler, 2012). Amphibian species, a taxon that is declining worldwide, are at risk to be significantly affected by EDCs, in part due to external egg development in aquatic habitats and also due to their highly permeable skin (Hayes et al., 2006). Agricultural chemicals have been found to compromise the health and reproduction of amphibians (reviewed in Mann et al., 2009). For example, pesticides at low concentrations are known to interfere with the thyroid or stress hormone axes and neurological system to induce growth and developmental abnormalities as well as immune dysfunction. Chemicals with estrogenic action (e.g., bisphenol A, 4-nonylphenol, 17α-ethinylestradiol, and atrazine) have been shown to alter growth and development, feminize and to bias sex ratios (i.e., increased female phenotype) in amphibian species (Kloas et al., 1999; Langlois et al., 2010a; Levy et al., 2004). In contrast, compounds with an androgenic mode of action such as 17β-trenbolone and 17α-methyldihydrotestosterone have been associated with growth and developmental alteration (Finch et al., 2012), changes in mating behaviour (Hoffmann and Kloas, 2012), altered gonadal histomorphology (i.e., spermatogenic nests and slight oocyte atresia; Cevasco et al., 2008), masculinization and to bias sex ratios (Bögi et al., 2002) in the African clawed frog (*Xenopus laevis*). Therefore, amphibian populations are sensitive to EDCs that act through different steroid receptor signalling cascades.

Finasteride (FIN) is a pharmaceutical with androgen–disrupting properties. This compound is currently approved to treat benign prostatic hyperplasia by inhibiting the Srd5α that converts T to 5α-DHT. Recently, adverse effects of using FIN have become well established and data suggests that, in humans, it not only impairs sexual function, but also that it is associated with male breast cancer, various skin conditions, and depression, while in rodents, FIN is linked to erectile dysfunction and change in prostate tissue histoarchitecture (reviewed in Traish, 2012). Aquatic amphibian species exposed to this Srd5α inhibitor also showed disrupted spermatogenesis, intersex animals, and a female biased sex ratio (Duarte-Guterman et al., 2009; Urbatzka et al., 2009). At the molecular level, gene expression endpoints have shown that FIN alters the mRNA levels of gonadotropins-, androgen-, and thyroid hormone-related genes in the Western clawed metamorphosing frogs (*Silurana tropicalis*; Duarte-Guterman et al., 2009; Langlois et al., 2010c; Urbatzka et al., 2009). Although it is established that FIN induces sex reversal in amphibians, it is currently unknown which molecular signalling pathways are activated/inhibited by FIN prior to morphological changes in the gonad. Therefore, an in depth analysis of testicular changes at the molecular level could help to better understand the mechanisms involved in androgen synthesis in testes after treatments.

Many studies using androgens focus on general effects pertaining to reproduction, but it has been found that both T and 5α-DHT exert crucial roles in other vital biological functions including immune response, growth, osmoregulation, apoptosis, transport and oxidation of lipids, synthesis and transport of hormones, protein metabolism, cell proliferation, and nervous system (Águila et al., 2013; Lieberman et al., 2001; Martyniuk and Denslow, 2012; Sangiao-Alvarellos et al., 2006; Sparks et al., 2003). This suggests that an imbalance of androgens can affect sexual development and reproduction as well as several other biological functions. Thus, a

comprehensive investigation making use of high throughput gene expression screening technologies would allow a better understanding of the complex EDC-responsive regulatory network of multiple cellular processes.

Transcriptomics is one approach used to characterize molecular, cellular and physiological effects of endogenous and exogenous steroid treatments. Furthermore, gene expression analysis in conjunction with short-term steroid bioassays has been successful for studying molecular signatures in the gonad. Assays to screen for estrogenic environmental compounds using *ex vivo* approaches in fish gonad or amphibian liver slices have been used for assessing excreted hormones during and after exposures to EDCs and endogenous hormones (Chishti et al., 2013; Garcia-Reyero et al., 2013; Hurter et al., 2002; A Marca Pereira et al., 2011). The aim of this study was to investigate the effects of FIN exposure on brain, liver and testis tissues. First, an *ex vivo* assay was used for *S. tropicalis* to expose juvenile brain, liver, and testis to FIN to test the effects of this chemical on sex steroid production. Secondly, microarray analysis was performed in the testes in order to explore the transcriptomic response to FIN inhibition and to examine the biological functions altered by Srd5 inhibition.

3.2 MATERIALS AND METHODS

3.2.1 EXPERIMENTAL ANIMALS

Maintenance of juvenile male *S. tropicalis* (12 months post-metamorphosis) occurred in dechlorinated and aerated water at the Queen's University Animal Care facilities (Kingston, ON, Canada) in accordance with guidelines of the Canadian Council on Animal Care. Animals were kept in a 12:12 h light:dark cycle with a water temperature of 26 ± 1 °C.

The *ex vivo* assay was first optimized in Chapter 2 (section 2.2.3) and was used for this study with a few alterations. Briefly, eight male juvenile frogs were anesthetized in 0.1% MS-222. Brain, liver, and testes were carefully dissected and placed in separate 1.5 mL centrifuge tubes filled with 500 µL ice cold L-15 media (Sigma, Oakville, ON, Canada). Once all animals were dissected, the brain, testes, and two pieces of each liver (cut in 10-30 mg pieces) were transferred into designated wells in a 96-well plate containing 100 µL ice cold L-15 media. The weight of each organ (brain: n = 8, liver: n = 16, testis: n = 16) was recorded in order to correct for steroid production. Prior to start of the incubation, the media in each well of the 96-well plate was replaced by either 100 µL L-15 media (control samples) or L-15 media containing 5 µM FIN (treated samples; F1293; Sigma, Oakville, ON, Canada). This concentration was chosen based on an *in vivo* study conducted in *S. tropicalis* (Duarte-Gutermann et al., 2009). The 96-well plate was incubated for 6 h at 26 °C on an orbital shaker. After 6 h, the media and the organs were frozen on dry ice and stored at -80 °C for subsequent hormone and microarray analysis, respectively.

3.2.2 SEX STEROID MEASUREMENT

Concentrations of T, 5α-DHT, and E2 excreted from the organs into the media were measured using commercially available enzyme-linked immunosorbent assays (T and E2: Cedarlane, Burlington, ON, Canada; 5α-DHT: Diagnostics Biochem Canada, Dorchester, ON, Canada). Media samples were thawed on ice and diluted in the immunoassay buffer. The immunoassay protocol was followed as described by the manufacturer. All samples were measured in duplicate. The absorbance of samples after the designated incubation time of 1 h for 5α-DHT and E2 and 2 h for T were measured using an iMark microplate absorbance reader (Bio Rad, Mississauga, ON, Canada) at 415 nm for T and E2, and at 450 nm for 5α-DHT. The limit of detection according to the manufacturer was 6 pg/mL, 19 pg/mL, and 6 pg/mL for T, E2, and 5α-DHT, respectively. Hormone concentrations were normalized to tissue weight and total hormone mass produced was calculated. Testosterone, E2, and 5α-DHT concentrations in brain, liver, and testes were analyzed using one-way ANOVA followed by a Tukey's post-hoc test using Graph Pad Prism v5.0 software (Graphpad Software Inc., La Jolla, USA). The effects of finasteride exposure on hormone concentration in each organ was analyzed using an unpaired t-test. Significant differences were reported when $p < 0.05$.

3.2.3 RNA ISOLATION

Total RNA from testis tissue was isolated using Trizol (Life Technologies Inc., Burlington, ON, Canada) according to the manufacturer's protocol. All RNA was column purified before microarray labelling using the RNeasy Mini Kit to minimize genomic DNA contamination (Qiagen, Toronto, ON, Canada). RNA quantity was determined using a NanoDrop 2000 (Nanodrop Technologies, Wilmington, DE, USA) and RNA quality was evaluated using a 2100 BioAnalyzer (Agilent Technologies, Mississauga, ON, Canada). Four control and four finasteride-treated samples with a RNA integrity number (RIN) average ± SD of 7.9 ± 0.5 were used for microarray analysis.

3.2.4 MICROARRAY AND BIOINFORMATICS

Custom *S. tropicalis* 4 x 44K oligonucleotide microarrays (GPL15626) were used to identify transcripts differentially regulated by FIN. The microarray was manufactured with Agilent Sure Print Technology by Agilent and has been described and validated previously by Langlois and Martyniuk (2013). RNA labeling, microarray hybridization, and microarray analysis were performed according to Agilent's One-Color Microarray-Gene Based Expression Analysis protocol (Version 6.5, May 2010; Agilent, Mississauga, ON, Canada). Raw expression data were extracted from *tif* images using Feature Extraction Software 10.7.3.1. Microarray data have been deposited into Gene Expression Omnibus (GSE46366) and are MIAME compliant (http://www.ncbi.nlm.nih.gov/geo/info/MIAME). Intensity data were imported into JMP® Genomics (version 5.1) and data were normalized using Quantile normalization. Differently expressed genes were identified using one-way ANOVA and a false-discovery rate of 5% (FDR = 0.05). To reduce noise, probes that showed a signal intensity below 3.8 were assigned a value of 3.8. The limit of detection of the microarray was based upon the lowest standard curve points and Agilent negative controls. Two-way hierarchical clustering of all expression data was performed using the Fast Ward algorithm. Rows were centered to a mean of zero prior to clustering and were also scaled to a variance of one prior to clustering. Gene set enrichment on gene ontology (GO) terms was conducted using the Parametric Analysis of Gene Set Enrichment (PAGE) algorithm which is a two-sided *z*-score for gene ontology categories. Pathway Studio 9.0 (Ariadne, Rockville, MD, USA) and ResNet 9.0 were utilized for sub-network enrichment analysis (SNEA). SNEA uses known relationships (*i.e.*, based on expression, binding, and common pathways) between genes to build networks focused around gene hubs. This approach has been applied in biomarker discovery in mammals (Kotelnikova et al., 2012) and for gene and protein networks in teleost fishes (Christopher J Martyniuk et al., 2013; Martyniuk et al., 2012). GenBank ID was used for mapping human homologs in Pathway Studio and approximately 4,763 genes were successfully mapped. For all analyses, annotated pathways were expanded to include cell processes and functional classes in target gene seeds. Enrichment *p*-value cut-off was set at $p < 0.05$.

3.3 RESULTS

3.3.1 HORMONE SECRETION OF BRAIN, LIVER, AND TESTIS

The total amount of sex steroids produced, *i.e.*, T, 5α-DHT, and E2 was measured in the media of control and FIN-exposed brain, liver, and testes tissues. As expected, the testes produced androgens (T and 5α-DHT) at a significantly higher amount than either liver or brain (ANOVA; T: $p = 0.001$; 5α-DHT: $p = 0.023$); whereas E2 was most prominently synthesized in the liver (ANOVA; $p = 0.006$; Figure 6). Production of T in the finasteride-treated samples increased significantly in the liver (3.5X, $p = 0.038$) and testis (1.5X, $p = 0.001$), but not in the brain ($p = 0.693$). Interestingly, 5α-DHT did not differ in any of the tissues (brain: $p = 0.490$; liver: $p = 0.229$; testes: $p = 0.231$). Production of E2 was also not affected by the presence of the 5α-reductase inhibitor (brain: $p = 0.108$; liver: $p = 0.959$; testes: $p = 0.176$).

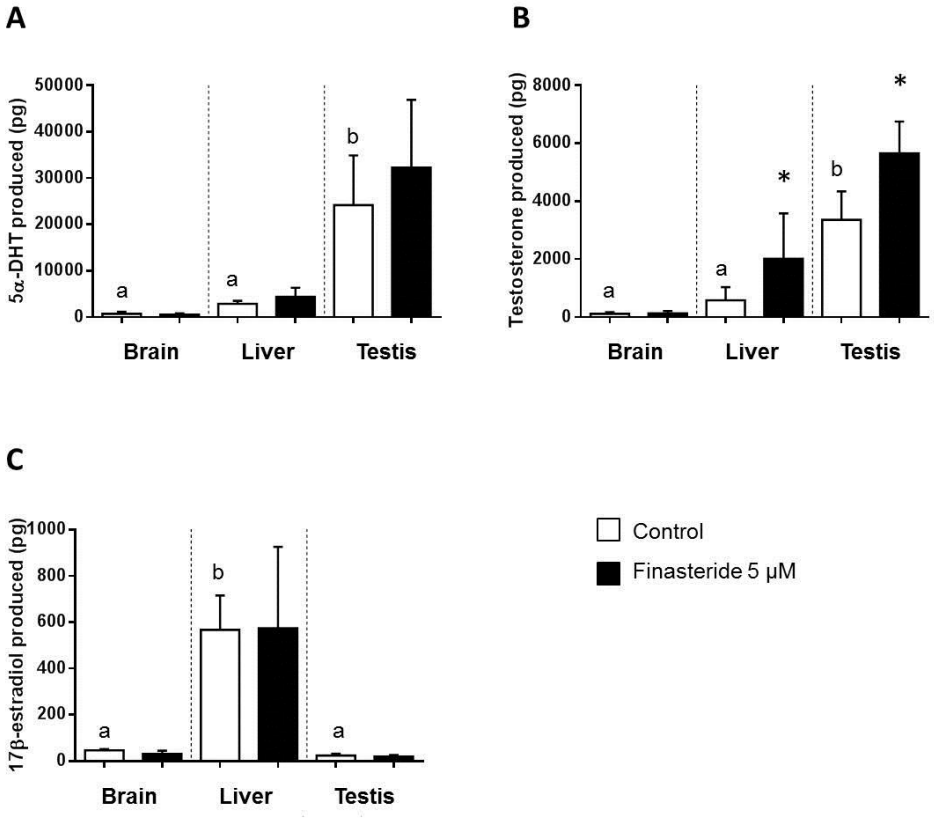

Figure 6 Total hormone produced (normalized by organ weight) during an 6 h ex vivo incubation by the male brain, liver or testis (A) 5α-dihydrotestosterone (5α-DHT) (B) testosterone and (C) 17β-estradiol. Significant differences ($p < 0.05$) between control and treated samples for a given organ is designated with an * and with letters (a and b) for control samples between different organs (n = 8, mean + SD are provided)

3.3.2 GENE EXPRESSION PROFILING FOLLOWING FINASTERIDE EXPOSURE

The microarray analysis performed on testes revealed a total of 1,434 gene probes differentially expressed in samples exposed to FIN compared to control samples ($p < 0.05$). Using only the probes that showed differential expression at $p < 0.05$, a cluster analysis revealed clear separation for FIN-exposed samples versus control samples (Figure 7). A comparable level of transcripts was found to be increased (n = 789) and decreased (n = 645). Transcripts with the largest increase in relative abundance (>3-fold) included *abhydrolase domain-containing protein 3*, *cytochrome P450 C21*, *S1 RNA binding domain protein*, *ATPase Na+/K+ transporting beta 1 peptide*, and *serine protease inhibitor Kazal-type 10*. Transcripts with the largest decrease in relative abundance (>3-fold) included *ubiquitin ISG15 ligase TRIM 25*, *C-reactive protein pentraxin*, *G protein-coupled receptor 144*, *epsilon 3 p17 subunit*, and *protein kinase C theta type*.

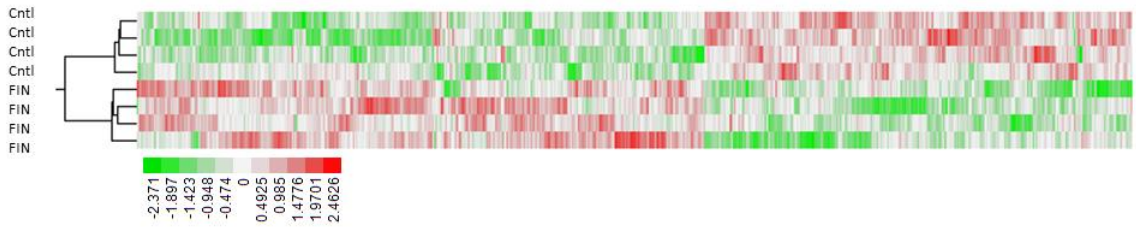

Figure 7 Hierarchical clustering of gene expression data using gene probes that were differentially expressed ($p < 0.05$) in *S.tropicalis* testes exposed to 5µM finasteride (FIN) during an 6 h *ex vivo* incubation versus control (Cntl) samples.

3.3.3 GENE ONTOLOGY AND ENRICHMENT ANALYSIS

PAGE analysis provided novel insight into biological processes, molecular functions, and cellular components by revealing a number of GO terms that were enriched after finasteride exposure. In total, 68 biological processes, 48 molecular functions, and 23 cellular components were significantly enriched after FIN exposure ($p < 0.05$). Transcripts involved in the biological processes of reproduction, apoptosis, metabolism, biosynthesis, immune response, cell communication, cell growth and differentiation, and epigenetics were preferentially affected by FIN and summarized in Table 6. Moreover, glucose metabolic process, and regulation of cell growth were GO terms significantly enriched. Molecular functions that were affected in FIN-treated samples included nucleotide binding and ATP activity. In particular, ATPase activity and double stranded DNA binding were gene ontology terms significantly impacted by FIN. Cellular components such as the rough endoplasmic reticulum, small ribosomal unit, and nuclear inner membrane preferentially localize transcripts/proteins that were affected by FIN.

Table 6 Summary of significantly altered gene ontology terms involved in reproduction, immune response, cell signalling, cell differentiation, cell death, metabolism and biosynthesis, and epigenetics using PAGE analysis ($p < 0.05$).

Function	Biological Process or Molecular Function	PAGE *z*-Score	PAGE Raw *p*-value
Reproduction	sterol biosynthetic process	-3.754	0.0002
	hormone biosynthetic process	-2.698	0.007
	hormone activity	-2.246	0.024
	steroid biosynthetic process	2.061	0.039
	gonadotropin-releasing hormone receptor activity	-2.025	0.043
Immune response	viral reproduction	3.010	0.003
	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	2.158	0.031
Cell signalling	negative regulation of rho protein signal transduction	-2.753	0.006
	regulation of arf protein signal transduction	-2.572	0.010
	cell communication	-2.485	0.013
	wnt receptor signalling pathway, calcium modulating	-2.456	0.014
	signal transducer activity	-2.219	0.026
	regulation of g-protein coupled receptor protein	2.103	0.035
	cell surface receptor linked signalling pathway	-2.088	0.037
Cell differentiation	regulation of cell growth	-3.416	0.0006
	positive regulation of smooth muscle cell proliferation	2.012	0.044
	neuron fate specification	-1.976	0.048
	lung epithelial cell differentiation	-1.976	0.048
	m phase of mitotic cell cycle	1.965	0.049
Cell death	positive regulation of apoptosis	3.055	0.002

Table 6 Continued

Metabolism and biosynthesis	l-serine biosynthetic process	-3.777	0.0002
	sterol biosynthetic process	-3.754	0.0002
	shingolipid metabolic process	-3.336	0.0009
	urate metabolic process	-3.225	0.001
	carbohydrate metabolic process	2.812	0.005
	hormone biosynthetic process	-2.698	0.007
	biosynthetic process	2.610	0.009
	regulation of glucose transport	2.549	0.011
	glucose transport	2.549	0.011
	cellular amino acid metabolic process	2.469	0.014
	folic acid-containing compound biosynthetic process	-2.338	0.019
	urea cycle	2.236	0.025
	steroid biosynthetic process	2.061	0.039
	retinoid metabolic process	-2.209	0.027
Epigenetics	chromatin assembly or disassembly	-2.611	0.009

SNEA was used to identify gene regulatory networks by using predefined gene sets with genes or proteins belonging to the same biological pathway. Our data set showed both increased and decreased networks after treatment with FIN (Table 7).

Table 7 Sub-network enrichment analysis (SNEA) in the testis of S. tropicalis. All gene set seeds that had > 10% overlap with the annotated pathways are listed ($p < 0.05$). The total number of neighbors refers to the total number of known entities in a sub-network.

Biological process	Gene set seed	Total # of neighbors	# of Measured neighbors	Percent overlap	Median change	*p*-value
Reproduction	penile erection	67	9	13	-1.233	0.001
	male meiosis	22	5	23	-1.232	0.029
	sperm entry	25	7	28	1.074	0.030
	hormone biosynthesis	29	7	24	1.172	0.002
Immune response	phagocyte activity	167	16	10	-1.143	0.012
Cell signalling	respiratory gaseous exchange	79	12	15	-1.148	0.010
	synaptic vesicle transport	58	16	28	-1.107	0.018
	striatal dopamine release	36	9	25	-1.107	0.044
	cell communication	119	22	18	1.125	0.039
Cell differentiation	stem cell development	34	6	18	-1.065	0.038
	heart morphogenesis	34	7	21	1.085	0.041
Organ function	liver uptake	72	11	15	-1.152	0.011
	kidney excretion	58	11	19	1.131	0.032
	renal reabsorption	89	20	22	-1.077	0.036
Other functions	parturition	165	32	19	1.065	0.005
	tolerance induction	103	10	10	1.094	0.006
	iron ion homeostasis	56	11	20	1.111	0.047
	muscle blood flow	26	5	19	1.133	0.048
	tropism	90	12	13	-1.126	0.024
	drug transport	38	8	21	1.208	0.027

Networks associated with male reproduction such as male meiosis, penile erection, hormone biosynthesis, and sperm entry were significantly affected by FIN exposure (Figure 8). Interestingly, significantly altered expression networks also included cell communication, phagocyte activity and drug transport. A limitation of using SNEA is that it uses only genes that have a human homologue, whereas the PAGE analysis that was used for gene enrichment analysis used all genes that were annotated for *S. tropicalis*, an approach that provided us with additional data as there was a larger number of annotated genes for gene ontology. However, the methods were complimentary in many ways, each identifying common processes such as hormone biosynthesis and cell communication as well as pathways involved in immune function, cellular regulation, and reproduction.

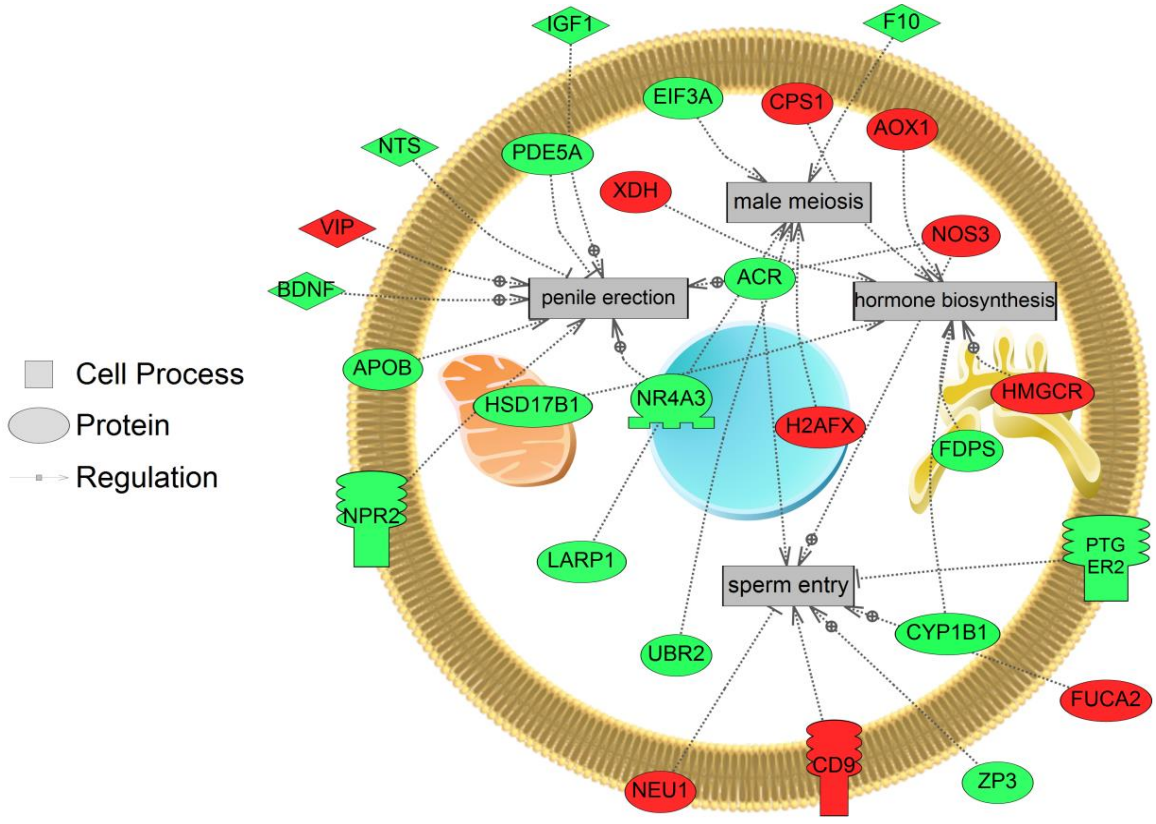

Figure 8 A reproductive related gene network showing altered regulation of genes after 5 µM finasteride treatment during an 6 h *ex vivo* incubation. Red indicates genes increased and green indicates genes decreased in the network.

3.4 DISCUSSION

Sex steroids exert crucial roles in reproductive developmental processes in vertebrates. Currently, there are only limited studies that investigate the organ-based sex hormone production in amphibians. This is the first study that investigates the sex steroid production in brain, liver, and testes in *S.tropicalis* and provides novel insight in sex steroid excretion for various tissues. Hormones produced in the male brain of *S. tropicalis* showed a higher concentration of androgens (T and 5α-DHT) compared to E2. Previous studies also demonstrated that the androgen level in the amphibian brain is higher than the E2 level. Similar to our study, Santillo et al. (2013) showed that the concentration of T was approximately twice as high as the E2 level in reproductive male brain in the green frog (*Pelophylax esculentus*). Testosterone is the precursor for the synthesis of both E2 and 5α-DHT. The higher concentration of T is potentially to maintain a metabolic pool of steroid precursors, as E2 and 5α-DHT may have more potent effects in the CNS. The hormones produced by the male liver exhibited a similar pattern compared to the brain for sex steroid levels; E2 was lower than both androgens. As expected, testes of *S. tropicalis* produced much higher androgens when compared to E2 (1000X). Furthermore, 5α-DHT was found to be ~ 7X higher compared to T in the testes. In *in vivo* experiments the circulating 5α-DHT level would be expected to be lower than T due to a constant feedback response from the hypothalamus. The observed higher level of 5α-DHT compared to the prohormone T could be explained by the lack of feedback inhibition from the hypothalamus in *ex vivo* assays. Similar hormonal trends were observed in Burrone et al. (2012) as they demonstrated that T was much higher in the frog *Pelophylax esculentus* testes than E2 (~1000X). In a study performed by Muller and Licht (1980) using an *ex vivo* approach to determine hormone concentrations in adult *Xenopus laevis*, it was shown that 5α-DHT was 15-50X higher than T in testes after incubation for 4 h. Data discrepancy with our study can be explained by differences in species and/or stage of development (*S. tropicalis*: juvenile; *X. laevis*: adult). Differences in androgen levels and androgen ratios have been well documented in amphibians, including frogs, toads, and salamanders (Muller and Licht, 1980). Interestingly, even though the testes are the male reproductive organ, estrogens also have vital biological roles in males as demonstrated by other researchers investigating estrogens and androgens in frog testes.

It was originally hypothesized that FIN exposure would increase T level and decrease the production of 5α-DHT. Indeed, both liver and testis tissues produced significantly higher levels of T compared to controls following treatment. This is likely due to the ability of FIN to inhibit the reduction of T into 5α-DHT. However, there was no significant change of 5α-DHT levels for any of the tissues tested. Based on these results, an alternate hypothesis is proposed (Figure 9). Finasteride specifically targets the enzyme Srd5α2; however there are also type 1(Srd5α1) and 3 (Srd5α3) isoforms that can convert T into 5α-DHT (Figure 9). Although, Srd5α2 is the predominant form of Srd5α in the male reproductive organ in mammalian species (reviewed in Langlois et al., 2010b), it is likely that after being inhibited, the tissue increases the amount of Srd5α1 and / or Srd5α3 to compensate for the inactive type 2 enzyme and to overcome the decreasing 5α-DHT concentration. Furthermore, the conversion of T into 5β-DHT by Srd5β is known as an integral part of the T metabolism and is known to be inhibited by finasteride in amphibians (Duarte-Guterman et al., 2009). Taken together, the increase of Srd5α1 and / or Srd5α3 could maintain the level of 5α-DHT, while the potential decrease in 5β-DHT synthesis could lead to an increase in T. Previously assumed to be not active in reproduction, 5β-DHT may have a crucial function in regulating the availability of T in the frog testes. Early studies

suggested that in the bird brain, 5β-reduction is used as a T inactivation pathway that is engaged in the control of brain sensitivity to androgens (Steimer and Hutchison, 1981). The present study provides insight into the importance of 5β-DHT within reproduction in amphibians. Further experiments should be undertaken to investigate this finding in details. It is also noteworthy that FIN was developed for human applications and may have other effects in amphibians than the ones observed in rodents and humans. Nevertheless, FIN has been shown previously to successfully inhibit *srd5α2* and *srd5β* mRNA levels as well as the srd5β enzyme activity (Langlois et al., 2010c). Thus, the observed response could also be explained by the utilization of *ex vivo* tissue culture assay. *Ex vivo* tissue cultures lack a hypothalamus-driven feedback inhibition mechanism to regulate hormone production and therefore may respond differently than *in vivo* tissues to regulate the imbalanced hormone ratio. Taken together, the significant increase of T observed validates the *ex vivo* inhibition assay in *S. tropicalis* and confirms that FIN alters Srd5 abundance in frog tissues and interferes with T metabolism.

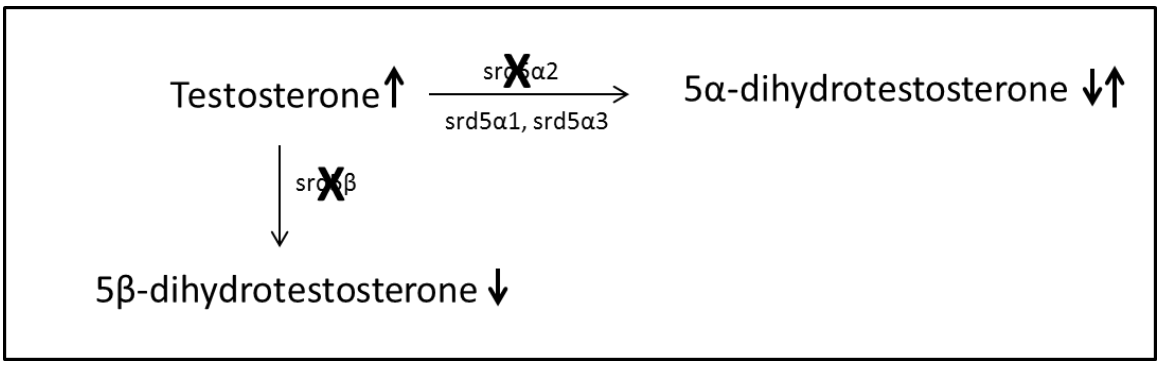

Figure 9 Proposed hypothesis on how finasteride elicits its action on the testosterone metabolism. Arrows indicate potential increases or decreases of hormone synthesis (Legend: srd5α1, steroid 5α-reductase type 1; srd5α2, steroid 5α-reductase type 2; srd5α3, steroid 5α-reductase type 3; srd5β, steroid 5β-reductase).

The accumulation of T in the male reproductive tissue was of particular interest due to the highest levels of secreted steroids and the importance of the T metabolism in the testes. Thus, the increase of T in testis tissue after FIN treatment is expected to result in various effects at the molecular level; therefore, a microarray investigation was conducted on this tissue. Microarray data suggested that FIN has significant impacts on pathways related to reproduction. Sub-network enrichment analysis identified several reproductive-related gene sub-networks being altered by Srd5 inhibition, e.g., genes involved in male meiosis, penile erection, hormone biosynthesis, and sperm entry. The synthesis of all hormones starts with cholesterol, which is subsequently converted by various enzymes to other steroids. The inhibition of one enzyme can lead to a cascade effect in affecting the synthesis of the subsequent steroids. Male meiosis is important for sperm maturation and subsequent reproduction. Disrupted meiosis could ultimately lead, for example, to infertility and therefore an inability to reproduce. Sub-networks related to reproduction are consistent with the literature as many studies have reported reproductive defects following FIN treatment or exposure. In humans, FIN induced side effects involved overall reproductive health effects such as infertility; whereas in rodents, erectile dysfunction and change in prostate tissue histoarchitecture were observed after treatment (Traish, 2012). Both Duarte-Guterman et al. (2009) and Urbatzka et al. (2009) showed that amphibians exposed to FIN

displayed disrupted spermatogenesis, intersex phenotype, and female-biased sex ratio. Hence, FIN induces adverse effects on the reproductive system at the molecular, cellular and physiological levels.

Finasteride treatment also induced the expression of genes associated with Gonadotropin-releasing hormone (GnRH). GnRH is involved in FSH and LH release, which ultimately leads to sex hormone production. Although GnRH is the main target in hypothalamus and pituitary gland, research studies have suggested that GnRH and GnRH receptors are also present in non-neural reproductive tissues such as the testes (Ramakrishnappa et al., 2005). We also point out that the GnRH network can include genes that are localized to both brain and testes. In fish, it is well established that T can act as a feedback mechanism to either stimulate or inhibit GnRH release from the hypothalamus (Habibi and Huggard, 1998). Furthermore, Urbatzka et al. (2006) found that the exposure of synthetic androgen (methyldihydrotestosterone) led to a decreased mRNA level of LH. In contrast, FIN which is a compound with known properties to disrupt the T metabolism results in increase in T levels (this study) which ultimately could lead to an increase in GnRH from the hypothalamus. Thus, chemicals that interfere with the androgen biosynthesis can also affect the feedback mechanism of GnRH, which can eventually lead to an imbalanced hormone ratio.

Noteworthy is that FIN altered the expression of genes associated with 25-hydroxycholesterol production and phagocyte activity. 25-hydroxycholesterol, an oxysterol converted by the enzymes 24-hydroxylase, 25-hydroxylase, and 27-hydroxylase is known to be involved in inflammatory response and the regulation of the lipid metabolism (Bielska et al., 2012; Diczfalusy, 2013; Honda et al., 2011). Previously, it has been shown that an accumulation of oxysterols are linked to chronic diseases involving inflammation such as atherosclerosis, neurodegenerative disease, and inflammatory bowel disease due to changes in cholesterol uptake or metabolism (Poli et al., 2013) and have been related to phagocyte functions of macrophages (Spann and Glass, 2013). Testosterone is known to function as a key molecule for local production of immunoregulatory factors that regulates the overall immune environment in the testes (Arck et al., 2013). This finding suggests that FIN impacts the immune system and could potentially lead to a chronic inflammation or a change in the response to antigens.

Cell proliferation and apoptosis are key functions that are tightly regulated in each cell. In our study, processes related to both were affected, suggesting an interruption in the normal cell cycle. BPA, a contaminant found in the environment has also been shown to alter early development and apoptosis regulation in the central nervous system in *X. laevis* (Oka et al., 2003). Similar to FIN, BPA is also an anti-androgenic compound with adverse effects on steroidogenesis. A change in normal regulation of apoptosis has been linked to an increase in oxidative stress (Martindale and Holbrook, 2002) and has also been found to be associated to immunological and developmental disorders, neurodegeneration, and cancer (Fuchs and Steller, 2011). Thus, a change in apoptotic regulation after FIN treatment could explain the histological changes in testicular tissue as seen, for example, in rodents by altered prostate tissue histoarchitecture after FIN treatment (Traish, 2012).

Epigenetics refers to changes in the genome that do not result in change in the DNA nucleotides. Gene expression is regulated by DNA methylation, histone modification, miRNA, or chromatin remodelling, which are all changes at the epigenomic level (Tammen et al., 2013). Epigenetic regulation is tremendously important during murine gametogenesis (Maclean and Wilkinson, 2005; Shirakawa et al., 2013). Modified epigenetic profiles have been linked to

reproductive diseases in humans (Calicchio et al., 2014; Dada et al., 2012). Our study revealed one GO term with a biological process associated to epigenetic regulation that was enriched after FIN treatment: chromatin assembly or disassembly. It has been demonstrated that environmental stimuli, hormones, or drugs can induce DNA methylation or histone alteration that have been linked to changes in chromatin dynamics and have been correlated to certain human diseases (Hirst and Marra, 2009). For example, maternal exposure of DEHP induced testicular dysgenesis syndrome in mice due to changes in the DNA methylation profile (Wu et al., 2010). Manikkam et al. (2013) found that BPA, DEHP, and DBP lead to transgenerational inheritance of altered epigenetic profiles in mice. The F1 generation suffered from kidney and prostate disease whereas the F3 generation suffered from pubertal abnormalities, testis disease, obesity, and ovarian disease due to a large amount of differentially methylated DNA in gene promoters identified in the sperm epigenome. These compounds are known to exert anti-androgenic properties (Albert and Jégou, 2014). There is increasing evidence that epigenetic regulation is important in enzymes, transcription factors and nuclear receptors that play a role in steroidogenesis (reviewed in (Martinez-Arguelles and Papadopoulos, 2010; García-Carpizo et al., 2011; Piferrer, 2013). Hence, the present study presents evidence that FIN changes the normal epigenetic profile of genes by interfering with steroidogenic enzymes. Epigenetic profile changes in steroidogenic enzymes could lead to transgenerational inheritance; however this hypothesis must be rigorously tested.

3.5 CONCLUSION

In this study, we observed elevated T concentration in the media after exposure to the 5-reductase inhibitor FIN in liver and testes of *S. tropicalis*. Our data also provides insights into the transcriptomic responses to FIN at the differentiated testicular level revealing disruption of functions involved in maintenance and maturation of testis tissue. Exposure of FIN to testes during sexual differentiation may differ as functions of this tissue change with testis maturation. Many pathways associated with sexual reproduction were significantly affected in the differentiated testes, consistent with the mode of action of FIN. Other significantly affected pathways included oxysterols, apoptosis, epigenetic regulation, and immune response. Thus, the transcriptomics data supports our hypothesis that Srd5 inhibition also alters the transcriptomic responses of pathways not associated to sexual reproduction. The disruption of 5α-DHT synthesis is believed to result in physiological effects as androgens are well-known to be involved in crucial biological functions within and outside of sexual reproduction (reviewed in Martyniuk et al., 2013). Furthermore, androgens including 5α-DHT are recognized to have crucial functions during embryogenesis (reviewed in Martyniuk et al., 2013). However, current studies do not provide data on the tissue distribution of Srd5 isoforms, in particular during frog embryogenesis. The localization of tissue distribution of Srd5 throughout embryogenesis could provide valuable insight about the roles of 5α- and 5β-metabolites in various tissues.

ACKNOWLEDGMENTS

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CHAPTER 4: STEROID 5-REDUCTASES ARE FUNCTIONAL DURING EARLY FROG DEVELOPMENT AND ARE REGULATED VIA DNA METHYLATION

Chapter adapted from **Sonja Bissegger** [1] and Valerie S. Langlois [2] (2016) Mechanisms of Development *doi: 10.1016/j.mod.2016.06.005*

Main contributions of each co-author:

[1] Contributed to original ideas, performed animal exposure, designed probes for *in situ* hybridization, optimized *in situ* hybridization assay, designed primers for specific DNA methylation analysis, optimized qMethyl assay for frogs, data analysis, and manuscript preparation.

[2] Contributed to original ideas and revised manuscript.

4.1 INTRODUCTION

One of the most important functions of Srd5 in adult vertebrates is androgen biosynthesis. Specifically, Srd5α or Srd5β convert the main circulating androgen T into the more potent androgen 5α-DHT or 5β-DHT, respectively. Precisely, there are a total of three Srd5α isoforms (i.e., Srd5α1, Srd5α2 and Srd5α3) responsible of converting delta 4,3-keto steroids into their respective 5α-metabolites. In mammals, Srd5α1 is mainly present in non-androgen tissues, including skin; Srd5α2 is predominantly found in reproductive tissues such as testes, while Srd5α3 is ubiquitously distributed throughout the body (Langlois et al., 2010b). In contrast, Srd5β is mostly associated to the liver in mammals due to its crucial role in bile biosynthesis (Chen and Penning, 2014). However, previous research also demonstrated that Srd5β is involved in neurosteroid synthesis, initiation of steroid hormone clearance, erythropoiesis, and vasorelaxation (Langlois et al., 2010b; Chen and Penning, 2014). While the involvement of Srd5 in crucial biological functions is known in adulthood, the presence of Srd5 and its associated gene regulation during embryogenesis and early development remains unclear.

Expression of *srd5* has been shown to be activated by hormones (Chang, 2002), but studies have suggested that DNA methylation regulates genes throughout development (Ehrlich, 2003), leading to the assumption that this epigenetic mechanism could also be involved in regulating *srd5* throughout early development. As a part of epigenetics, DNA methylation is crucial for tissue-specific gene expression (Chen and Riggs, 2005). The addition of methyl groups to the cytosine in a CpG is often associated with gene silencing, by either blocking the access of transcription factors to the promoter site, or forming a tight chromatin structure that creates an inactive region (Casati et al., 2015). With regards to the influence on *srd5* expression regulation, a high DNA methylation level of *SRD5α2* in human liver corresponds to a low mRNA level of the enzyme (Moribe et al., 2008). Moreover, the transcript level of *SRD5α2* in human lymphocytes is also linked to its DNA methylation state (Rodríguez-Dorantes et al., 2002). Taken together, this suggests that *srd5* could also be regulated by DNA methylation in early frog development.

Accordingly, the primary objective was to investigate the regulation of *srd5* during early frog development, and for this, enzyme inhibition and DNA methylation imprinting were assessed as potential mechanisms of regulation of *srd5* mRNA levels. The secondary objective of this study was to localize all four *srd5* using whole mount *in situ* hybridization during this critical developmental period in order to help elucidate the early roles of Srd5 in localizing their transcriptional activity in the various organ systems.

4.2 MATERIALS AND METHODS

4.2.1 ANIMALS

Male and female *S. tropicalis* were kept in dechlorinated and aerated water at the Queen's University Animal Care facilities (Kingston, ON, Canada) in accordance with the Institution's animal care protocols and the Canadian Council on Animal Care guidelines. Animals were kept in a 12:12 h light:dark cycle with a water temperature of 26 ± 1 °C. Fertilized eggs were obtained by injection of 12.5 IU human chorionic gonadotropin (hCG, Fisher Scientific, Toronto, ON, Canada) into the dorsal lymph sac of both male and female *S. tropicalis* followed by a boosting injection of 200 IU hCG after 24 h. The fertilized eggs were dejellied using 2% (w/v) L-cysteine and kept in FETAX (625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4 \cdot 2H_2O$, 30 mg KCl, and 15 mg $CaCl_2$/L distilled dechlorinated water) solution containing 50 mg/L gentamicin. The Nieuwkoop and Faber (NF) developmental table was used to stage the development of the embryos.

4.2.2 ENZYMATIC INHIBITION

Once 600 eggs reached NF 13, the eggs were exposed to either 1% ethanol, 100 µM FIN, 100 µM CA, 100 µM ursodeoxycholic acid (UA), or no additive (water control). At stage NF 34, 100 embryos of each treatment were washed in distilled water and fixed in MEMFA solution (pH7.4, 0.1M MOPS, 2mM EGTA, 1mM MgSO4, and 3.7% formaldehyde) for 1 h. The MEMFA solution was then exchanged with absolute ethanol and the embryos were stored at -20 °C before further analysis by *in situ* hybridization.

4.2.3 DEVELOPMENTAL PROFILE AND EMBRYO PREPARATION

Approximately 100 embryos for DNA isolation and subsequent DNA methylation analysis were collected at NF stages 2, 13, 21, 25, 31, 34, 41, and 46 and flash frozen on dry ice in pools (20 embryos for NF 2 to NF 34, 15 embryos for NF 41, and 10 embryos for NF 46). The samples were stored at -80 °C before further analysis.

In addition, 100 embryos were collected at NF stages 7, 13, 17, 21, 25, 31, 34, 41, and 46 to establish the localization profile of *srd5α1*, *srd5α2*, *srd5α3*, and *srd5β* transcripts. Once the embryos reached the targeted developmental stages, the embryos were washed in distilled water and fixed in MEMFA. The MEMFA solution was then exchanged with absolute ethanol and the embryos were stored at -20 °C before further analysis.

4.2.4 DNA METHYLATION ANALYSIS

DNA from frog embryos was isolated using the Qiagen Blood and Tissue kit (Qiagen, Toronto, ON, Canada). Concentration and quality were assessed by NanoDrop 2000 (Nanodrop Technologies, Wilmington, DE, USA). Primers to assess the specific DNA methylation in the promoter region of *srd5α1*, *srd5α2* and *srd5α3* in *S. tropicalis* gonads using the qMethyl kit (Zymo Research, Irvine, CA, USA) were designed according to the manufacturer's guidelines and are shown in Table 8. Non-methylated frog DNA standard was prepared by amplification of the genomic DNA using the illustra™ Ready-To-Go GenomiPhi V3 DNA Amplification Kit (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada). Methylated DNA standard was prepared by subsequent methylation of 800 ng of the non-methylated DNA for 4 h at 37 °C using 4 units of the CpG methylase (*M. SssI*, Cedarlane, Burlington, ON, Canada). A standard curve containing 0, 25, 50, 75, and 100% methylated DNA was prepared by mixing the non-methylated and methylated DNA to test the linearity of the designed primers for *srd5α1*, *srd5α2,* and *srd5α3* when using the qMethyl kit. The actual methylation percentage was calculated for each gene and compared to the predicted value.

DNA isolated at different stages of development (NF 2, 13, 21, 25, 29, 34, 41, and 46) from three individual samples was analyzed using the qMethyl kit. The DNA methylation percentage was calculated using the C_t difference between a reference (no enzymes) and a test (including methylation sensitive restriction enzymes) reaction sample. Each experiment included a methylated and non-methylated DNA standard as a control to ensure the qPCR reaction was working properly.

Table 8 qPCR primers and assay conditions to assess the specific DNA methylation in *Silurana tropicalis* gonads in the promoter region of *srd5α1, srd5α2 and srd5α3* using the qMethyl kit. Complete list of target genes, primer sequences, amplicon size, optimized primer conditions, and number of methylation sensitive restriction enzyme (MSRE) sites in the amplicon.

Target gene	Primer	Sequence (5' - 3')	Annealing temperature (°C)	Amplicon size (bp)	Primer (nM)	# MSRE
srd5α1	Forward	CCGTACAAACACAGAAAAGC	54	161	500	3
	Reverse	ACTAACAAACATGGCCTCCT			500	
srd5α2	Forward	AGGAAACCACGTGGAGCCTAAGA	55	135	600	3
	Reverse	TCCTCACAAGTCTGGCAATGGT			600	
srd5α3	Forward	GAGAATAACCTGCCTAGTGG	54	327	500	2
	Reverse	CTTTATTGGACTGCTCCTTC			500	

4.2.5 PROBE SYNTHESIS FOR *IN SITU* HYBRIDIZATION

Primers were designed using NCBI for the four *srd5* (*srd5α1, srd5α2, srd5α3,* and *srd5β*), and *eukaryotic elongation factor 1α1* (*eef1α1*) as a positive control. PCR was used to confirm the correct targeting of the primers using cDNA obtained from *S. tropicalis* at NF 46. Optimized conditions for PCR can be found in Table 9. Each gene was then ligated into the pGEM vector (Promega, Madison, WI, USA) and cloned into JM109 competent *E. coli* (Promega, Madison, WI, USA). Plasmid was extracted using a plasmid mini kit (VWR, Mississauga, ON, Canada) and linearized using *NdeI* restriction enzyme (Fisher Scientific, Toronto, ON, Canada). *In vitro* transcription of each gene was performed using the T7 RNA Polymerase (Roche Applied Science, Laval, QC, Canada) to receive antisense probes labeled with digoxigenin to be used in *in situ* hybridization.

4.2.6 WHOLE MOUNT *IN SITU* HYBRIDIZATION

The *in situ* hybridization protocol was adapted from Sive et al. (2000). Briefly, the embryos were rehydrated, treated with 10 μg/ml proteinase K (embryos after stage NF 32) or treated with 10 μg/ml proteinase K, 2 mg/ml collagenase A, and 20 units/ml hyaluronidase (embryos before stage NF 32), fixed in 4% paraformaldehyde, and hybridized with the desired probe (*eef1α1*, *srd5α1*, *srd5α2*, *srd5α3*, or *srd5β*). After extensive washing of the embryos, the preadsorbed anti-digoxigenin Fab fragments coupled to alkaline phosphatase antibody (Roche Applied Science, Laval, QC, Canada) were added at a 1: 2000 dilution overnight at 4 °C. The staining with nitro blue tetrazolium (Sigma, Oakville, ON, Canada) / 5-bromo-4-chloro-3'-indolyphosphate p-toluidine (Sigma, Oakville, ON, Canada) was then carried out for 6 h for all *srd5* probes, and 45 min for the *eef1α1* probe. The embryos were cleared in a mix of 2:1 benzyl benzoate/benzyl alcohol and then stored in methanol in the dark at 4 °C. Embryos were rehydrated and mounted in glycerol before taking pictures using a Nikon SMZ-10 stereomicroscope.

Table 9 PCR primers and assay conditions of genes in *Silurana tropicalis* used to synthesize probes by *in vitro* transcription used in subsequent whole mount *in situ* hybridization protocol. Complete list of target genes, accession numbers, primer sequences, amplicon size, and optimized primer conditions.

Target gene	Accession No.	Primer	Sequence (5' - 3')	Annealing temperature (°C)	Amplicon size (bp)	Primer (nM)
eef1α1	NM_203970	Forward	AGTTGGTCGTGTGGAGACTG	55	628	500
		Reverse	GACTGAGGTGAGAGATGCTGG			
srd5α1	BC076920.1	Forward	GTGTTTAGCCTGTGACGCTTT	60	519	500
		Reverse	GCACCTGATTGGCGATGCT			
srd5α2	NM_001017113.2	Forward	CCCACGAAAAAGGAAAGCCC	58	478	500
		Reverse	GCATCTTTAGGGACAGCACTC			
srd5α3	NM_001079071.1	Forward	TGGGTGTGGCTTATGGGATG	60	822	500
		Reverse	AGGGCAGGACACTCTCTCAA			
srd5β	NM_001030438.1	Forward	TCTCCCCGAACTACACCCAA	58	536	500
		Reverse	AGCAGTTTGGGCTGAGTGAA			

4.2.7 QUANTIFICATION OF THE mRNA LEVEL AFTER WHOLE MOUNT IN SITU HYBRIDIZATION

To visually quantify the amount of mRNA expression after the exposure to FIN, CA, and UA in exposed and control embryos (n = 5) a scoring system to compare staining intensity (0 = no stain, 1 = light stain, 2 = medium stain, and 3 = strong stain) was established and compartmentalized in head, stomach, and tail regions as shown in Figure 10. The staining score of head, stomach, and tail regions of each embryo was added up for an overall staining score for each treatment. One-way analysis of variance (ANOVA) was used to analyze any significant changes in mRNA expression. p-value < 0.05 was accounted for as significant.

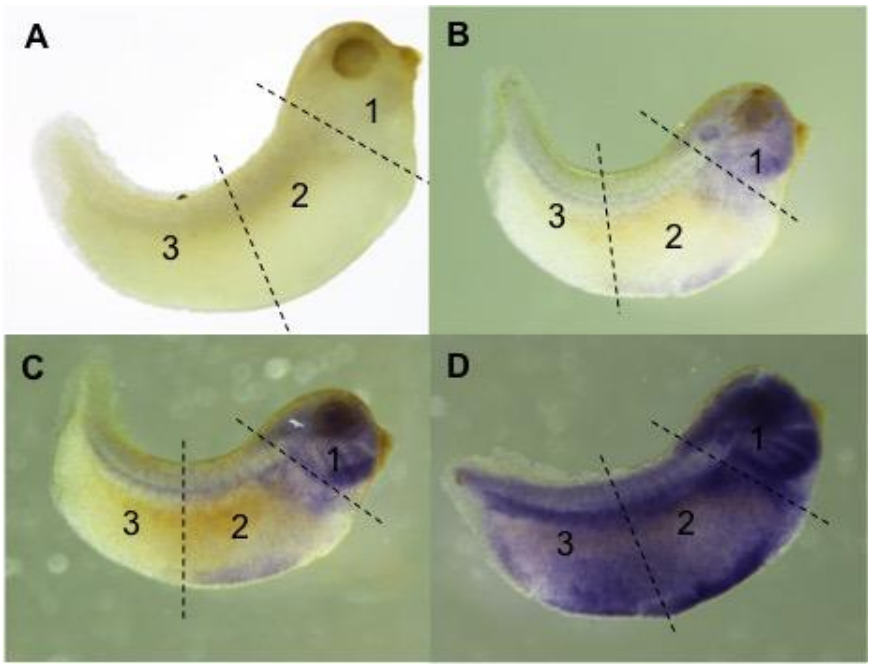

Figure 10 Assessment for gene expression intensity in a Nieuwkoop Faber stage 34 embryo split in three parts (1: head; 2: middle section; and 3: tail). Example embryos showing A) no staining; B) light staining in head, middle section, and tail region; C) medium staining in head and middle section, and light staining in tail region; D) strong staining in head, middle section, and tail region.

4.3 RESULTS

4.3.1 ENZYMATIC INHIBITION

In order to confirm that *srd5* transcripts are being converted into active enzymes, specific enzymatic inhibitors for *srd5α1, srd5α2, srd5α3,* and *srd5β* (e.g., FIN, CA, or UA) were tested during early development. The exposure lasted until the embryos reached stage NF 34. At this stage, the tissue differentiation is clearly apparent and clear staining was achieved in control embryos when using whole mount *in situ* hybridization (Figure 11). Treatment with FIN significantly decreased the mRNA level of all *srd5* (*srd5α1:* $p = 0.0159$; *srd5α2:* $p = 0.0004$; *srd5α3:* $p < 0.0001$; *srd5β*: $p = 0.0001$) as evaluated by a scoring system visually established (Figure 12). The expression of *srd5α2* was not detectable anymore after FIN treatment. The mRNA level of *srd5α1* decreased in particular in the head region. The expression of *srd5α1* in the stomach region was not decreased by FIN. In contrast, staining attributed to *srd5α3* and *srd5β* mRNA expression decreased in all areas but not completely. Embryos exposed to UA did not show any decrease in the mRNA level of *srd5*. Embryos exposed to 100 µM CA were shown to result in a significant *srd5β* reduction ($p = <0.0001$) across the body whereas all other enzymes did not change in expression abundance.

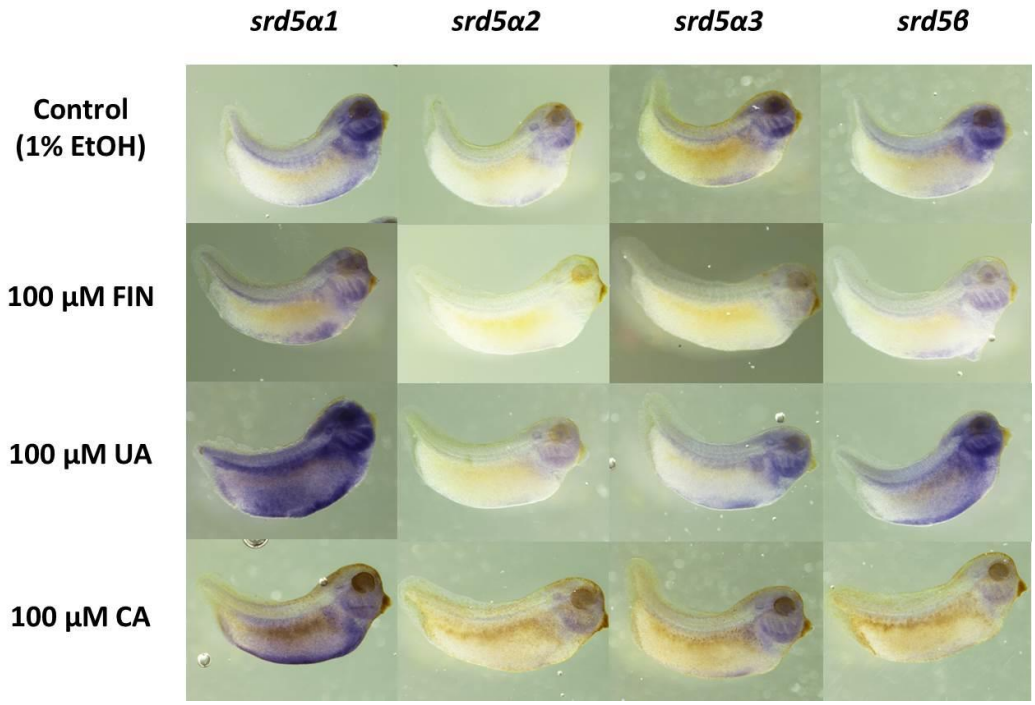

Figure 11 Localization of 5α-reductase type 1, 2, 3 (*srd5α1, srd5α2, srd5α3*) and 5β-reductase (*srd5β*) mRNA level localized by in situ hybridization at Nieuwkoop Faber stage 34 with and without inhibition to 100 µM finasteride (FIN), 100 µM ursodeoxycholic acid (UA), or 100 µM chenodeoxycholic acid (CA).

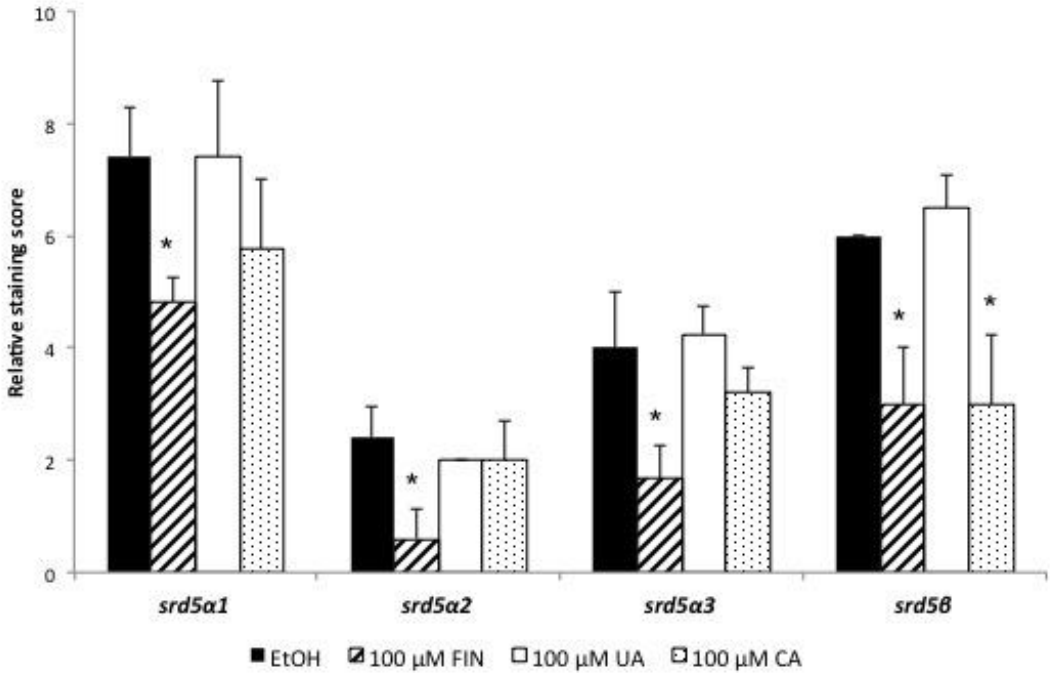

Figure 12 Relative gene expression observed by whole mount in situ hybridization of 5α-reductase type 1, 2, 3 (*srd5α1, srd5α2, srd5α3*) and 5β-reductase (*srd5β*) expressed as a relative staining score of each embryo (n = 5). Nieuwkoop Faber stage 34 embryos were exposed to finasteride (FIN), ursodeoxycholic acid (UA), chenodeoxycholic acid (CA). Significant differences are marked with * ($p < 0.05$) and analyzed by ANOVA.

4.3.2 SPECIFIC DNA METHYLATION IN THE PROMOTER REGION OF SRD5A DURING EARLY DEVELOPMENT

Specific DNA methylation analysis in the promoter region of the three *srd5α* isoforms provided novel insight into the regulation mechanism of the gene expression at various stages of early development. The results suggested specific DNA methylation pattern for the genes encoding the three different *srd5α* isoforms. Specifically, *srd5α1* was the isoform with the least amount of DNA methylation in the promoter region when comparing to the other two isoforms (Figure 13A). A significant increase in DNA methylation occurred between stages NF 2 and NF 13 ($p = 0.002$). The DNA methylation profile of *srd5α1* did not change significantly in the following stages until the beginning of feeding at NF 46. The isoform found mostly in reproductive tissues, *srd5α2,* was found to be highly methylated at all stages of development and did not change significantly throughout embryogenesis (Figure 13B). The DNA methylation state of *srd5α3* is highly methylated at most stages but changed during the development. The methylation pattern of *srd5α3* significantly increased to around 90 % at stage NF 13 ($p = 0.005$) from a very low methylated state at NF 2 (Figure 13C). At stage NF 34 the DNA methylation significantly decreased ($p = 0.0335$) and is in line with increased mRNA expression detected by *in situ* hybridization.

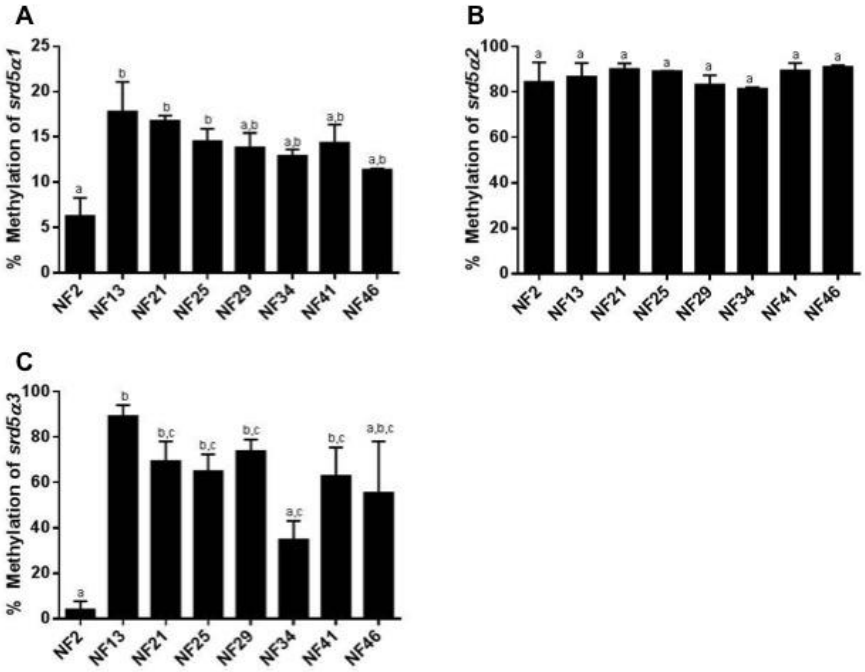

Figure 13 Specific DNA methylation (%) in the promoter region of *srd5α1, srd5α2,* and *srd5α3* during early development of S. tropicalis from Nieuwkoop Faber (NF) stage 2 through 46.

4.3.3 DEVELOPMENTAL EXPRESSION OF SRD5 LOCALIZED USING WHOLE MOUNT IN SITU HYBRIDIZATION

In order to localize the expression pattern of *srd5α1*, *srd5α2*, *srd5α3*, and *srd5β* mRNA, whole-mount *in situ* hybridization during blastula (Figure 14), neurula (Figure 14), tailbud (Figure 15), and tadpole development until the start of feeding at stage 46 (Figure 15) was performed by visually observing the intensity of the staining. Although Srd5α1, Srd5α2, Srd5α3 share the same function, the mRNA sequence similarity between all three isoforms is between 50 and 60%, whereas the Srd5β shares approximately 50% of sequence similarity with the Srd5α isoforms (Table 10). Specific mRNA sequences were targeted when designing probes to eliminate cross reactivity between probes.

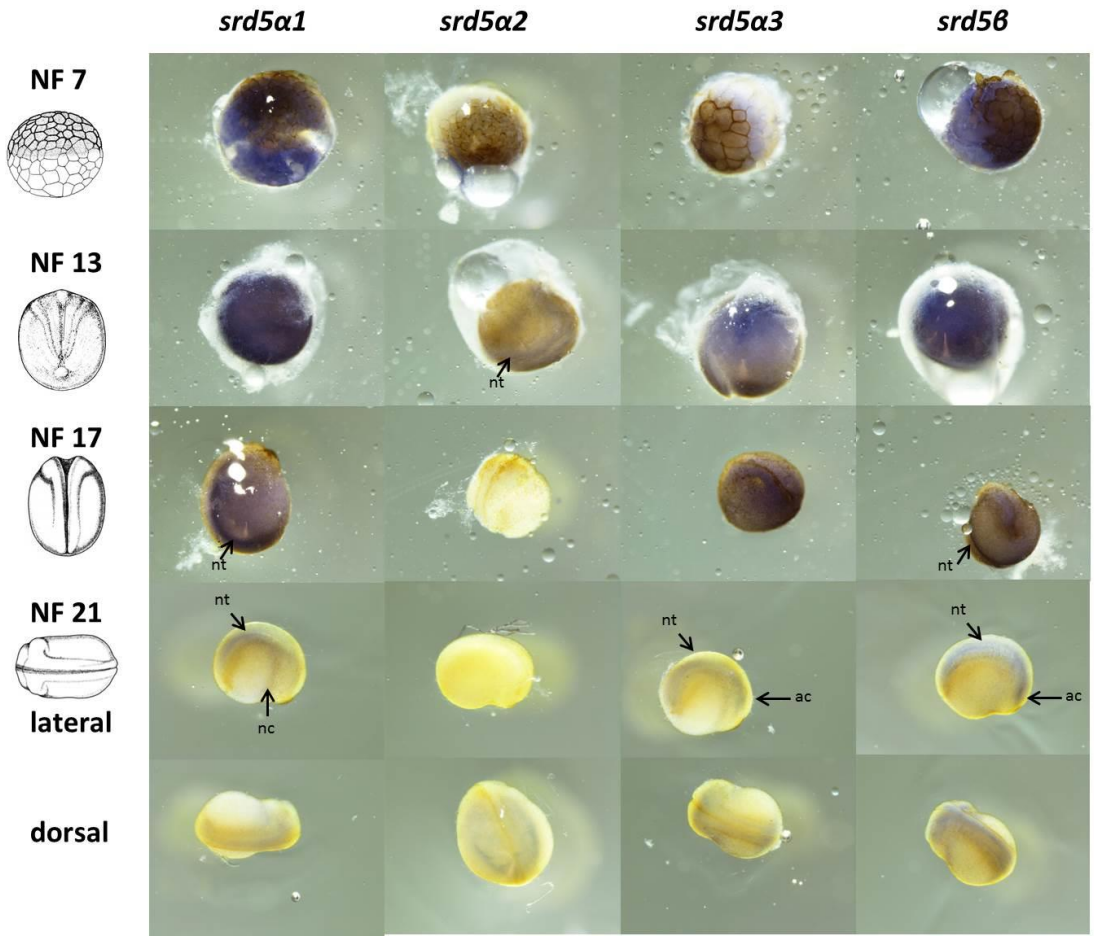

Figure 14 Gene expression of 5-alpha reductase type 1, 2, 3 (*srd5α1, srd5α2, srd5α3*) and 5-beta reductase (*srd5β*) during S. tropicalis early development at blastula and neurula in Nieuwkoop Faber (NF) stages 7 through 21 localized by whole mount in situ hybridization. Legend: ac: archencephalon; nc: notochord; nt: neural tube.

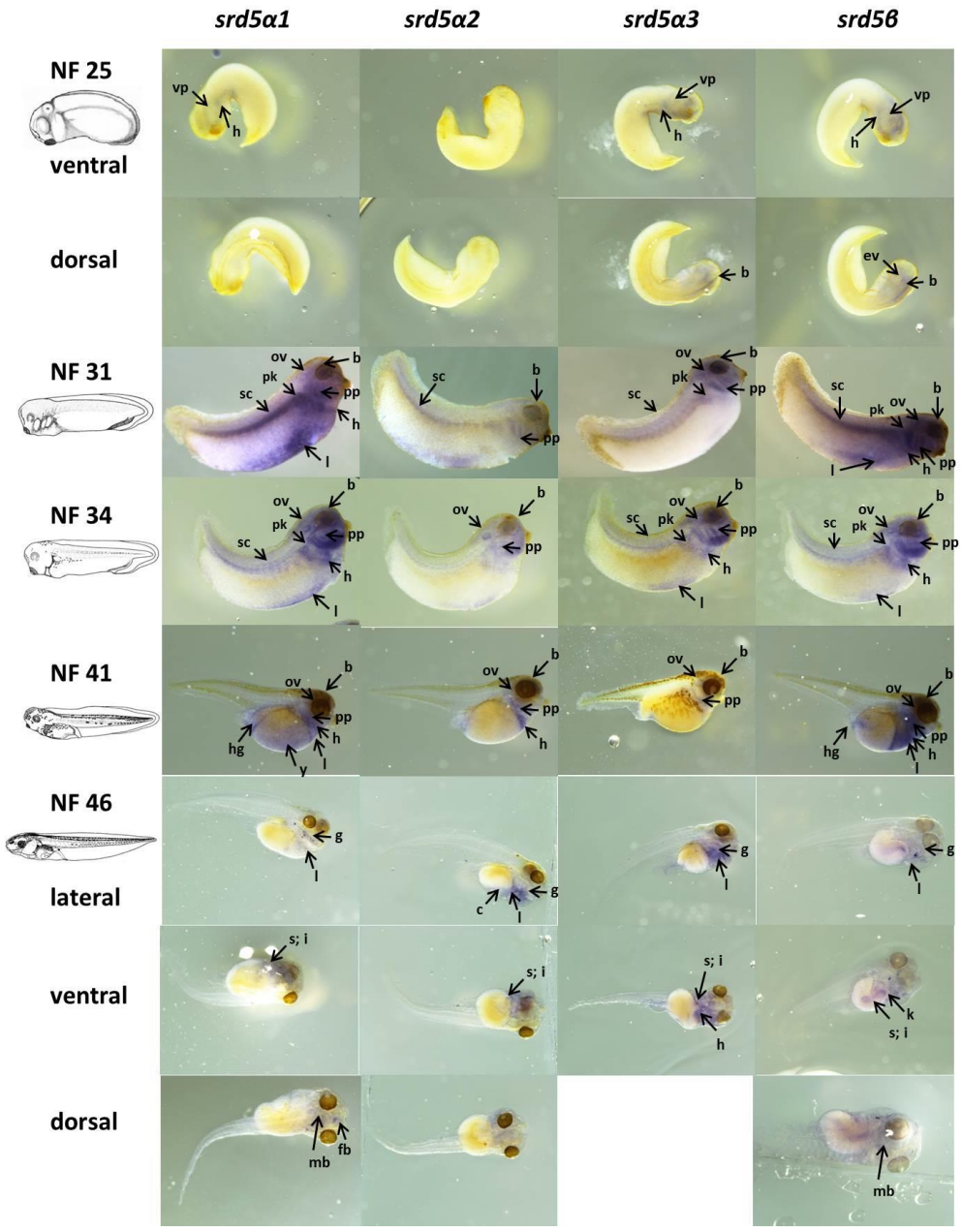

Figure 15 Gene expression of 5-alpha reductase type 1, 2, 3 (*srd5α1, srd5α2, srd5α3*) and 5-beta reductase (*srd5β*) during S. tropicalis early development in early and late tailbud Nieuwkoop Faber (NF) stages 25 through 41 and early tadpole stage NF 46 localized by whole mount in situ hybridization. Legend: b: brain; c: cloaca; ev: ear vesicle; fb: forebrain; g: gills; ha: heart anlage; h: heart; hg: hind gut; i: intestine; k: kidney; l: liver; mb: midbrain; ov: otic vesicle; pk: pronephric kidney; pp: pharyngeal pouch; s: stomach; sc: spinal cord; vp: visceral pouch; y: yolky endoderm.

Table 10 Percentage of mRNA identity of *srd5α* and *srd5β* calculated by the online tool ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

	srd5α2	*srd5α3*	*srd5β*
srd5α1	58.9	50.3	48.9
srd5α2		55.2	52.2
srd5α3			48.2

4.3.4 EXPRESSION PATTERN OF SRD5α1

Early on, in blastula stage NF 7, *srd5α1* transcript is present in the vegetal yolk mass. During neurulation (NF 13 to NF 21), it was observed that the distribution of *srd5α1* transcript gradually became more visually distinguishable between tissues: whereas at NF 13 the expression profile is not clearly visible yet, the expression along the neural plate becomes more pronounced at NF 17. The expression of *srd5α1* along the neural tube and the notochord is visible at NF 21, which marks the start of organogenesis. At the early tailbud stage (NF 25) expression is localized to the head for *srd5α1*. Both the spinal cord and the brain showed a slight staining. During the late tailbud stage NF 31, the expression becomes clearly distinct and is visually observable in the spinal cord and brain. In addition, the *srd5α1* transcript was also observed in the otic vesicle as well as in the pharyngeal pouch. Furthermore, expression was also visible in the heart, pronephric kidney, stomach, and liver rudiment suggesting a vital role of steroid production in these organs. As the development progresses to NF 34, the expression of *srd5α1* is similar to NF 31 regarding to localization of the expression, but decreases in the amount. In terms of NF 41, the expression is localized to the heart, liver, intestine, stomach, otic vesicle, pharyngeal pouch, brain, hindgut, hepatic diverticum and yolky endoderm. NF 46 marks an early tadpole stage and the start of feeding. High *srd5α1* expression is observed in the brain and can be localized to the telencephalon and midbrain. Other areas with lower *srd5a1* expression are located to the gill arches, liver, stomach, and intestine.

4.3.5 EXPRESSION PATTERN OF SRD5α2

srd5α2 transcript is present early on in blastula stage NF 7. At the start of neurulation at NF 13, the expression of *srd5α2* is observable along the neural plate. At the end of neurulation (NF 17) the expression decreases below the detection limit. Organogenesis starts at stage NF 21 and expression is marginally visible on the dorsal head region at that stage, but decreases below detection limit again at the early tailbud stage NF 25. The late tailbud stage NF 31 shows increased and localized expression of *srd5α2* in spinal cord, stomach, and pharyngeal pouch. As the development progresses to NF 34, expression is also starting to become noticeable in the otic vesicle and brain. Expression of *srd5α2* is observed in the otic vesicle, brain, liver, pharyngeal pouch, and heart once reaching NF41. At the early tadpole stage NF46, *srd5α2* expression continues to increase and is observed in stomach, intestine, and cloaca.

4.3.6 EXPRESSION PATTERN OF SRD5α3

The expression of *srd5α3* follows a similar pattern as *srd5α1*. mRNA of *srd5α3* is present at the earliest investigated stage NF 7. At the end of neurulation at NF 17 the expression along the neural tube becomes apparent and staining is clearly visible at the start of organogenesis (NF 21) in different organ systems. Apparent expression is observed in the archencephalon, which later develops into the midbrain and forebrain. NF 25, an early tailbud stage, the expression of *srd5α3* is observed in the heart anlage, visceral pouches, and the brain. Expression of *srd5α3* becomes more distinct at the late tailbud stage 31 and is present in the otic vesicle, brain, pharyngeal pouches, and spinal cord. Later on, at stage 34, expression is also observed in the heart and liver. *Srd5α3* expression decreases in NF 41 larvae but is still detectable in the brain, otic vesicle, and the pharyngeal pouches. In NF 46, the expression level increases and is present in liver, stomach, intestine, gills, and heart.

4.3.7 EXPRESSION PATTERN OF SRD5β

Expression of *srd5β* largely follows a similar trend compared to *srd5α1* and *srd5α3*, with the exception that the observed expression level of *srd5β* increases at the beginning of organogenesis at NF 21. High expression level is visible along the neural tube as well as in the archencephalon. The early tailbud stage NF 25 does not show any expression along the spinal cord but shows expression in the heart anlage, visceral pouch, ear vesicle, and the brain. The expression level of *srd5β* experiences a dramatic increase in the late tailbud stage NF 31. A high transcript level of *srd5β* is observed in the spinal cord, pronephric kidney, otic vesicle, brain, pharyngeal pouches, heart, and the liver. The expression of *srd5β* continues to be present in all these organs until late tailbud stage NF 41. The early tadpole stage NF 46 shows a more distinctive expression pattern in liver, intestine, stomach, kidneys, gills, and midbrain.

4.4 DISCUSSION

Previous *in silico* analysis suggested that the *srd5* genes contain androgen responding elements, indicating the expression of these enzymes are subject to the regulation from androgens (Flood et al., 2013). Moreover, it was previously revealed that exposure to the Srd5 inhibitor FIN led to significantly low levels of *srd5α2* and *srd5β* mRNA in *S. tropicalis* tadpoles (Langlois et al., 2010c). As such, we validated that the *srd5* transcripts observed are being converted into active proteins (enzymes), as their enzymatic inhibition was successful. The *in situ* hybridization data showed that FIN reduced the mRNA level of all *srd5* in *S. tropicalis* at the developmental stage NF 34. It is worth noting that the type 2 isoform exhibited the strongest inhibition, consistent with the previous findings where FIN has demonstrated more inhibitory effect on the expression of Srd5α2 than Srd5α1 (Tian et al., 1994). Furthermore, similar to our finding, recent data demonstrated that FIN inhibited *SRD5α3* at a similar potency as *SRD5α2* in transfected HEK-293 cells (Yamana et al., 2010). Inhibition exposure to Srd5 in amphibians previously showed impaired sexual functions, including decreased spermatogenesis, a female biased sex ratio, accelerated ovarian differentiation, and intersex animals (Duarte-Guterman et al., 2009; Petrini and Zaccanti, 1998; Urbatzka et al., 2009). In depth analysis of adverse effects of Srd5α inhibition in mammals provided evidence that unfavorable outcomes are not limited to sexual functions (Traish et al., 2015). For example, an increase in insulin resistance, an increased risk of cardiac failure, an increased rate of osteoporosis, and changes in neurobehavioral functions were observed (Traish et al., 2015). Thus, a dysfunction of Srd5α can result in a broad range of adverse effects and shows evidence that Srd5α are enzymes producing steroid metabolites that have many crucial roles in an organism. In our study, the localization of *srd5α* in varies organ systems throughout development in *S. tropicalis* points to the fact that these enzymes are crucial in embryogenesis for proper development as inhibition does occur by the same mechanism as in mammals. In addition, FIN also decreased the mRNA level of *srd5β*, agreeing with previous reports (Drury et al., 2009). In order to find a specific inhibitor for Srd5β only, we exposed fertilized frog eggs to CA and UA. CA and UA are bile acids that have been previously shown to inhibit human SRD5β (Chen et al., 2011). Here, we discovered that CA but not UA decreased mRNA levels of *srd5β* but not *srd5α* in *S. tropicalis*. A dysfunction of Srd5β has been linked to hepatic failures in infants (Lemonde et al., 2003). The presence of *srd5β* in tissues other than liver suggests adverse effects if Srd5β activity is lower than normal. With biological functions including neurosteroid synthesis, initiation of steroid hormone clearance, erythropoiesis, and vasorelaxation, it would be expected that dysregulation of Srd5β results in adverse effects other than hepatic failures (reviewed in Langlois et al., 2010b; Chen and Penning, 2014). Thus, a dysregulation of Srd5 potentially leads to detrimental developmental defects. According to our results, the regulation mechanism of *srd5α* and *srd5β* in the frog embryo are self-regulated when exposed to specific Srd5 inhibitors, with decreasing mRNA levels after exposure. These findings correspond to a previous study that exposed frog embryos to FIN (Langlois et al., 2010c). In contrast, *in vivo* FIN exposure to frog tadpoles until after metamorphosis showed tissue-dependent regulation mechanisms of Srd5. The *Xenopus* brain was not affected by Srd5 inhibition, while the liver experienced a decrease in *srd5α2* mRNA and testicular tissue resulted in an increase in *srd5α2* mRNA (Duarte-Guterman et al., 2009; Langlois et al., 2011; Urbatzka et al., 2009). These findings suggest that the regulation of Srd5 is tissue-specific and may be due to a differing response on gonadotropin hormone feedback in different tissues.

Currently, little is known regarding such regulations of Srd5 enzymes during embryogenesis. In particular, no previous research has shown that the transcriptional regulation of the three *srd5α* isozymes is different. The presence of isoform-specific expression pattern of *srd5α* in various tissues proposes a distinctive regulatory mechanism for the three isozymes. We explored the possibility of DNA methylation being part of the transcriptional control for the *srd5α* enzymes. DNA methylation is a key regulator for gene expression and is tightly regulated during normal embryonic development (Chen and Riggs, 2005; Meehan et al., 2005). In *Xenopus* embryos, DNA methylation is typically associated to gene silencing prior to transcriptional initiation (Stancheva and Meehan, 2000). After onset of acquiring the ability of gene transcription at the mid-blastula transition stage, global DNA methylation level sees decreases, possibly leading to changes in DNA methylation of individual gene promoters (Stancheva et al., 2002). In *Xenopus* embryos, the three *srd5α* isoforms exhibited different specific DNA methylation profiles. First, the lowest DNA methylation level observed in *srd5α1* coincides with the highest expression of this isoform throughout development, especially in the later stages. It should be noted that as opposed to the localized mRNA observed by *in situ* hybridization, the DNA methylation pattern was analyzed in the whole body and is compared best to mRNA data obtained by quantitative RT-PCR from whole body animal. Langlois et al. (2010c) quantified mRNA of the three *srd5α* isoforms by quantitative RT-PCR. In this study, the general trend of DNA methylation pattern of *srd5α1* coincides with the abundance of mRNA level in whole larvae suggesting that DNA methylation is involved in regulating the expression level of *srd5α1*. Interestingly, *srd5α2* (the isoform mostly found in reproductive tissues of adults) was found to be highly methylated at all stages of development. In contrast, mRNA of *srd5α2* detected by *in situ* hybridization and quantitative RT-PCR (Langlois et al., 2010c) revealed changing mRNA levels. However, due to the lower expression level of *srd5α2* compared to *srd5α1 and srd5α3,* a change in DNA methylation may be more difficult to observe. Thus, DNA methylation control of *srd5α2* is not ruled out but needs further investigation. In contrary, the DNA methylation pattern of *srd5α3* changed throughout development and is consistent with the results from *in situ* hybridization, as well as quantitative RT-PCR results (Langlois et al., 2010c). Taken together, it appears that DNA methylation is involved in regulating *srd5α1* and *srd5α3* expression in *S. tropicalis* during early development. Our findings expand on the previous report that the expression of both, *srd5α1* and *srd5α2,* are regulated by DNA methylation in rat testis and human tissues (Moribe et al., 2008; Reyes et al., 1997; Rodríguez-Dorantes et al., 2002; Tsunedomi et al., 2010). DNA methylation imprinting is inherited. Thus, a transgenerational effect in the regulation of *srd5α* is expected via maternal transfer. A modified DNA methylation pattern may lead to changes in gene regulation and subsequently lead to developmental defects. Studies have previously shown that an altered DNA methylation pattern in the germ line induced by endocrine disrupters (vinclozolin and methoxychlor) is associated to decreased spermatogenesis in rats and promotes transgenerational diseases (Anway et al., 2005). Thus, the study of transgenerational effects on the regulation of Srd5 is important to understand possible implications by interference with environmental compounds.

The developmental profile data show that maternally transferred mRNA was present for all *srd5* in the stages prior to NF 8. This correlates with another study, which quantified the expression of *srd5* in *S. tropicalis* (Langlois et al., 2010c). Maternal mRNA has been previously detected in mammals (Telford et al., 1990), amphibians (Heasman, 2006a; King et al., 2005; Kloc et al., 2001), and fish (Aegerter et al., 2004). Maternally transferred mRNA is essential before the onset of transcription for embryonic patterning, controlling cell division, and basic biosynthetic processes (Heasman, 2006b; Lee et al., 2014; Tadros and Lipshitz, 2009). The

presence of *srd5* early on suggests that these enzymes are involved in synthesizing steroids during embryogenesis before the onset of transcription. For *Xenopus spp*, the ability to transcribe genes is developed during mid-blastula transition (NF 8; Newport and Kirschner, 1982), which correlates to this present study. Once passing the onset of the start of gene transcription, specific expression of *srd5α1*, *srd5α2*, *srd5α3*, and *srd5β* is present and changes in concentration and location, indicating the ability of transcribing genes. *srd5* transcripts were present throughout early development in organs associated with the CNS, digestive tract, sensory-, cardiac-, respiratory-, and detoxifying systems in the anuran *S. tropicalis* (Table 11).

srd5 mRNA transcripts were observed early on in tissues related to the CNS suggesting that these enzymes might play a role in the development of the CNS in amphibians. Presence of *srd5α1* in the notochord suggests a potential role for 5α-reduced steroids in this tissue. The notochord is involved in the signalling and coordination of development (Malikova et al., 2007). Moreover, throughout early frog development we found that, depending on the stage, transcripts of *srd5* are also present at different transcript levels in the neural plate, neural tube, and spinal cord. Other studies also observed Srd5α in human, rat, and frog spinal cord (Jurman et al., 1982; Poletti et al., 1998; Pozzi et al., 2003). In addition, transcripts of all *srd5* isoforms were detected in the developing frog brain, suggesting that 5α-reduced steroids are necessary during the brain development. Previous studies have shown that the development of the brain during adolescence responds to sex steroids and show sex specific differences (Koolschijn et al., 2014). Moreover, in the bullfrog, 5α-DHT is responsible for maintaining the sexual dimorphism of the pretrigeminal nucleus (Boyd et al., 1992). Other studies revealed that Srd5 isoforms were observed in the endoplasmic reticulum of neurons and play an important role in the development of sexually-dimorphic structures in the brain (Behan et al., 2003). In addition, isoform-specific distribution is present in the brain during development and adulthood for Srd5 enzymes. In particular, Srd5α2 was localized only in specific tissues and time periods. In both rat and guinea pig brains, the mRNA level of *Srd5α2* was significantly higher before birth than after, also with region-specific distribution patterns (Kelleher et al., 2013; Poletti and Martini, 1999). Moreover, the level of *srd5α2* mRNA was shown to gradually decrease throughout metamorphosis in brains of *S. tropicalis* and *X. laevis*, with the lowest mRNA level in NF 66 frog brains (Urbatzka et al., 2007; Duarte-Guterman and Trudeau, 2010). Similarly, our data revealed decreasing expression levels of *srd5α2* in brain throughout development, suggesting the expression pattern of *srd5α2* in *S. tropicalis* being consistent to other species. *srd5α1*, however, was detected in high quantities in the frog brain, agreeing with the previous studies in rat, human, and frog that have shown that *srd5α1* is more abundant in brain than *srd5α2* (Poletti et al., 1998; Poletti et al., 1997; Urbatzka et al., 2007). Similar to *srd5α1, srd5β* was also observed at high expression levels in the frog brain throughout development. Although Srd5β is known for its major function in bile biosynthesis in the liver (Chen and Penning, 2014), it has also been found in brain. Studies have observed Srd5β in avian brain and suggested an important role of Srd5β in inactivating T to 5β-DHT to protect the brain from androgenic action (Balthazart et al., 1986; Tobet and Handa, 2009). Thus, it seems that the presence of *srd5β* throughout early development in the frog may be functionally consistent with the observations from previous studies.

Table 11 Summary of gene expression of *srd5* grouped to organ function. Legend: – not expressed, + light expression, ++ medium expression, +++ strong expression, n/a not applicaple.

Stage	**Organs**	***srd5α1***	***srd5α2***	***srd5α3***	***srd5β***
NF 21	CNS	+	-	+	++
	Sensory system	n/a	n/a	n/a	n/a
	Cardiac system	n/a	n/a	n/a	n/a
	Respiration	n/a	n/a	n/a	n/a
	Detoxifying system	n/a	n/a	n/a	n/a
	Digestive tract	n/a	n/a	n/a	n/a
NF 25	CNS	-	-	+	+
	Sensory system	-	-	-	+
	Cardiac system	+	-	+	+
	Respiration	+	-	+	+
	Detoxifying system	n/a	n/a	n/a	n/a
	Digestive tract	n/a	n/a	n/a	n/a
NF 31	CNS	+++	+	++	+++
	Sensory system	+	-	++	+++
	Cardiac system	++	-	-	+++
	Respiration	+++	+	++	+++
	Detoxifying system	+++	-	+	+++
	Digestive tract	n/a	n/a	n/a	n/a

Table 11 Continued

Stage	Organs	*srd5α1*	*srd5α2*	*srd5α3*	*srd5β*
NF 34	CNS	++	+	++	++
	Sensory system	++	+	+	++
	Cardiac system	++	-	+	++
	Respiration	+++	+	++	+++
	Detoxifying system	++	-	+	+
	Digestive tract	n/a	n/a	n/a	n/a
NF 41	CNS	++	+	+	++
	Sensory system	+	+	+	++
	Cardiac system	+	-	-	-
	Respiration	++	+	+	+++
	Detoxifying system	+	-	-	+++
	Digestive tract	+	-	-	+
NF 46	CNS	+	-	-	+
	Sensory system	-	-	-	-
	Cardiac system	-	-	++	-
	Respiration	+	++	+	+
	Detoxifying system	+	++	+	++
	Digestive tract	+	++	+	+

Androgens also contribute to heart health. An imbalance of androgens such as low T levels and high 5α-DHT levels promote progression of certain cardiovascular diseases (Bianchi and Mezzani, 2013; Dusková and Pospísilová, 2011). Testosterone and 5α-DHT increase cardiac hypertrophy, as well as stimulate the proliferation of cardiac myocytes (Kienitz and Quinkler, 2008). Furthermore, androgens have significant regulatory function in cardiac repolarization (Brouillette et al., 2005). The presence of AR in cardiovascular cells indicates the potential involvement of androgenic action in the cellular function of this cell type (Kienitz and Quinkler, 2008; Pierdominici et al., 2011). Given the role of androgens in heart tissue, Srd5 might be required to ensure proper androgenic function. Our results revealed that in frogs the transcripts of *srd5β, srd5α1,* and *srd5α3* were all present in the heart anlage as well as in the fully developed heart. Srd5 has also been detected in the heart tissue of other species (Chávez et al., 2015; Valérie S. Langlois et al., 2010b). However, in the frog, *srd5α3* was the only gene that was expressed in heart tissue at the most developed stage tested (i.e. NF 46), suggesting a predominant presence of this particular isoform in the heart later in development. Interestingly, however, *srd5α2* expression was not observed in the tissues related to the cardiac system, which corresponds to previous studies suggesting that Srd5α2 is mostly present in reproductive tissues (Langlois et al., 2010b). Srd5α3 is the most recently discovered isoform in the Srd5α family and little is known about its exact functions. While some studies propose that human and hamster Srd5α3 have no catalytic capability to convert T and other 3-keto-4-ene steroids *in vitro* (Chávez et al., 2014), other research shows the ability of Srd5α3 to reduce T and other 3-keto-4-ene steroids *in vitro* (Titus et al., 2014).

In addition to functions related to the heart, *srd5α3* was also detected in the respiratory system along with the other *srd5* enzymes. Specifically, *srd5* enzymes are expressed in the pharyngeal pouch during early developmental stages, which would eventually develop into gill arches where the transcripts for all *srd5* are also observed in the corresponding stages. This observation indicates that in amphibians, Srd5 enzymes and possibly androgens might be part of the respiratory system development process. In mice and humans, *srd5α1* and, although to a lower extent, *srd5α2* are present in the developing lung, whose development has also been demonstrated to be heavily influenced by administration of synthesized androgens (Aumüller et al., 1996; Mikkonen et al., 2010; Seaborn et al., 2010). In particular, androgens stimulate airway branching morphology and cell proliferation (Volpe et al., 2013). Moreover, T reportedly attributes to assisting the control of breathing in humans, as well as modulating cellular respiration and intracellular oxygen level in cancer cell lines (Behan et al., 2003; Prior et al., 2014; Saaresranta and Polo, 2002). Thus, it is not surprising that Srd5 enzymes might also hold significant roles in the development of the respiratory system in amphibians.

In addition, expression of *srd5* enzymes were detected in detoxification and digestive tissues. For example, *srd5* was expressed in liver, starting at stage NF 31, which coincides with the start of liver formation. The liver is responsible for detoxification and metabolizing hormones. A high level of *srd5α1* is found in rat hepatocytes, for inactivating androgens (El-Awady et al., 2004). Similarly, *srd5α1* is expressed continuously, spanning from stage NF 31 to NF 46. In contrast, *srd5α2* mRNA was only detected at NF 41, indicating an isoform-specific expression pattern. *srd5β* transcript is also abundant in liver, which is not a surprise as the enzyme features pivotal function in bile acid synthesis (Chen and Penning, 2014).

4.5 CONCLUSION

With the help of *in situ* hybridization, the current study demonstrated for the first time that steroid metabolites synthesized by Srd5α and Srd5β are expressed in multiple tissues during early amphibian development. Moreover, our results revealed that specific DNA methylation may serve as a part of the regulation of the expression for *srd5α1* and *srd5α3*. In addition, inhibition studies showed that gene regulation of *srd5* in early frog development is based on a positive feedback loop mechanism. Taken together, our study provides novel insight on *srd5* transcript tissue localization and potential mechanisms behind *srd5* expression regulation during frog early development, and provides evidence that Srd5 are functional during the critical early developmental period. Considering that *srd5* is present in varies tissues during embryogenesis, it would be interesting to examine the tissue distribution in male and female adult frog tissues. Moreover, transcriptional changes of *srd5* by androgen stimulation would elucidate gene regulation of *srd5* in different tissues.

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CHAPTER 5: ANDROGENS MODULATE GENE EXPRESSION AND SPECIFIC DNA METHYLATION PATTERN OF STEROID 5A-REDUCTASES IN THE FROG *SILURANA TROPICALIS*

Chapter adapted from **Sonja Bissegger** [1] and Valerie S. Langlois [2] (2016) General and Comparative Endocrinology 234: 123-132

Main contributions of each co-author:

[1] Contributed to original ideas, performed animal exposures, real-time RT PCR, specific DNA methylation analysis, data analysis, and manuscript preparation.

[2] Contributed to original ideas and revised manuscript.

5.1 INTRODUCTION

EDCs present in our aquatic ecosystems have resulted in adverse effects on the endocrine system in vertebrates (reviewed in Vos et al., 2000). Some of these compounds are known to have androgenic or anti-androgenic properties mediating their actions through nuclear receptors or by interfering with enzymes that synthesize steroids. A group of enzymes known as Srd5 (involved in the synthesis of androgens) has been shown to be affected by certain compounds present in the environment. For example, plastic compounds (in rats, Castro et al., 2013), organotins (in humans, Alléra et al., 2004; Doering et al., 2002), polychlorinated biphenyls (in rats, Colciago et al., 2009, 2006; Ulbrich and Stahlmann, 2004), pharmaceuticals (in frogs, Duarte-Guterman et al., 2009) and pesticides (in rat, (Cooke et al., 1998; Kniewald et al., 1979; in frog, Langlois et al., 2010a) have been shown to modulate mRNA and/ or protein expression of Srd5 in different species. It has been established that the dysregulation of Srd5 leads to a variety of diseases in humans, including acne, hirsutism, male pattern baldness, benign prostate hyperplasia, prostate cancer, congenital glycosylation disorder, and hepatic disorders (Azzouni et al., 2012; Cantagrel et al., 2010; Clayton et al., 1996; Mindnich et al., 2011). In amphibians, specific inhibition of Srd5 resulted in altered sexual development by inducing a female biased sex ratio, intersex animals with altered *srd5* mRNA transcripts, and disrupted spermatogenesis (Duarte-Guterman et al., 2009; Urbatzka et al., 2009). These observations demonstrate that Srd5 are crucial enzymes involved in a variety of biological functions, including erythropoiesis, bile acid formation, vasorelaxation, parturition, drug metabolism, neuroactive steroid production, and most significantly, androgen biosynthesis (reviewed in Langlois et al., 2010b).

Androgen biosynthesis is one of the most known functions for this group of enzymes. Indeed, androgens are produced by a series of steroidogenic enzymes. Cholesterol is first converted into progestagens by cholesterol side-chain cleavage enzyme and 17α-hydroxylase and then further transformed into the androgen T by 17, 20 lyase, 3β-hydroxysteroid dehydrogenase (3β-HSD), and 17β-HSD. Testosterone is then converted into the more potent androgen 5α-DHT by Srd5α type 1, 2, and 3 (Srd5α1, Srd5α2, and Srd5α3). 5α-DHT is known to be actively involved in sexual differentiation, particularly in the development and regulation of male secondary characteristics. Alternatively, T can be converted into 5β-DHT by Srd5β, which is considered a crucial metabolite in initiation of steroid clearance (Chen and Penning, 2014). However, the effect of these androgens on the regulation of Srd5 is still unclear.

In vertebrates, the level of androgens is controlled by the hypothalamus and pituitary gland that act to induce positive or negative feedback mechanisms depending on T levels (reviewed in Jin and Yang, 2014). Androgens, in particular 5α-DHT and T, bind to the AR that in turn activates gene expression of specific genes by binding to AREs (Chang, 2002). All four *srd5* are known to have AREs, which indicates that these enzymes could be directly regulated by androgens (Flood et al., 2013). In addition to hormonal activation, transcription factors are essential for gene expression. For example, *srd5α1* and *srd5α2* depend on Sp1 (zinc finger transcription factor) in rat and sterol regulatory element binding proteins in mice to regulate their gene expression (Blanchard et al., 2007; Seenundun and Robaire, 2005; Seo et al., 2009). Another type of gene regulation that could affect the four *srd*5*s* is based on epigenetic processes and is a research area that has gained much interest over the last decade. DNA methylation is a type of epigenetic regulation that is crucial for tissue-specific gene expression (Chen and Riggs, 2005). Methyl groups are attached to the cytosine in a CpG and is often associated with gene silencing. This methylation process can suppress gene expression by either eliminating access of

transcription factors to the promoter site or by forming a tight chromatin structure that creates an inactive region (Casati et al., 2015). Reyes et al. (1997) demonstrated that differential DNA methylation of *Srd5α1* and *Srd5α2* in rat testis controls their expression level. In humans, studies have also shown that DNA methylation regulates the *SRD5* mRNA level in lymphocytes (*SRD5α1* and *SRD5α2)* and in liver (*SRD5α2*; Rodriguez-Dorantes et al., 2002; Moribe et al., 2008; Tsunedomi et al., 2010). Altogether, this suggests that androgens and androgenic-like compounds may mediate their effects through enzymatic inhibition, AR binding, and/or DNA methylation alteration.

An e*x vivo* approach was used to elucidate tissue- and sex-specific changes of *srd5* before and after androgen exposures in the frog *Silurana tropicalis*. Brain, liver, and gonads were exposed to T, 5α-DHT, and 5β-DHT and further analyzed for *srd5* expression and DNA methylation. This study presents novel insight in regards to the regulation of *srd5* and provides valuable information on the potential interactions between EDCs exhibiting androgenic properties and *srd5* transcription in amphibians.

5.2 MATERIALS AND METHODS

5.2.1 EXPERIMENTAL ANIMALS

Maintenance of male and female *S. tropicalis* occurred in dechlorinated and aerated water at the Queen's University Animal Care facilities (Kingston, ON, Canada) in accordance with guidelines of the Institution's animal care protocols and the Canadian Council on Animal Care. Animals were kept in a 12:12 h light:dark cycle with a water temperature of 26 ± 1 °C.

The *ex vivo* assay was used as optimized and described in Chapter 2 (section 2.2.3) with a few alterations. Concisely, 16 male and 16 female juvenile frogs were anesthetized in 0.1% MS-222 (Sigma, Oakville, ON, Canada). Brain, liver, and gonads were carefully dissected and placed in separate 1.5 mL centrifuge tubes filled with 500 µL ice cold L-15 media (Sigma, Oakville, ON, Canada) at pH 7.4. Once all animals of the same sex were dissected, the brain, two pieces of each liver (10-30 mg pieces), and testes or two pieces of each ovary (10-30 mg pieces) were transferred into designated wells in 24-well plates containing 500 µL ice cold L-15 media. The weight of each organ (brain: n = 4, liver: n = 8, testis: n = 8, ovary: n = 8 per treatment group) was recorded. Prior to the start of the incubation, the media in each well of the 24-well plate was replaced by 500 µL of L-15 media containing 0.05% ethanol (control samples) or L-15 media containing 1 µM of one of the three androgens T, 5α-DHT, or 5β-DHT (Steraloids, Newport, RI, USA). The 24-well plates were incubated for 6 h at 26 °C using an orbital shaker. After 6 h, the organs were snap-frozen on dry ice and stored at -80 °C for subsequent RNA isolation and gene expression analysis. The same experimental design was followed for a second *ex vivo* assay with male and female gonads only. These gonads were incubated for 6 h at 26 °C and then snap-frozen on dry ice and stored at -80 °C for subsequent DNA extraction and DNA methylation analysis.

In addition, three male and three female frogs were carefully dissected after anesthetizing the frogs in 0.1% MS-222. One testis (15-25 mg) or a ~30 mg piece of ovary of each frog was snap-frozen on dry ice and stored at -80 °C for subsequent DNA isolation and DNA methylation analysis of non-exposed animals.

5.2.2 RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was isolated from the brain, liver, and gonads using the Trizol reagent (Life Technologies Inc., Burlington, ON, Canada). Sample homogenization and disruption was done using a sonicator (Fisher Scientific, Toronto, ON, Canada). RNA was resuspended in 20 µL nuclease free water and the concentration and quality was assessed using the NanoDrop-2000 spectrophotometer (Fisher Scientific, Toronto, ON, Canada). Residual genomic DNA was eliminated by the TURBO DNA-free kit (Life Technologies Inc., Burlington, ON, Canada). Total cDNA was obtained from 1 µg RNA and 0.5 µg random primers utilizing the GoScript reverse transcriptase (Fisher Scientific, Toronto, ON, Canada).

5.2.3 REAL-TIME RT-PCR

The expression level of *srd5α1*, *srd5α2*, *srd5α3*, and *srd5β* was determined relative to the reference gene *odc* by real-time RT-PCR using the SYBR Green detection system. Each sample was diluted 1:80 and analyzed in duplicate using the GoTaq qPCR Master Mix (Fisher Scientific, Toronto, ON, Canada) with the optimized concentration of forward and reverse primer (0.1 to 0.65 µM, refer to Table 4 in Chapter 2, section 2.2.6) on a CFX96 Touch™ real-time RT-PCR machine (Bio Rad, Mississauga, ON, Canada). The program used to run all samples included an enzyme activation step at 95 °C for 2 min followed by 40 cycles with 95 °C for 15 sec and 58 or 62 °C (depending on target gene) for 1 min. After the amplification phase, a dissociation curve was established in order to ensure the presence of a single amplicon. Reaction efficiencies were 100 ± 10 % with an $R^2 > 0.990$ and calculated by the CFX Manager Software (Bio Rad, Mississauga, ON, Canada).

In each assay, a standard curve (0.048 to 50 ng), a no template control, and a no reverse transcription control (to ensure the absence of genomic DNA in the samples) were run with the samples. The standard curve was generated by pooling equal amounts of the treated and control samples and was then serially diluted 1:4 to obtain concentrations from 50 to 0.048 ng. The standard curve was then used to interpolate and calculate the mRNA level of target and reference gene in each sample. The mRNA level of each target gene was calculated relative to the reference gene *odc*.

5.2.4 DNA METHYLATION ANALYSIS OF SRD5A

DNA from frog gonads was isolated using the Qiagen Blood & Tissue kit (Qiagen, Toronto, ON, Canada). Concentration and quality were assessed by NanoDrop-2000 (Nanodrop Technologies, Wilmington, DE, USA). Primers to assess the specific DNA methylation in the promoter region of *srd5α1*, *srd5α2* and *srd5α3* in *S. tropicalis* gonads using the qMethyl kit (Zymo Research, Irvine, CA, USA) were designed according to the manufacturer's guidelines (refer to Table 8 in Chapter 4, section 4.2.4). Non-methylated frog DNA standard was prepared by amplification of the genomic DNA using the illustra™ Ready-To-Go GenomiPhi V3 DNA Amplification Kit (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada). Methylated DNA standard was prepared by subsequent methylation of 800 ng of the non-methylated DNA for 4 h at 37 °C using 4 units of the CpG methylase (*M. SssI*, Cedarlane, Burlington, ON, Canada). A standard curve containing 0, 25, 50, 75, and 100% methylated DNA was prepared by mixing the non-methylated and methylated DNA to test the linearity of the designed primers for *srd5α1*, *srd5α2,* and *srd5α3* when using the qMethyl kit. The actual methylation percentage was calculated of each gene and compared to the predicted value.

A triplicate of testis and ovary DNA was then analyzed using the qMethyl kit. The DNA methylation percentage was calculated using the C_t difference between a reference (no enzymes) and a test (including methylation sensitive restriction enzymes) reaction sample. Each experiment included a methylated and non-methylated DNA standard as a control to ensure the qPCR reaction worked properly.

5.2.5 STATISTICAL ANALYSIS

All statistical analysis was performed using the software GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Gene expression results were analyzed after removing outliers using ANOVA with a subsequent post-hoc test (Dunnett's) to determine significant differences between treatments. One-way ANOVA was also used to compare DNA methylation percentages between testes and ovaries. A p-value of < 0.05 was selected as significant.

5.3 RESULTS

5.3.1 STEROID 5-REDUCTASE GENE EXPRESSION IN MALE AND FEMALE FROG BRAIN, LIVER, AND GONAD

The mRNA levels of *srd5α1*, *srd5α2*, *srd5α3*, and *srd5β* were measured in the brain, liver, and gonads of control organs to establish the expression profile in non-exposed tissues (Figure 16). All three *srd5α* isoforms showed significantly higher mRNA levels (ANOVA: *srd5α1*: 1.2-fold increase, $p = 0.0001$; *srd5α2*: 12-fold increase, $p = 0.0002$; *srd5α3*: 8-fold increase, $p < 0.0001$) in the testes compared to the ovaries, but the *srd5β* mRNA level was not different between male and female gonads. While ovaries contain a similar amount of each of the three *srd5α* isoforms, the testes contain a higher mRNA level of *srd5α2* and *srd5α3* than *srd5α1 (srd5α2*: 2.6-fold increase, $p < 0.0001$; *srd5α3*: 2.4-fold increase, $p < 0.0001$). No expression of *srd5α2* was detected in the brain or liver of male and female *S. tropicalis*. mRNA levels of *srd5α1* and *srd5α3* were significantly higher in liver than in brain in both male (ANOVA: *srd5α1*: 4-fold increase $p < 0.0001$; *srd5α3*: 4.5-fold increase, $p < 0.0001$) and female frogs (ANOVA: *srd5α1*: 4.5-fold increase, $p < 0.0001$; *srd5α3*: 2.7-fold increase, $p = 0.0024$), but did not result in a sex difference. The mRNA level of *srd5β* was observed at a much higher concentration than *srd5α* ($p < 0.0001$) in female liver and *srd5β* was higher in female than male frogs (3-fold increase, $p = 0.0237$).

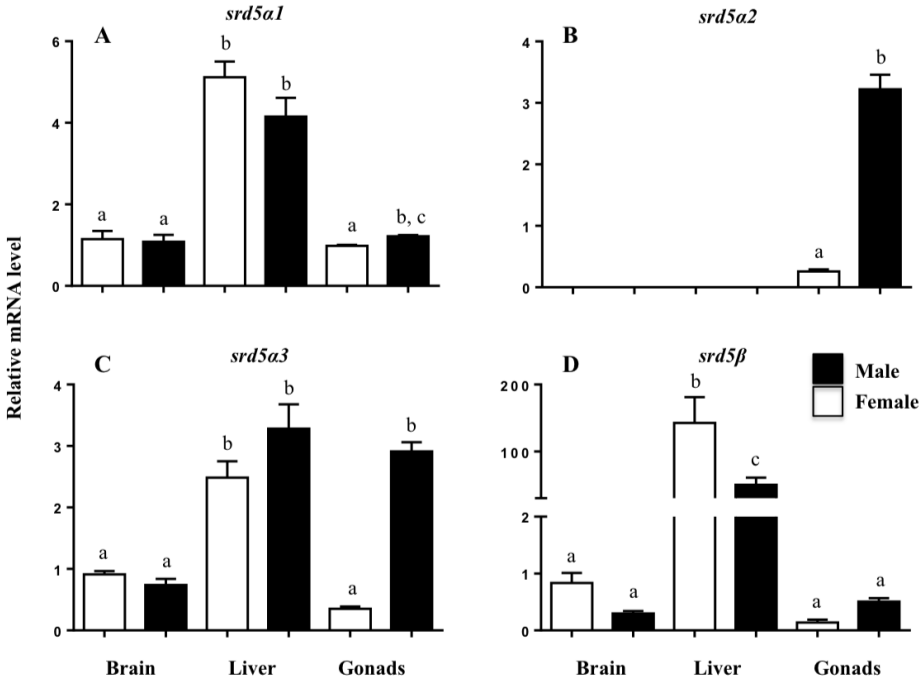

Figure 16 mRNA levels of A) *srd5α1*, B) *srd5α2*, C) *srd5α3*, and D) *srd5β* in male and female frog brain, liver, and gonads. Data are expressed relative to the reference gene *odc*. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance (n = 4-8; $p < 0.05$).

5.3.2 GENE EXPRESSION OF *SRD5* IN MALE AND FEMALE BRAIN, LIVER, AND GONADS OF *S. TROPICALIS* AFTER ANDROGEN TREATMENT

Gene expression of steroidogenic enzymes involved in the T metabolism was analyzed to assess the response to androgens in specific organs in order to gain a better understanding of the regulation of these enzymes. Table 12 presents a summary of all gene expression changes in male and female brain, liver, and gonads following exposure to the androgens.

Table 12 Gene expression changes in male and female frog brain, liver, and gonad following an exposure to androgens. Statistically significant fold changes are expressed as arrows indicating a decrease or increase of the mRNA level. (n = 4 for brain; n = 8 for liver and gonads) Legend: -, no significant fold change; 5α-DHT, 5α-dihydrotestosterone; 5β-DHT, 5β-dihydrotestosterone; n.d, not detectable, T, testosterone

			srd5α1	*srd5α2*	*srd5α3*	*srd5β*
Male	**Testes**	Ctr	-	-	-	-
		T	↑	-	↑	-
		5α-DHT	-	-	↑	-
		5β-DHT	↑	-	↑	-
	Brain	Ctr	-	n.d.	-	-
		T	-	n.d.	-	↑
		5α-DHT	-	n.d.	-	-
		5β-DHT	-	n.d.	-	-
	Liver	Ctr	-	n.d.	-	-
		T	-	n.d.	-	-
		5α-DHT	-	n.d.	-	-
		5β-DHT	-	n.d.	↑	-
Female	**Ovary**	Ctr	-	-	-	-
		T	-	-	↓	-
		5α-DHT	↓	-	↓	-
		5β-DHT	-	-	↓	-
	Brain	Ctr	-	n.d.	-	-
		T	-	n.d.	-	-
		5α-DHT	-	n.d.	-	-
		5β-DHT	-	n.d.	-	-
	Liver	Ctr	-	n.d.	-	-
		T	-	n.d.	-	-
		5α-DHT	-	n.d.	-	-
		5β-DHT	-	n.d.	-	-

In testes, T and 5β-DHT increased the mRNA level of *srd5α1* (ANOVA: T: 1.3-fold increase, $p = 0.0005$; 5β-DHT: 1.2-fold increase, $p = 0.0014$), and all three treatments increased *srd5α3* expression (ANOVA: T: 1.3-fold increase, $p = 0.0404$; 5α-DHT: 1.3-fold increase, $p = 0.0230$; 5β-DHT: 1.4-fold increase, $p = 0.0026$) (Figure 17). Interestingly, no treatment had an effect on *srd5α2*. In the male frog brain, T significantly increased the expression of *srd5β* (ANOVA: 3.7-fold increase, $p = 0.0476$, Figure 18). The male liver produced an increase of *srd5α3* expression after exposure to 5β-DHT (ANOVA: 1.6-fold increase, $p = 0.0305$, Figure 19). On the contrary, the female brain and liver did not result in any significant mRNA level changes by any treatment. Exposure of T to the ovaries resulted in a decreased mRNA level of *srd5α3* (ANOVA: 1.6-fold decrease, $p = 0.0223$, Figure 20); whereas 5α-DHT decreased *srd5α1* and *srd5α3* mRNA levels (ANOVA: *srd5α1*: 1.3-fold decrease $p = 0.0056$; *srd5α3*: 1.6-fold decrease, $p = 0.0181$). Ovaries exposed to 5β-DHT decreased the *srd5α3* (ANOVA: 1.6-fold decrease, $p = 0.0279$) mRNA level.

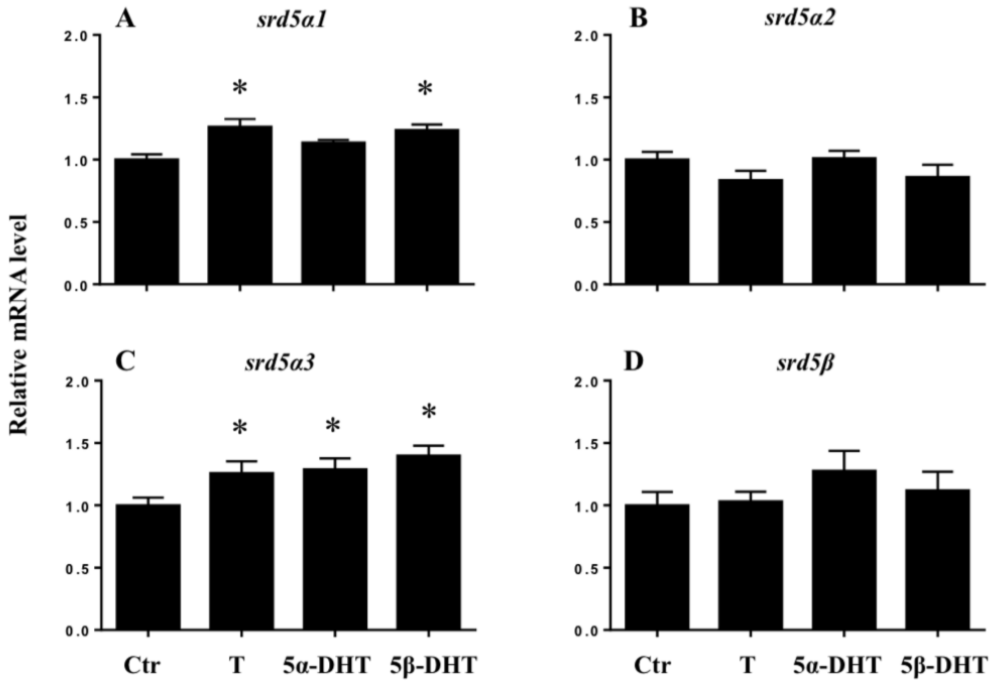

Figure 17 mRNA levels of A) *srd5α1*, B) *srd5α2*, C) *srd5α3*, and D) *srd5β* in frog testes after exposure to testosterone, 5α-dihydrotestosterone, and 5β-dihydrotestosterone. Data are expressed relative to the control sample and normalized to the reference gene *odc*. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance (n = 8; $p < 0.05$). Stars indicate statistically significant differences between treated and control tissues.

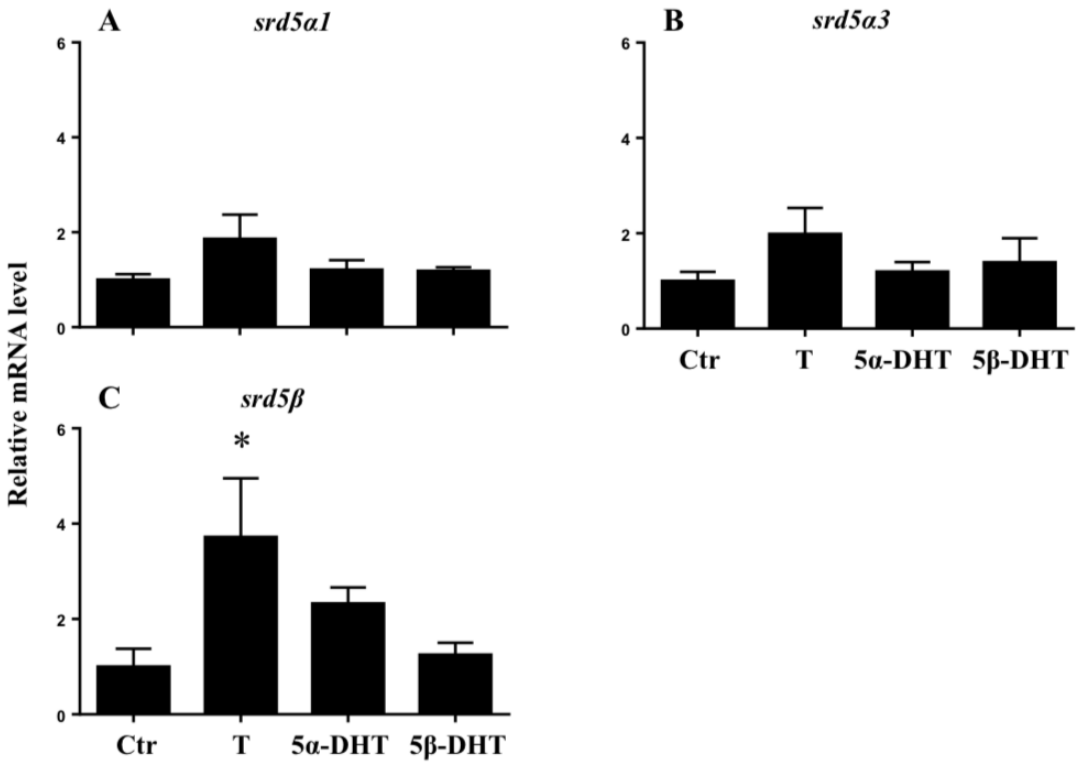

Figure 18 mRNA levels of A) *srd5α1*, B) *srd5α3*, and C) *srd5β* in male frog brain after exposure to testosterone, 5α-dihydrotestosterone, and 5β-dihydrotestosterone. Data are expressed relative to the control sample and normalized to the reference gene *odc*. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance (n = 4; $p < 0.05$). Stars indicate statistically significant differences between treated and control tissues.

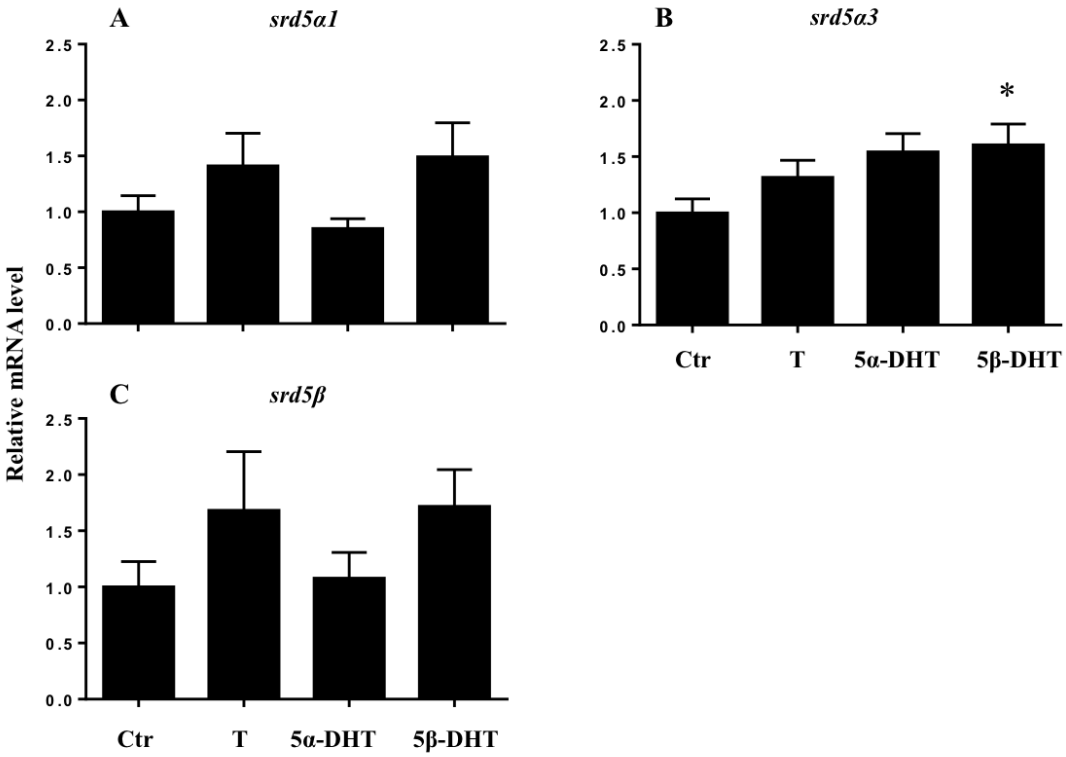

Figure 19 mRNA levels of A) *srd5α1*, B) *srd5α3*, and C) *srd5β* in male frog liver after exposure to testosterone, 5α-dihydrotestosterone, and 5β-dihydrotestosterone. Data are expressed relative to the control sample and normalized to the reference gene *odc*. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance (n = 8; $p < 0.05$). Stars indicate statistically significant differences between treated and control tissues.

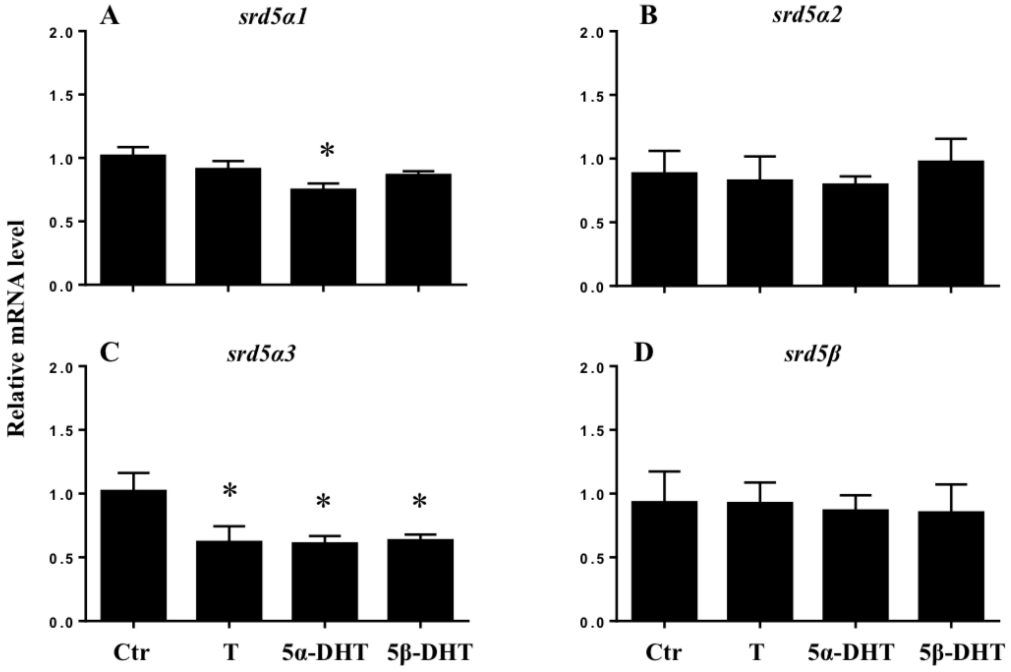

Figure 20 mRNA levels of A) *srd5α1*, B) *srd5α2*, C) *srd5α3*, and D) *srd5β* in frog ovaries after exposure to testosterone, 5α-dihydrotestosterone, and 5β-dihydrotestosterone. Data are expressed relative to the control sample and normalized to the reference gene odc. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance ($n = 8$; $p < 0.05$). Stars indicate statistically significant differences between treated and control tissues.

5.3.3 DNA METHYLATION PROFILE IN TESTES AND OVARIES OF S. TROPICALIS BEFORE AND AFTER ANDROGEN TREATMENT

Frog testes and ovaries were the only examined tissues that contained all three *srd5α* isoforms. Thus, specific DNA methylation analysis in the promoter region using methylation sensitive restriction enzymes, followed by quantitative RT-PCR, was used to gain insight into the regulation mechanism of the three *srd5α* isoforms at the epigenetic level. The differential regulation of *srd5α* in ovaries and testes after androgen exposure lead to the hypothesis that DNA methylation may be involved in their transcriptional regulation. As a baseline, the DNA methylation profile of frog gonads was assessed before androgen treatment (Figure 21).

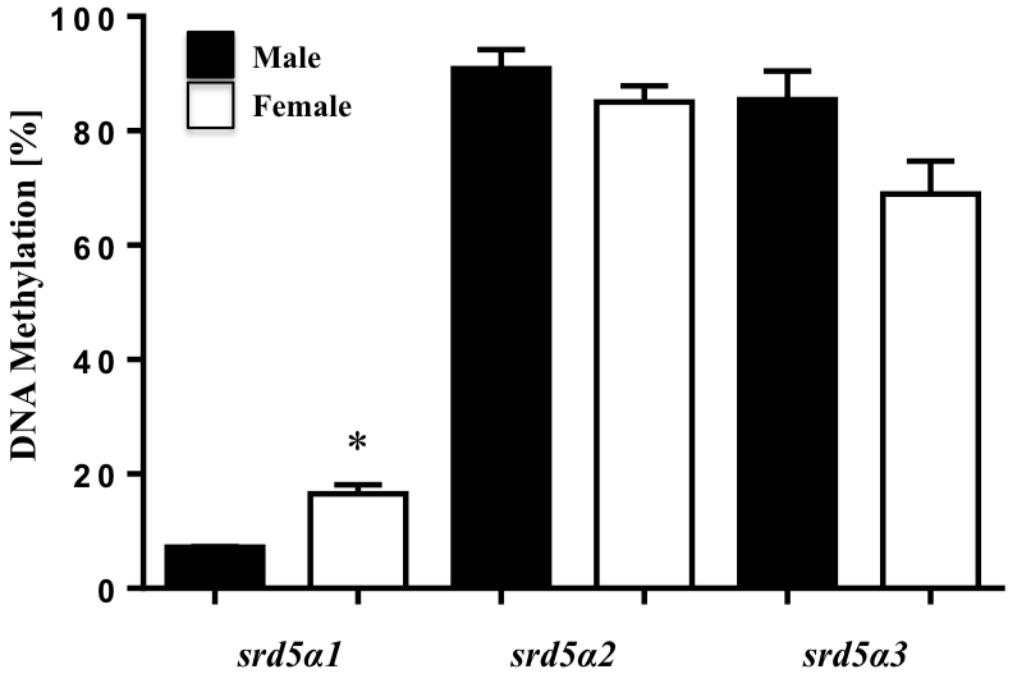

Figure 21 Specific DNA methylation % in the promoter region of *srd5α1*, *srd5α2*, and *srd5α3* analyzed by methylation sensitive restriction enzymes followed by quantitative RT-PCR in frog testes and ovaries. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance (n = 3; $p < 0.05$). Stars indicate statistically significant differences between male and female gonads.

The DNA methylation percentage of *srd5α1* was low (7%) compared to *srd5α2* (91%) and *srd5α3* (85%) in testes and a similar pattern was observed in ovaries (*srd5α1:* 17%, *srd5α2:* 85%, *srd5α3:* 69%). *Srd5α1* was the only isoform that showed a sex specific DNA methylation profile (ANOVA: $p = 0.0299$). The higher DNA methylation percentage in ovaries compared to testes of *srd5α1* corresponds to the lower mRNA level found in ovaries compared to testes. However, the DNA methylation profile of frog gonads after androgen exposure only showed a significant difference in the testes after being exposed to 5α-DHT (Figure 22). Indeed, DNA methylation of *srd5α2* significantly increased 1.1-fold compared to controls ($p = 0.0210$).

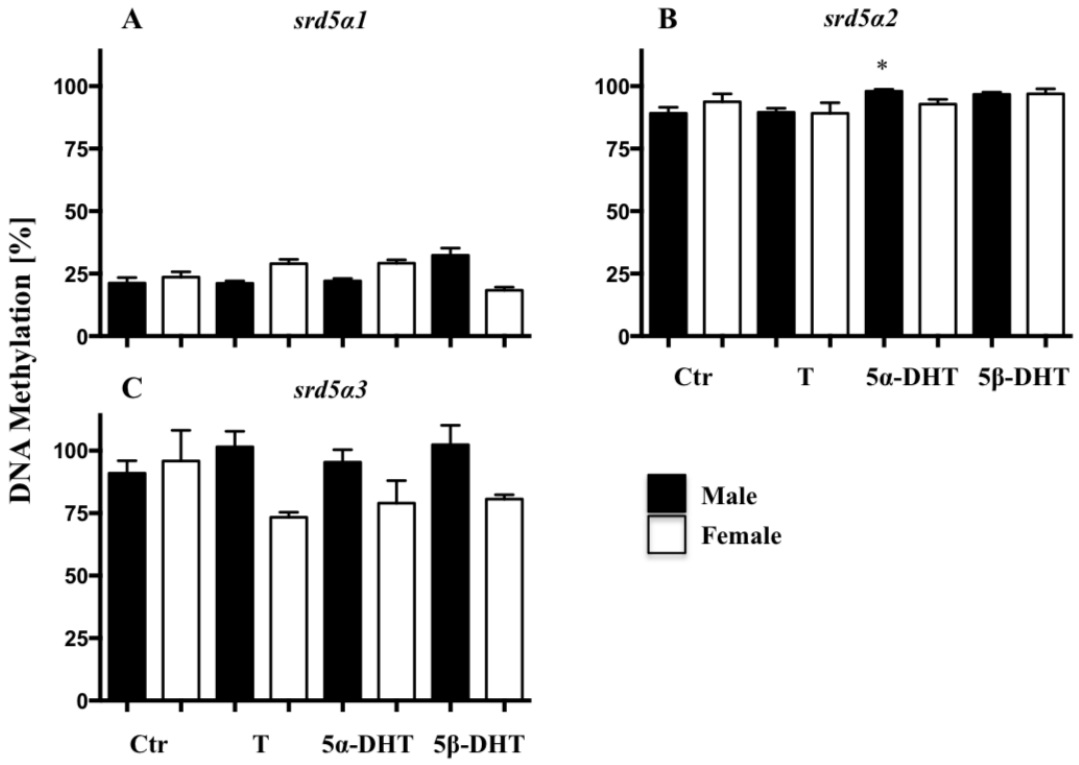

Figure 22 Specific DNA methylation % in the promoter region of A) *srd5α1*, B) *srd5α2*, and C) *srd5α3* analyzed by methylation sensitive restriction enzymes followed by quantitative RT-PCR in frog testes and ovaries after exposure to testosterone, 5α-dihydrotestosterone, and 5β-dihydrotestosterone. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance ($n = 3$; $p < 0.05$). Stars indicate statistically significant differences between treated and control tissues.

5.4 DISCUSSION

Steroid 5-reductases synthesize steroid hormones that exert crucial roles in vertebrates and were shown to be a target for EDCs. However, the current knowledge is limited on *srd5* expression and regulation, particularly in lower vertebrates such as amphibians. Therefore, this study investigated the regulation of androgens on *srd5* expression in frogs using an *ex vivo* approach to elucidate potential mechanisms of action of EDCs with androgenic-like action in organs most associated with steroid synthesis.

Our data confirmed that transcription of *srd5* occurs in the frog brain, liver, and gonads in an isoform- and sex-specific pattern suggesting that male and female frogs can be differently affected by endogenous and likely exogenous androgens. The mRNA level of *srd5α1* was detected in all organs with the liver containing the highest amount of transcripts. Likewise, other vertebrates, including mammals, birds, and fish also present Srd5α1 in adult brain, liver, and gonads with predominant occurrence in peripheral tissues (reviewed in Langlois et al., 2010b). In contrast, *srd5a2* was not detected in frog brain or liver. Localization of Srd5α2 in the liver has been found to be species-specific. This isoform is detected in human and mouse liver, but not in rat liver suggesting that in certain species, including frogs, androgen synthesis in the liver depends on the other Srd5α isoforms (Berman and Russell, 1993; Reyes et al., 1997; Seo et al., 2009; Thigpen et al., 1993). *srd5a3*, which has been discovered during the last decade, was observed in frog brain, liver, and gonads. Little is still known about the tissue distribution and the biological roles of Srd5a3, but others also reported it to be ubiquitously distributed in human and hamster tissues (Chávez et al., 2015; Yamana et al., 2010). Interestingly, frog testes and ovaries showed a significantly different mRNA level of this isoform. The higher abundance of *srd5a3* transcripts in the testes suggests that this isoform is involved in male steroid synthesis at testicular maturity. However, the catalytic capability of Srd5α3 is debated. Srd5α3 has been demonstrated to synthesize androgens in human prostate and peripheral tissues in vitro (Uemura et al., 2008; Yamana et al., 2010, Titus et al., 2014), but other researchers failed to show that human SRD5α3 can catalytically reduce T to 5α-DHT *in vitro* (Cantagrel et al., 2010; Chávez et al., 2015). In comparison to the three isoforms for Srd5α, the enzyme Srd5β is the only protein found in humans with steroid β-reduction capability and its major role is the synthesis of bile acids in the liver (Tsutsui, 2011, Chen and Penning, 2014). Thus, the high transcript level of *srd5β* found in frog liver is likely explained by its role in bile biosynthesis. In frogs, a sex specific pattern of *srd5β* expression was observed with female liver exceeding significantly the amount in the male liver. Other studies have also observed a higher Srd5β activity in female rat liver as compared to their male counterparts (Babelova et al., 2015; Langlois et al., 2010a). Interestingly, *srd5β* mRNA was also detected in the brain and gonads of male and female frogs, suggesting that this enzyme is involved in synthesizing 5β-metabolites in these tissues. Currently, the role of Srd5β in reproduction is not yet acknowledged in any species, but the presence of *srd5β* in frog gonads also suggests a role in controlling the level of androgens.

In addition to isoform- and tissue-specific distribution of *srd5* in adult frog, our data suggest that androgens regulate the transcription of *srd5* in a sex-, tissue-, and isoform-specific manner. While the brain of both male and female *S. tropicalis* did not respond to T or 5α-DHT treatments by modulating the *srd5α* transcription, gonads responded to androgens by modulating the *srd5α1* mRNA level. While T enhanced the *srd5α1* level in the testes, 5α-DHT inhibited *srd5α1* expression in the ovaries, suggesting that androgens regulate gonadal *srd5α1* expression via positive or negative feedback mechanisms in male and female frogs, respectively. Previous

studies observed sex specific responses to androgen treatment. For example, *in vivo* exposure to T decreased SRD5α activity in male rat liver but female rat liver did not respond to T (Dieringer et al., 1979). Furthermore, a tissue-specific regulation mechanism was also found in rat. *In vivo* exposures demonstrated that T negatively regulates *Srd5α1* in the brain, positively controls *Srd5α1* in the liver, and negatively modulates *Srd5α1* in the testes proposing that T also alters *Srd5α1* level in a sex- and tissue-specific manner in rat (Pratis et al., 2003; Sánchez et al., 2006; Torres et al., 2003; Torres and Ortega, 2003a; Torres and Ortega, 2003b). Interestingly, both T and 5α-DHT did not alter *srd5α2* mRNA, suggesting that this isoform is not auto-regulated directly by androgens at sexual maturity. This finding corresponds to other studies that demonstrated that T did not affect the mRNA level of *Srd5α2* in rat testes, but the same study revealed that *Srd5α2* responded to follicle stimulating hormone (Pratis et al., 2003). Since *ex vivo* tissue cultures lack a hypothalamus-driven feedback mechanism, this could be a reason why androgens did not respond to *srd5α2* in the frog gonads. However, other experiments revealed that T down-regulates *Srd5α2* in rat brain and up-regulates *SRD5α2* in human prostate suggesting that *srd5α2* responds to androgens in some organs. (Bonkhoff et al., 1996; Torres and Ortega, 2003b). Isoform specific regulation by androgens was also observed in rats (Poletti and Martini, 1999; Torres and Ortega, 2003b). Moreover, BPA exposure in rat prostate resulted in a isoform specific response, where SRD5α1 and SRD5α2 decreased and SRD5α3 increased in both mRNA and protein level (Castro et al., 2013). Similarly, the *srd5α3* level in frog liver responded to T by stimulating the mRNA level. In addition, T also led to an increase in *srd5α3* mRNA in testes, whereas in the ovary, the *srd5α3* transcript level decreased, suggesting that T positively controls the level of *srd5α3* in testes and liver but in ovaries T regulates *srd5α3* via inhibition feedback. Similar to T, 5α-DHT induced the mRNA level of *srd5α3* in the testes and reduced the transcriptional level of *srd5α3* in the ovary indicating a positive or negative self-regulation mechanism by androgens in the male and female gonad, respectively. The stimulation of the mRNA level of srd5α3 by T suggests that in frog testes as well as in rat prostate Srd5α3 contributes to the synthesis of 5α-DHT and has an important role in reproduction. Recent studies have shown that cancer tissues over-express Srd5α3 in comparison to non-malignant tissues (Godoy et al., 2011). These findings mark the importance of this newly discovered isoform. More research should be dedicated to elucidate its exact function. The difference in response to androgen treatment in frog brain, liver, and gonads indicates that the regulation of *srd5α* by androgens is sex-, isoform-, and tissue-specific.

In addition of *srd5α* being capable of responding to T and 5α-DHT, *srd5β* mRNA levels were also altered following androgen treatment. Indeed, *srd5β* responded differently to androgens than *srd5α.* In the male frog brain, T increased the mRNA level of *srd5β*. It was suggested that the presence of Srd5β in avian brain contributes to the synthesis of 5β-DHT, a metabolite essential for initiating steroid clearance (Tsutsui, 2011, Chen and Penning, 2014). The synthesis of 5β-DHT from T by Srd5β is a process described to eliminate excess T (Barski et al., 2008). Early research has suggested that in birds, an increase in Srd5β activity in the brain protects the avian brain from androgenic action by inactivating T to 5β-DHT (Balthazart and Ottinger, 1984; Hutchison and Steimer, 1981). Furthermore, the ligand access to AR is regulated through the abundance of short-chain dehydrogenases / reductases and aldo-keto reductases including Srd5β suggesting that it is a crucial enzyme in controlling the androgen level in mammals (Bauman et al., 2004; Penning and Drury, 2007). These observations suggest that the increase of *srd5β* may protect the frog brain from excessive androgenic action by eliminating T to 5β-DHT. Presently, 5β-DHT is not believed to be an active androgen. However, in this study we show that 5β-DHT was able to induce similar transcriptional changes as T. 5β-DHT increased *srd5α1* and *srd5α3* in

the testes, whereas *srd5α3* transcription in the ovary was suppressed. This provides novel evidence that 5β-DHT is active in amphibians. The detailed functions of 5β-metabolites in amphibians have yet to be elucidated. In mammals, 5β-metabolites contribute to many biological functions including bile acid formation, erythropoiesis, vasorelaxation, parturition, drug metabolism, neuroactive steroid production, and steroid clearance (Chen and Penning, 2014). The regulation of *srd5α* by 5β-DHT can potentially imbalance the androgen levels, which in turn can initiate biological functions and perhaps also lead to adverse effects. Thus, understanding the gene regulation of *srd5α* is of tremendous importance because of their role in key reproductive functions.

Gene regulation is a complex process and little is currently known as to how the three *srd5α* isoforms are regulated in the gonads where sex steroid synthesis is high. Given that androgens modulated *srd5α* differently in male and female frog gonads, it is suspected that the regulation is affected by other intrinsic mechanisms. DNA methylation is one type of gene regulation involved in tissue-specific gene expression, which was tested in this study to determine if it was involved in controlling the expression of the three different isoforms of *srd5α* in frog gonads. Unexposed gonads showed a low DNA methylation percentage of *srd5α1* and resulted in a sex- specific methylation pattern that coincides with the significantly higher mRNA level present in the frog testes. DNA methylation has been associated with transcriptional repression in mammals leading to low mRNA levels of highly methylated genes (Chen and Riggs, 2005). Thus, the lower mRNA level and higher DNA methylation of *srd5α1* in ovaries compared to testes follows this mechanism. On the contrary, both *srd5α2* and *srd5α3* were found to be highly methylated in both testes and ovaries and not affected by sex. Only a few studies have looked at DNA methylation of *srd5α*. Reyes et al. (1997) demonstrated that DNA methylation also modulates gene expression of *Srd5α1* in rat testis, but they found that *Srd5α2* expression was also modulated by changes in the DNA methylation. Likewise in humans, studies have shown that DNA methylation regulates the mRNA level of *SRD5α1* and *SRD5α2*, but in other cell and tissue types, e.g., in lymphocytes (i.e., *SRD5α1* and *SRD5α2*) and in liver (i.e., *SRD5α2*, Rodriguez-Dorantes et al., 2002; Moribe et al., 2008; Tsunedomi et al., 2010). However, our data suggest that DNA methylation of the three isoforms does not show complete correlation to the mRNA profiles of *srd5α*. While the DNA methylation percentage correlates with the mRNA level in the ovary, DNA methylation in testes does not coincide with the mRNA level observed. These data show that *srd5α* isoforms yield tissue- and isoform-specific regulation mechanisms in *S. tropicalis*.

Can the presence of androgens in the nucleus modify the DNA methylation state of the promoter region of genes? Numerous studies have indeed observed altered DNA methylation profiles after androgen exposure. For example, exposure to 500 ng/L T of zebrafish during two time periods (i.e., 26 to 56 hours post fertilization or 21 to 28 days post fertilization) decreased the global methylation levels in the ovaries (Xu et al., 2014). DNA methylation of 82 specific promoters in genes associated to masculinization of the liver was either up or down regulated in mice by exposure to T (Dkhil et al., 2015). Liu et al. (2014) provided evidence that exposure to EE2 and 17α-methyltestoterone (MT) changed the transcriptional activity of *cyp17a1* in the gonads of male and female rare minnow by altering the DNA methylation pattern. EE2 exposure increased the DNA methylation of *cyp17a1* in the testes, while MT decreased the DNA methylation of *cyp17a1* in ovaries (Liu et al., 2014). In this present study, 5α-DHT increased DNA methylation of *srd5a2* in frog testes. The transcriptional regulation of *srd5a2* by 5α-DHT leads to the potential of modulating the 5α-DHT level by altering the conversion capability of T into 5α-DHT, which can affect the androgen level in the cells. In the testes for example, a proper

androgen level is essential for normal spermatogenesis. Therefore, a modulation of the androgen level can result in adverse effects of biological functions that require androgenic action. In addition, the ability of androgenic compounds to interact with the epigenome in gonads has significant implications for evolutionary biology by promoting transgenerational diseases and the potential to reprogram germ lines (Anway et al., 2005). Thus, it is imperative to understand how androgens interfere with transcription at the epigenetic level. We provide a first insight that androgens potentially change DNA methylation pattern in frogs.

5.5 CONCLUSIONS

Taken together, our results show that androgens regulate the three *srd5α* isoforms in a sex- and tissue-specific manner, suggesting that male and female frogs can be differently affected by endogenous and likely exogenous androgens. While androgens modulated the transcriptional level of *srd5α1* and *srd5α3*, the commonly investigated *srd5α2* isoform was altered at the DNA methylation level. Thus, we suggest that the Srd5 enzyme family is a target for EDCs and provide a mechanism to modulate the androgen biosynthesis in frogs, which can potentially result in adverse effects by altering important biological functions.

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CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

The overall aim of this thesis was to gain a better understanding of the enzymes Srd5α and Srd5β in relation to their molecular regulation and biological functions during development and reproduction in amphibians.

6.1 PHTHALATES MODIFY THE TRANSCRIPTIONAL LEVEL OF ENZYMES INVOLVED IN TESTOSTERONE METABOLISM

Past research studies have demonstrated that EDCs can impede enzymes involved in steroid synthesis. In particular, mRNA and/or protein levels of Srd5α and Srd5β were shown to be altered by EDCs in various species, and thus, pose a risk for reproductive dysfunction. Phthalates are known to interfere with the male reproductive system (Kay et al., 2014); however, the exact mechanism of action has not been clearly demonstrated. One of the possible mechanisms of action suggested is the modification of the gene expression of enzymes involved in steroid production, which may lead to altered steroid levels, that in turn interfere with normal reproductive functions. Experimental data of the presented thesis, established for the first time that both *srd5α* and *srd5β* mRNA levels are modified when exposed to the phthalates DEHP, DBP, and DEP in frogs. This confirms that EDCs can affect the molecular level of crucial genes required in the reproductive axes in amphibians. Therefore, future research should focus on this newly observed mechanism of action by which phthalates may interfere with development and reproduction (i.e., Srd5β) in order to assess consequences of *srd5* alteration in frogs, but also other vertebrates.

6.2 SRD5 INHIBITION ALTERS STEROID LEVELS AND GENE NETWORKS IN FROG TESTES

Chapter 3 investigated the consequences of Srd5 inhibition in frogs. As androgen production is known to be very important to the male gonad, a human Srd5 inhibitor (i.e., FIN) was used to inhibit Srd5 *ex vivo* in testes. FIN significantly modified steroid levels as well as gene networks associated to biological functions involved in the reproductive system. This finding verified that similar to other species, Srd5 is involved in vital functions related to reproduction in frogs. In addition, gene networks correlated to biological functions beside reproduction were altered as well. These data propose that Srd5 is also involved in essential functions unrelated to reproduction, such as oxysterol synthesis, apoptosis, and epigenetic regulation. This indicates that several biological functions would be altered in frogs if EDCs (e.g., phthalates, pharmaceuticals) were to target these enzymes.

6.3 SRD5 ARE EXPRESSED AND FUNCTIONAL DURING EARLY DEVELOPMENT

Little is currently known about the tissue distribution of Srd5 in frogs. Srd5 have been detected in whole larvae during early frog development, but the tissue localization was not established. Tissue localization of these enzymes is important to better understand their roles in different tissues outside of reproduction as highlighted in Chapter 3. During the course of this Ph. D., specific probes for *in situ* hybridization were designed and the localization of *srd5α1, srd5α2, srd5α3,* and *srd5β* expression was characterized throughout frog embryogenesis. I found that all four isoforms are maternally transferred and expressed during the early stages of development in the central nervous, sensory, cardiac, respiratory, and detoxifying systems. However, isoform- and tissue-specific expression patterns were observed. These findings demonstrate that EDCs that target Srd5 (e.g., phthalates, pharmaceuticals) could potentially interfere with the endocrine system of the developing frog embryo. Thus, it is of tremendous importance to understand the fundamental regulation of Srd5. One of such possible regulating mechanisms is through epigenetic regulation. To test this hypothesis, I designed primers to assess the specific DNA methylation of the three *srd5α* isoforms. Indeed, I found that both *srd5α1* and *srd5α3* are regulated by DNA methylation during embryogenesis, while *srd5α2* did not seem to be regulated via this epigenetic mechanism. This study provides the first evidence of specific DNA methylation of *srd5α* in frogs and also proposes that *srd5* are present in various tissues during early frog development.

6.4 ANDROGENS REGULATE SRD5 IN FROGS

One objective of this thesis was to investigate the hormonal regulation of *srd5* in frog tissues. Hormone regulation is the mechanism most known for regulating Srd5 in mammals. Thus, I conducted androgen exposures to the frog brain, liver, and gonad tissues. My data confirmed that this regulating mechanism was also valid in frogs, but only for *srd5α1* and *srd5α3*. Furthermore, I analyzed specific DNA methylation of *srd5α1, srd5α2,* and *srd5α3*, and that again, only *srd5α1* and *srd5α3,* but not *srd5α2*, were regulated by DNA methylation. Next, it would be interesting to understand the epigenetic regulation of these enzymes with regards to histone and chromatin modifications.

6.5 NEW ADVANCES FOR *SRD5α3*, *SRD5β*, AND 5β-DHT

One of the major research aims of this thesis was to gather more knowledge about the most recently discovered isoform *srd5α3.* Even though the ability for *srd5α3* to synthesize steroids is still debated, my data provided evidence that *srd5α3* transcripts are highly abundant in the frog testes, suggesting a possible role in steroid synthesis. Data also showed that *srd5α3* is widely expressed throughout development, which suggests that Srd5α3 has potentially vital function during this critical period. Moreover, results have demonstrated that the regulation of *srd5α3* is similar to *srd5α1*, which is the isoform with the highest turnover rate and capable in synthesizing 5α-DHT. Thus, I suggest that *srd5α3* is able to synthesize steroids, such as 5α-DHT in frogs. Further research assessing steroid synthesis capability by Srd5α3 in amphibians is warranted.

As compared to Srd5α, the regulation and biological functions of the enzyme Srd5β and its metabolite 5β-DHT are not well explored, in particular in frogs. Mammalian studies have shown that 5β-DHT has no androgenic effect; however, experiments in birds demonstrated that 5β-DHT has a crucial role in clearing androgens from the brain (Steimer and Hutchison, 1981). Similarly, my *ex vivo* exposure to the frog brain showed that *srd5β* mRNA level was induced after T exposure. This provides evidence that sex steroids, including T, regulate *srd5β* transcription in frogs and could potentially be a mechanism to protect the brain from too high levels of androgens. Interestingly, this regulating mechanism was only seen in male brain and not in the female brain. Therefore, this dimorphic observation could be explained by a protective mechanism to control aromatization in the male brain. Additionally, gene expression of *srd5β* was different in male and female liver tissues, suggesting that a dimorphic expression of this enzyme is important to maintain the androgen:estrogen ratio. In addition, *ex vivo* exposure of both 5β-DHT and 5α-DHT resulted in increased mRNA levels of *srd5α*, which implies that 5β-DHT has androgenic properties in frogs and possibly in other vertebrates. Further experiments using androgen exposures in frogs and other species are required to completely understand the regulation of *srd5β* in other organs and during embryogenesis. The data presented in this thesis provides novel data on the distribution and regulation of *srd5α* and *srd5β* in amphibians.

6.6 CONCLUSIONS AND NEXT RESEARCH QUESTIONS

Overall, the experimental data presented in this thesis demonstrated that *srd5α* and *srd5β* are expressed in various tissues throughout frog embryogenesis and adulthood, and are regulated by androgens and DNA methylation. The gained knowledge on expression, regulation, and functions of *srd5α* and *srd5β* are a step forward in understanding a potential mechanism of action of how EDCs can interfere with the reproductive system in frogs. Improving the knowledge on mechanisms of action of EDCs helps in population management and to stop the decline of amphibians.

Future research should in particular investigate the exact mechanism of action by which phthalates interfere with *srd5α* and *srd5β* to adversely affect development and reproduction in frogs and other vertebrates. The creation of knock-out models (*srd5α* and/or *srd5β* deficient) using morpholinos (during embryogenesis) or transcription activator-like effector nucleases (TALEN, during embryogenesis and maturation) would help establish adverse developmental effects and consequences at the physiolocial, cellular, and molecular levels in the absence of Srd5α or Srd5β. Using these knock-out models, not only reproductive functions, but other health issues connected to heart, respiratory, central nervous, and digestive systems could be assessed. Recent research has shown that EDCs (e.g., phthalates) can induce epigenetic modifications. Thus, a more depth analysis of histone and chromatin modification in gene regions of *srd5* would provide evidence if EDCs also interfere with *srd5* at the epigenetic level, which may be another mechanism of action of how phthalates mediate their effect on Srd5 enzymes.

Another area of future research is furthering the fundamental knowledge on Srd5 enzymes. Protein distribution and cellular distribution during embryogenesis and in organs would help identify if mRNA and protein levels of Srd5 are correlated and expressed in the same tissues. Mammals could also be assessed for the possibility of androgen availability and clearance by *srd5β* by *in vivo* or *ex vivo* (i.e., brain) exposure of the animal to T and analyze protein and mRNA levels in the brain. Understanding regulation and expression levels of Srd5

could contribute and enhance our knowledge on how EDCs mediate their action in reproductive organs.

APPENDIX A: LIST OF PUBLICATIONS

LIST OF PUBLICATIONS APPEARING AS CHAPTERS

Bissegger, S., Martyniuk, C.J., Langlois, V.S. 2014. Transcriptomic profiling in *Silurana tropicalis* testes exposed to finasteride. Gen. Comp. Endocrinol. 203, 137–145.

Bissegger, S., Langlois, V.S. 2016.Androgens modulate gene expression and specific DNA methylation pattern of steroid 5α-reductases in the frog *Silurana tropicalis*. Gen. Comp. Endocrinol. 234, 123-132.

Bissegger, S., Langlois, V.S. Steroid 5-reductases are functional during early frog development and are regulated via DNA methylation. Mech. Dev. doi: 10.1016/j.mod.2016.06.005.

Bissegger, S., Langlois, V.S. Phthalates modulate mRNA levels of steroid 5-reductases in *Silurana tropicalis*. In preparation. To be send to the journal Reproductive Toxicology.

ADDITIONAL PUBLICATIONS

Martyniuk, C.J., **Bissegger, S.**, Langlois, V.S. 2013. Current perspectives on the androgen 5 alpha-dihydrotestosterone (DHT) and 5 alpha-reductases in teleost fishes and amphibians. Gen. Comp. Endocrinol. 194, 264–274.

Gibson, L.A., Lavoie, R.A., **Bissegger, S.**, Campbell, L.M., Langlois, V.S. 2014. A positive correlation between mercury and oxidative stress-related gene expression (GPX3 and GSTM3) is measured in female Double-crested Cormorant blood, Ecotoxicol. 23, 1004-1014.

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