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Effects and modes of action of exogenous androgens on gonadal steroidogenesis in male  
mummichog (*Fundulus heteroclitus*)

By

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**DISSERTATION**

**Submitted to the Biological and Chemical Sciences Program**

**Faculty of Science**

**in partial fulfilment of the requirements for the**

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**Wilfrid Laurier University**

**2020**

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## Declaration of Previous Publication and Co-Authorship

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## Declaration of co-authorship

I hereby declare that this thesis contains no materials which have been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference is made within the text.

This thesis includes two original papers published in peer reviewed journals and two unpublished publications. The core theme of the thesis is determining if correlation exists between steroidogenic gene transcript changes and periods of plasma hormone depression induced by exposure to exogenous androgens in the male mummichog (*Fundulus heteroclitus*). The ideas, development and writing of all papers included herein were the principal responsibility of myself, the student, working within the Department of Biology under the supervision of Dr. Deborah MacLatchy.

The inclusion of co-authors reflects the fact that this work came from active collaboration between myself and Dr. Andrea Lister, Research Coordinator of the MacLatchy lab. Dr. Lister aided in training me in specific laboratory protocols, such as the use of Experion to quantify RNA integrity and assisted in structuring and editing manuscripts prior to submission for publication. In the case of chapters two and three, my contribution to the work consisted of concept, design, implementation, data collection and analysis, manuscript drafts and submission for peer review, comprising approximately 90% of the overall workload. I have not renumbered sections of published papers during incorporation into the current thesis.

Signed: Robert Rutherford

Date: March 10, 2020

## Abstract

Androgens are a recognized class of endocrine disrupting compounds that are introduced into the aquatic environment through a variety of anthropogenic sources. Exogenous androgens can interact with the hypothalamus-pituitary-gonadal (HPG) axis in fish and alter its normal function, resulting in effects such as skewed sex ratios, intersex individuals, reduced fecundity, or altered mating behaviours. In fish, these apical effects are commonly correlated to depression of the terminal sex hormones testosterone, 17 $\beta$ -estradiol and 11-ketotestosterone (11KT). It is currently thought that the reduction in plasma sex hormones is an effect of negative feedback inhibition of steroid synthesis within the HPG axis after androgen receptor agonism. The specific point(s) of this inhibition are currently unknown. To begin to identify specific areas of the steroidogenic pathway which may be altered during exposure to exogenous androgens, I utilized male mummichog (*Fundulus heteroclitus*), an estuarine species inhabiting the eastern coast of North America. Using model androgens 5 $\alpha$ -dihydrotestosterone (DHT) and 17 $\alpha$ -methyltestosterone, I conducted a series of gonadal exposures to determine if specific genes, coding for critical enzymes within the steroidogenic pathway, fluctuate after incubation with exogenous androgens. Tissue processed through *in vitro* incubation expressed gene response profiles that were inconsistent and counterintuitive to established effects of plasma hormone depression. A series of follow-up exposures was conducted, with fish injected with 1 pg/g, 1 ng/g or 1  $\mu$ g/g body weight DHT to induce plasma hormone depression and quantify steroidogenic gene transcript levels at the same time period. Testis tissue was sectioned upon excision with one section immediately frozen and a second section processed through *in vitro* incubation before subsequently being frozen. Results from DHT injected fish indicate a correlation between gene expression in testis tissue frozen immediately and plasma hormone depression, with a decrease in transcript levels of steroidogenic acute regulatory protein (*star*), cytochrome P450 17a1 (*cyp17a1*), 3 $\beta$ -hydroxysteroid dehydrogenase (*3 $\beta$ hsd*), 11 $\beta$ -hydroxysteroid dehydrogenase and 17 $\beta$ -hydroxysteroid dehydrogenase occurring at the same time as plasma hormone depression. Response amplitudes were greatest for *3 $\beta$ hsd* and *cyp17a1*, with both of these genes being depressed immediately prior to plasma hormone depression and their recovery correlating to plasma hormone recovery. Gene responses following incubation did not display specific response patterns, indicating that analysis of gene transcript changes from *in vitro* samples is not reflective of actual *in vivo* responses. To test if the observed correlation between plasma hormone depression and gene transcript reduction in DHT injected fish was applicable to other androgens, I injected male mummichog to 11KT to induce plasma hormone depression and quantify steroidogenic genes. Incorporating various steroidogenic substrates into this protocol allowed me to test whether *3 $\beta$ hsd* and/or *cyp17a1* are responsible for depressed gonadal hormone production in 11KT-injected fish. Overall, steroidogenic genes correlated to plasma hormone depression in 11KT-injected fish, with *3 $\beta$ hsd* and *cyp17a1* still being the two most responsive genes. Use of precursors did not increase hormone production rate at any timepoint where transcripts of *3 $\beta$ hsd* and *cyp17a1* were depressed, confirming the important role these two genes play in steroid synthesis during exposure to exogenous androgens. This is the first work to clearly demonstrate the correlation between these genes and depression of plasma sex hormones, on an hourly scale. The data from this thesis will strengthen mechanistic understanding of molecular perturbation resulting in changes at higher levels of biological organization in androgen exposed fish.

## Acknowledgements

The completion of my thesis was in no small part dependant on the love and support of my family and friends. My parents, Gail and Gary, were always willing to lend a sympathetic ear when I felt the Science Gods were punishing me for some perceived slight. My husband Mark went above and beyond the call of duty. He sweetened the victories and diminished the losses through sharing them with me every step of the way. I owe him more than I can repay and know that I would not be where I am today without his unwavering support.

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## List of Abbreviations

*3βhsd*: 3β-hydroxysteroid dehydrogenase  
*11βhsd*: 11β-hydroxysteroid dehydrogenase  
11KT: 11-ketotestosterone  
17,20β-P: 17α,20β-dihydroxy-4-pregnen-3-one  
*17βhsd*: 17β-hydroxysteroid dehydrogenase  
AD: Androstenedione  
AMH: Anti-Mullerian hormone  
ANCOVA: Analysis of co-variance  
ANOVA: Analysis of variance  
AR: Androgen receptor  
CF: Condition factor  
*Cyp11a1*: Cytochrome P450 11A1  
*Cyp17a1*: Cytochrome P450 17A1  
*Cyp19a1*: Cytochrome P450 19A1  
DAX-1: Dosage-sensitive sex reversal, adrenal hypoplasia chromosome X, gene 1  
DBD: DNA binding domain  
DHEA: Dehydroepiandrosterone  
DHT: 5α-dihydrotestosterone  
*Dmrt1*: Doublesex and mab-3 related transcription factor 1  
DO: Dissolved oxygen  
E<sub>2</sub>: 17β-estradiol  
EDC: Endocrine disrupting compound  
EIA: Enzyme Immunoassay  
FSH: Follicle stimulating hormone  
GDP: Guanosine diphosphate  
GnRH: Gonadotropin releasing hormone  
GSI: Gonadosomatic index  
GTP: Guanosine triphosphate  
HPG: Hypothalamus-pituitary-gonadal  
HRE: Hormone response element  
HSI: Hepatosomatic index  
LBD: Ligand binding domain  
LH: Luteinizing hormone  
MeOH: Methanol  
MIS: Maturation inducing steroid  
MT: 17αMethyltestosterone  
P: Progesterone  
PKA: Protein kinase A  
*Sf-1*: Steroidogenic factor 1  
*Star*: Steroidogenic acute regulatory protein  
SU: SU-10603  
T: Testosterone  
TMS: Tricaine Methane sulfonate  
Trilo: Trilostane  
WWTPE: Wastewater treatment plant effluent

## **Chapter One:**

### **Exogenous androgens disrupt the endocrine system in male teleosts: how, where and what do we still need to know?**

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This chapter is intended to be submitted for publication as a review covering information on exogenous androgen interaction with the male teleost endocrine system.

## Abstract

Exogenous androgens are a recognized class of endocrine disrupting compounds and are introduced into aquatic environments through a variety of anthropogenic sources. These androgens negatively interact with the endocrine system of teleost fish and can induce masculinization in genetic females, impair fecundity, and/or reduce clutch sizes in exposed fish. Depression of plasma testosterone (T) and 11-ketotestosterone (11KT) are common effects of androgenic exposure and may lead to adverse effects at higher levels of biological organization. The causative mechanism(s) for this depression are currently unknown but hypothesized to result from negative feedback inhibition of the hypothalamus-pituitary-gonadal (HPG) axis after androgen receptor agonism. Some exogenous androgens alter the secretion of gonadotrophs from the pituitary, which results in depressed rates of gonadal sex hormone synthesis. This is not a universal response to exogenous androgen exposure, and inhibition of specific gonadal steroidogenic enzymes may also impact rates of sex hormone synthesis during exogenous androgen exposure. Investigations into steroidogenic gene expression as a means of identifying specific enzymes in the gonadal steroidogenic pathway altered by androgenic exposure has yielded conflicting results on sensitivity and response amplitude of each gene. Direct linkages between plasma hormone depression and changes in steroidogenesis and steroidogenic gene transcript levels are currently not described. Certain genes, such as *3 $\beta$ hsd* or *cyp17a1*, are sensitive to androgen exposure and may be responsible for depression of sex hormone synthesis. Future research needs to include a focus on more robust linkage(s) between changes in gene transcript levels, effects on enzymatic activity and steroid production, and circulating steroid levels. This review provides an overview of the current knowledge of aquatic androgen sources and impacts on teleosts, focusing on steroidogenesis and gonadal steroidogenic gene expression.

## 1. Introduction

Sex steroid synthesis occurs in various tissues throughout the teleost body (as reviewed in Tokarz et al., 2015) but gonadal tissue is a major source of synthesis, under control of the hypothalamus-pituitary-gonadal (HPG) axis (Golshan and Alavi, 2019). *De novo* synthesis of sex steroids from cholesterol occurs in gonadal cells, resulting in the terminal sex steroids  $17\beta$ -estradiol ( $E_2$ ) and 11-ketotestosterone (11KT; Figure 1.1). These sex steroids initiate effects primarily through receptor-mediated actions (Heinlein et al., 2002; Thomas et al., 2018).

Androgens are a group of sex steroids that include testosterone, androstenedione and 11KT (Figure 1.1). These hormones are involved in growth (Liu et al., 2017), testis differentiation (Devlin and Nagahama, 2002), development of male secondary sexual characteristics (Parks et al., 2001), controlling reproductive cycling (Alavi et al., 2012) and fecundity (Ankley et al., 2018; Lyu et al., 2019) in male fish. Changes in androgen production or maintenance can have negative impacts on these endpoints and may also impact overall population recruitment (Seki et al., 2004).

Various effluents have been reported to contain androgens (Table 1.1), which may act as endocrine disrupting compounds (EDCs) in exposed fish. Androgenic activity has been reported in pulp and paper mill effluents (Milestone et al., 2012), wastewater treatment plant effluents (Cavallin et al., 2016), runoff from agricultural lands (Huang et al., 2019a), and from aquaculture practices (Liu et al., 2017). The presence of androgens in these effluents can be the result of natural excretion (Gomes et al., 2009) or the use of androgens as growth promoters (Johnson and Hanrahan, 2010) or therapeutic drugs (Kaufman et al., 2019).

Anthropogenic effluents typically have low (parts per billion to parts per trillion) concentrations of androgens; however, high androgenic potency enables them to influence reproductive endpoints in fish by interacting with the HPG axis (Leet et al., 2011; 2015). Exogenous androgens affect the HPG pathway in a variety of ways including signal interference between and within sections of the axis (Hur et al., 2012; Karouna-Reiner et al., 2017), agonism/antagonism of hormone receptors (Ankley and Jensen, 2014; Ankley et al., 2018), or alteration of hormone synthesis and metabolism (Hoffman et al., 2008; Fetter et al., 2015; Ornostay et al., 2016). Via these mechanisms, exposure can result in male-skewed sex ratios (Gonzalez et al., 2015), reduced fecundity (Liu et al., 2018), intersex (Niemuth and Klaper, 2018), development of male secondary sexual characteristics in females (Hou et al., 2017), impaired immune function (Chaves-Pozo et al., 2018) and depressed plasma testosterone and 11KT (Rutherford et al., 2015; Table 1.2).

Depressed production and circulating levels of sex steroids are a commonly identified endpoint in fish exposed to exogenous androgens (Li et al., 2011; Margiotta-Casaluci and Sumpter, 2011; Schultz et al., 2013; Feswick et al., 2014; Rutherford et al., 2015; 2019b; Zheng et al., 2016; Lai et al., 2018; Passini et al., 2018). Plasma hormone depression is hypothesized to occur due to negative feedback along the HPG axis after androgen receptor agonism (Martyniuk and Denslow, 2012; Shao et al., 2013; Weber et al., 2019), but there is currently weak quantitative understanding of where within the HPG axis this negative feedback is initiated (Ankley et al., 2020). Exogenous androgen interference with gonadotropin release from the hypothalamus and pituitary is variable (Harding et al., 2016; Wylie et al., 2018), indicating that negative feedback within the HPG axis may occur within the gonad itself.



Despite the well-documented effects of exogenous androgens on the teleost endocrine system, there are no species-spanning biomarkers for androgen exposure. In the threespined stickleback (*Gasterosteus aculeatus*), the glycoprotein spiggin, normally produced by males for nest building, is induced in females exposed to androgens (Muldoon and Hogan, 2016). Spiggin has also been used as a biomarker for antiandrogen exposure in male stickleback, as antiandrogens will suppress its normal expression (Pottinger et al., 2013). The use of spiggin as a biomarker is limited, as it is not expressed in most teleost species.

Development of male secondary sexual characteristics in females, such as nuptial tubercles in fathead minnow (*Pimephales promelas*; Villeneuve et al., 2017), a gonopodium in western mosquitofish (*Gambusia affinis*; Huang et al., 2016) or male coloration in many fish species (Zhang et al., 2019; Ujhegyi and Bokony, 2020) have been used as indicators of androgen exposure. These attributes are normally only expressed in males and are under androgenic control (Ogino et al., 2018), which makes them useful biomarkers for androgen exposure in sexually dimorphic fishes. While no definitive biomarkers have been established for androgen exposure, the genes *3 $\beta$ hsd* or *cyp17a1* have been suggested as potential candidates (Kim et al., 2007; Zhai et al., 2018) as they are both androgen-sensitive and responsible for androgen synthesis in teleost steroidogenesis.

This chapter outlines the biosynthesis and function of endogenous androgens in male teleosts, identifies anthropogenic sources of androgens in the aquatic environment, outlines the current understanding of how these exogenous androgens interact with the HPG axis and impact multiple endpoints at many levels of biological organization and compares current knowledge of steroidogenesis in mammalian models to teleost models. The main focus of this chapter will be the effects of exogenous androgens on steroidogenic genes, as these genes are commonly used as

substitutes for enzymatic activity. Due to the integral functions sex steroids serve in teleost fish, it is necessary to better understand the interference exogenous androgens exhibit on sex hormone synthesis and circulation. Establishing relationships between changes in steroidogenic gene transcript levels and alterations of plasma sex hormone concentrations is the first step in linking gene expression to higher level endpoints. Improved mechanistic understanding also supports the development of appropriate biomarkers for ecotoxicological testing.

## **2. Androgen biosynthesis and functions in male fish**

### **2.1 Androgen biosynthesis in testis of male teleosts**

Androgens, and other sex steroids, are synthesized in the brain, interrenal and gonadal tissue of teleosts (Beitel et al., 2014; Petersen et al., 2015). In males, the Leydig cells of the testis are the major site of steroidogenesis and this review will focus on the sex steroid synthesis steps within them. Androgen biosynthesis is under the control of the HPG axis which incorporates endogenous and environmental cues to modulate sex steroid synthesis from cholesterol (Miller and Bose, 2011). Major products of steroidogenesis include the dominant teleost estrogen, E<sub>2</sub>, and the main teleost androgen 11KT (Olsson et al., 2005; Carnevali et al., 2018). The steroidogenic pathway (Figure 1.1) is highly conserved across vertebrate taxa and is a major target of endocrine disruption (Feswick et al., 2014).

Gonadal steroid production is initiated by gonadotropin releasing hormone (GnRH), secreted from the hypothalamus. GnRH secretion is controlled by various environmental cues such as temperature, food availability and photoperiod (Alok et al., 2000; Carrillo et al., 2015; Maitra and Hasan, 2016). The exact mechanism of hypothalamus control is not currently known but may include the saccus vasculosus, an environmental sensory organ in some fish species (Nishiwaki-Ohkawa and Yoshimura, 2016), or the presence of a kisspeptin ligand for the G

protein-coupled receptor 54, which may sense changes in insulin-like growth factor-1 or melatonin (Zohar et al., 2001). Kisspeptins are inhibited by high endogenous androgen levels, indicating the presence of a feedback loop which may modulate sex steroid production (Pinilla et al., 2012; Elakkanai et al., 2015). As a result of being stimulated by environmental cues, GnRH synthesis is coordinated among conspecifics during optimal environmental conditions for mating success (Ando et al., 2006; Di Yorio et al., 2019; Paullada-Salmeron et al., 2019).

GnRH stimulates the gonadotroph cells of the adenohypophysis in the pituitary to synthesize and release the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the blood (Peter et al., 1990; Whitlock et al., 2019). In fish, gonadotroph cells are localized along the median axis of the adenohypophysis, near blood vessels, allowing LH and FSH to quickly circulate to the gonads to stimulate steroidogenesis (Zohar et al., 2010).

Gonadal cells possess both LH and FSH receptors which initiate G-protein mediated signal transduction when bound (Levavi-Sivan et al., 2010). This results in the mobilization of cholesterol from the cytosol to the inner mitochondrial membrane, facilitated by steroidogenic acute regulatory protein (StAR). Once across the mitochondrial membrane, cholesterol is metabolized through a series of reactions including enzymatic hydrogenation, side chain cleavage, aromatization and  $\Delta^4/\Delta^5$ -isomerization (Piferrer and Guiguen, 2008; Ribas et al., 2019) to result in sex steroids (Jefcoate and Lee, 2018). The first step in steroidogenesis is the conversion of cholesterol to pregnenolone via the cytochrome P450 11A1 enzyme, which is subsequently metabolized to progesterone or  $17\alpha$ -hydroxypregnenolone via the enzymes  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ HSD) or cytochrome P450 17a (CYP17A), respectively (Simpson and Boyd, 1967; Hanukoglu and Jefcoate, 1980; Rajapaksa et al., 2016). Progesterone is converted to  $17\alpha$ -hydroxyprogesterone and then androstenedione via cytochrome P450 17a (a

hydroxylase reaction followed by a lyase reaction; Tokarz et al., 2015). Similarly,  $17\alpha$ -hydroxypregnenolone is metabolized to dehydroepiandrosterone via CYP17A1 and then to androstenedione via  $3\beta$ HSD (Zhou et al., 2007). Androstenedione can be converted to testosterone via  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ HSD) or to 11KT via  $11\beta$ -hydroxylase and  $11\beta$ -hydroxysteroid dehydrogenase ( $11\beta$ HSD; Rasheeda et al., 2010). Testosterone can also be aromatized to  $E_2$  via cytochrome P450 19a1 (CYP19A1 or aromatase; Bohne et al., 2013). Androgens produced from steroidogenesis include androstenedione and testosterone (Figure 1.1). The resulting apical sex steroids bind to their respective receptors to play integral roles in various physiological processes.

## 2.2 Androgen receptors (ARs)

Androgens initiate physiological effects mainly through binding to their receptors (as reviewed in Golshan and Alavi, 2019). Currently two types of androgen receptors, membrane-bound and nuclear, have been identified in teleosts (Thomas et al., 2012; 2018). The first type, membrane bound ARs, have been identified in and initiate rapid, nongenomic effects (Thomas, 2012) while the nuclear ARs act as transcription factors once bound to androgens.

Four distinct proteins, which appear to be unrelated to the nuclear AR isoforms, are currently identified as membrane ARs in teleosts (as reviewed in Thomas, 2019). These proteins have rapid association and dissociation kinetics and high affinity for DHT, T and 11KT (Braun and Thomas, 2004), and initiate secondary messenger cascades that elicit nongenomic hormonal responses in cells (Hatzoglou et al., 2005). Upregulation of intracellular calcium, inositol 3 phosphate and diacylglycerol and activation of the mitogen-activated protein kinases (MAPK) are commonly identified cell responses to membrane AR binding (Figure 1.2; Heinlein and Chang, 2002; Wunderlich et al., 2002; Sun et al., 2006). The zinc transporter protein Zrt- and Irt-

like protein 9 (ZIP9) is the best studied membrane AR in teleosts, and initiates secondary messenger cascades by activating the G protein-coupled to ZIP9 and increasing intracellular cAMP levels (Berg et al., 2014). While not as extensively characterized as nuclear ARs, membrane ARs have been identified in liver, brain and gonadal cells of fish (Gorczyńska and Handelsman, 1995; Braun et al., 2004; Fordorie and Handa, 2008).

The second AR type, nuclear ARs, were first cloned in fish in Japanese eel (*Anguilla japonica*; Todo et al., 1999), and are distributed in various tissues including liver, spleen, brain and testis (Gorelick et al., 2008). The testis is the major site of nuclear AR expression, primarily in the Leydig and Sertoli cells, which are responsible for modulating the biological activity of androgens (Schulz et al., 2010). There are two subtypes of nuclear AR,  $\alpha$  and  $\beta$ , which differ in tissue distribution and androgen binding affinity among species (Ikeuchi et al., 2001; Pu et al., 2013). Tissue distribution of the two AR subtypes can vary across teleost species. In female Japanese medaka (*Oryzias latipes*), AR $\alpha$  is highly expressed in the olfactory bulb, suggesting a potential role in detecting waterborne androgens as pheromones (Zempo et al., 2013), while the cichlid *Astatotilapia burtoni* has higher expression of AR $\beta$  in all tissues except the pituitary (Harbott et al., 2007). Differential tissue distribution is not a universal occurrence, as similar patterns of AR distribution were found in western mosquitofish (Ogino et al., 2004).

Transactivation studies examining the affinities of androgens to AR $\alpha$  or AR $\beta$  in Murray-Darling rainbowfish (*Melanotaenia fluviatilis*) identified differential affinities between the two AR subtypes for environmental androgens 17 $\beta$ -trenbolone and methyltestosterone (Bain et al., 2015). This indicates that sensitivity to certain environmental androgens may depend on tissue distribution of AR subtypes. Endogenous androgens possess similar binding affinities between

the two AR subtypes, with 11KT > DHT > T > androstenedione (Olsson et al., 2005), with ligand selectivity conserved across most teleost species (Ogino et al., 2009).

Both nuclear AR subtypes act as transcription factors once bound to a ligand. ARs are composed of three specific regions, the DNA binding domain (DBD), the ligand binding domain (LBD) and the NH<sub>2</sub>-terminal transactivation domain (NTD; Heinien and Chang, 2002). Receptor dimerization, a key step in permitting ARs to act as transcription factors, is facilitated by the DBD. This region also anchors the AR-ligand complex to DNA to permit transcription (McEwan, 2004). The LBD is the site of androgen binding, and once bound induces conformational change of the AR to expose the DBD for receptor dimerization (Pu et al, 2013). A transcriptional activation domain is present in the NTD region, allowing the AR-ligand complex to act as a transcription factor on genes possessing an androgen-response element in the promotor region (Figure 1.2; as reviewed in Golshan and Alavi, 2019). There is approximately 70% similarity in the DBD regions of the two AR subtypes, indicating that they may interact with different promotor sequences of target genes (Bain et al., 2015), although this is currently poorly understood in teleosts.

### 2.3 Roles of androgens

#### i) Spermatogenesis and reproductive cycling

Androgens are involved in supporting spermatogenesis in teleost fish, specifically during spermatogonial proliferation (Golshan and Alavi, 2019) and spermatocyte maturation (Schulz et al., 2010). Initial spermatogenesis is under FSH (and subsequently 11KT) control, while later spermiogenic stages are regulated by pituitary LH (Itoh et al., 1988). 11KT interacts with two members of the transforming growth factor-like super family to control spermatogenesis initiation (Miura et al., 2002). 11KT inhibits anti-Müllerian hormone (AMH), preventing it from

depressing spermatogonia proliferation (Miura et al., 1995). 11KT also activates transcription and translation of Activin B, which induces spermatogonial proliferation (Miura and Miura, 2011). Spermiogenic stages of spermatogenesis are controlled by maturation inducing hormones, e.g.,  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ( $17,20\beta$ -P), which regulate seminal plasma pH and induce meiosis via trypsin (Yaron et al., 2006). This production of  $17,20\beta$ -P occurs when the steroidogenic pathway shifts from predominantly synthesizing 11KT to preferentially synthesizing maturation inducing hormones (MIHs; Yaron and Levavi-Sivan, 2011).

Reproductive cycling in adult fish is linked with fluctuating levels of androgen synthesis (Weltzien et al., 2002). Seasonal variation in plasma 11KT levels are noted in several teleost species (Fine et al., 2004; Sisneros et al., 2004; Kachari et al., 2019), with peak levels occurring during the onset of the spawning period (Borg, 1994). FSH stimulates 11KT synthesis in testis tissue by binding to the FSH receptor on Leydig cells, increasing intracellular cAMP and thus initiating a cAMP-dependent protein kinase (Young et al., 2005; Schulz et al., 2010). Plasma testosterone levels also fluctuate seasonally (Borg, 1994) and are positively correlated to spermatogenesis, peaking during late stages (Rinhard et al., 2001). During late spermatogenesis, a reduction in testosterone and 11KT is attributed to a shift in the steroidogenic pathway towards  $17,20\beta$ -P or similar MIH production (Yaron and Levavi-Sivan, 2011).

## ii) Gonadal differentiation

Development of bi-potential gonadal cells into either testis or ovary is influenced by genetic and environmental factors (Conover and Kynard, 1981; Karube et al., 2007). Genetic sex determination is incredibly varied in teleost fish and can include monogenic or polygenic mechanisms (as reviewed in Devlin and Nagahama, 2002 and Martinez et al., 2014). During sex differentiation, androgens do not directly initiate gonadal differentiation, but their presence is

necessary to sustain the process (Schuppe et al., 2016; Anitha et al., 2019). In genetic males, testis formation is dependant on the retention of high levels of endogenous androgens, such as 11KT (Barannikova et al., 2004; Yarmohammadi et al., 2017), and the reduction of estrogen synthesis via the enzyme CYP19A1 (Wang et al., 2017). Androgens are suspected to interact with the sex-determining gene *dmrt1* to decrease the efficiency of CYP19A1, thereby lowering testis levels of E<sub>2</sub> (Leet et al., 2011). *Dmrt1* is a transcriptional regulator of AMH (Fernandino et al., 2008) which is responsible for male testis development in many teleosts (Wang et al., 2010; 2019a), although the specific relationship between AMH expression and testis development are not well understood (as reviewed in Pfennig et al., 2015). AMH may inhibit *cyp19a1* by binding to the promoter region of the gene and altering transcription/translation (Jamin et al., 2008), resulting in depressed E<sub>2</sub> synthesis.

iii) Mating behaviours and strategies

Androgens stimulate mating behaviours by acting as pheromones among conspecifics (as reviewed in Stacey, 2003; 2015). Free and conjugated androgens are released into the environment via the gills, through the bile or in the urine (van den Hurk and Resink, 1992; Sorensen and Stacey, 2004; Stacey, 2015), where they are detected in other fish by the olfactory system (Belanger et al., 2010). Female fish release androstenedione at the completion of vitellogenesis, which stimulates agonistic behaviour in males (van den Hurk and Lambert, 1983).

Androgens are associated with mating strategy in some teleost fish, such as Burton's mouthbrooder (*Astatotilapia burtoni*; Godwin, 2010). Males utilizing a 'sneaker' strategy, where they mimic female coloration and possess less developed secondary sexual characteristics, have lower concentrations of plasma 11KT compared to more aggressive, socially-dominant males which employ a 'bourgeois' mating strategy, investing in mate defense or displaying elaborate



secondary sexual characteristics or courting behaviour (Stacey, 2003). Some teleost species have set mating tactics, while others can transition between the two strategies (Fergus et al., 2013). In species that employ both mating strategies, plasma androgen levels fluctuate in correlation with the selected mating tactic (Godwin, 2010).

iv) Extragonadal roles of androgens

In addition to acting as pheromones for conspecific signalling and influencing mating strategy in some teleost species, androgens play integral roles in neurogenesis and neuroprotection (as reviewed in Diotel et al., 2018). Testosterone favors neurogenesis in song birds (Louissant et al., 2002) and mice (Heberden, 2017), acting to increase cell proliferation and increase neuron survival (Balthazart and Ball, 2016). A similar proliferation of neurons is found in tilapia (*Oreochromis mossambicus*) treated with 11KT, which the authors attribute to 11KT upregulating genes responsible for the cell cycle process (Narita et al., 2018). Androgens play a role in maintaining the blood brain barrier (BBB), a layer of specialized cells that maintain brain homeostasis by controlling molecular movements and protect neurons from pathogens in the blood (Banks, 2012). Depression of gonadal testosterone increases BBB permeability and inflammation (Si et al., 2014), an effect that is abolished by treatment with 11KT (Atallah et al., 2017).

Androgens also have roles in lipid transport and oxidation (Hoffman et al., 2008),  $\beta$  lymphocyte differentiation (Moens et al., 2007), xenobiotic clearance (Martyniuk and Denslow, 2012) and protein metabolism (Dorts et al., 2009). These effects are elicited by androgen receptor activation and can be altered in response to exogenous androgen exposure (Villeneuve et al., 2018). Androgens also regulate endothelial function, inflammation and oxidative stress in

teleosts (Krause et al., 2011; Gonzales et al., 2013; Si et al., 2014), although the specific mechanisms through which they accomplish this is currently unknown in teleosts.

### **3. Anthropogenic sources of exogenous androgens**

#### **i) Pulp and paper mills**

Androgenic substances have been reported in effluent from pulp and paper mills in multiple countries including Canada (Wartman et al., 2009), Chile (Hernandez et al., 2013), China (Hou et al., 2011) and the USA (Orlando et al., 2007) in a range of 40-700 ng/L dihydrotestosterone equivalents (Table 1.1). Phytoandrogens may be present in tree species used for pulping (Gabriel et al., 2017); however, comparison of androgen concentrations in feedstocks vs final effluent demonstrate that mill processes play a larger role in determining androgen concentrations within the final effluent (Milestone et al., 2012). Androgenic potency of pulp mill effluents has been tested using the *in vitro* human androgen receptor binding assay (Parks et al., 2001) but further identification of chemicals causing androgenic effects in fish has been unsuccessful (Jenkins et al., 2003). Androstenedione (Durhan et al., 2002), manool (Scott et al., 2011), and androstadienedione (Jenkins et al., 2004) have been identified in water systems receiving pulp mill effluent and may act as EDCs, but successful identification of specific bioactive compound(s) is hampered by effluent complexity (Singh and Chandra, 2019).

#### **ii) Municipal waste water**

Wastewater treatment plant effluent (WWTPE) is a major source of anthropogenic androgens (Tran et al., 2018; Luo et al., 2019). Recently androgenic activity, detected by yeast androgen screen or liquid chromatography/mass spectrometry, has been reported in sewage effluents from Egypt (Osman et al., 2015), Bosnia (Tousova et al., 2019), India (Williams et al.,

2019), China (Zhang et al., 2018a) and Serbia (Hashmi et al., 2018) in a range of 0.59-1368 ng/L dihydrotestosterone equivalents (Table 1.1).

Androgens are naturally excreted by humans (Gomes et al., 2009; Liu et al., 2009) and may undergo bacterial deconjugation during sewage treatment processes or soon after discharge into the aquatic receiving environment, resulting in increased androgenic bioactivity of WWTP effluent (Bartelt-Hunt et al., 2002; Zhang et al., 2016). In addition to natural excretion, androgens are commonly prescribed for hormone replacement therapies (Kaufman et al., 2019) and cancer treatments (Gao et al., 2019), which contributes to excreted levels.

Wastewater effluent treatment regimes affect the concentration of androgenic compounds present in final effluent (Luo et al., 2014). Aqueous phase removal, wastewater stabilization ponds, or constructed wetlands can all reduce the concentration of androgenic compounds from final effluent (Damkjaer et al., 2018; Herrera-Melian et al., 2018; Zhang et al., 2018b). Even with this decrease in androgen concentration, effluents are still capable of disrupting the HPG axis in fish (Truter et al., 2016). Extending anaerobic conditions during activated sludge treatment of sewage effluent also improves the removal of androgens, as determined by a series of *in vitro* androgen receptor-activation based bioassays (Volker et al., 2016).

Untreated sewage effluent is still found in many locations (Hashmi et al., 2018), which can result in larger concentrations of androgens (1560 ng/L androgen equivalents; Table 1.1) released into the environment, surpassing the predicted no effect limit of androgens in the aquatic environment (Williams et al., 2019). Even in areas where effluents are treated, overflow events due to extreme precipitation or processing error can result in untreated sewage being introduced to aquatic environments (Dhodapkar and Gandhi, 2019).

iii) Agriculture

Application of animal waste, termed biosolids, to agricultural fields is another source of androgenic EDCs (Yost et al., 2014). These biosolids contain both natural and synthetic androgens from beef and swine, in concentrations up to two  $\mu\text{g}/\text{kg}$  wet weight, and enter the aquatic environment through soil erosion, groundwater and runoff (Biswas et al., 2013). Androgenic hormones, such as  $17\beta$ -trenbolone and other metabolites of trenbolone acetate, have been detected in surface waters of environments receiving runoff from animal feedlots (Bartelt-Hunt et al., 2012; Ankley et al., 2018). While surface water concentrations of androgens are generally in the low ng/L range (Ankley et al., 2018), they have been found as high as 1020.8 ng/L downstream from a swine farm in China (Huang et al., 2019a) and at 1530 ng/L in a lagoon receiving effluent from a cattle feedlot in the USA (Khan and Lee, 2012).

Androgens are commonly used as growth promoters in beef, chicken and swine farming operations (Johnson and Hanrahan, 2010). Among the five approved steroid implants from the US Food and Drug Administration, two (testosterone propionate and trenbolone acetate) are androgenic (USFDA, 2011). Portions of these synthetic androgens and their metabolites are excreted into the environment via urine and fecal matter (Cavallin et al., 2014), with an estimated 5.4 tonnes of androgens excreted annually from feedlot animals (Biswas et al., 2013).

#### iv) Aquaculture

Aquaculture operations use androgens to create monosex fish stocks and as growth promoters without typically employing wastewater treatment regimes (Liu et al., 2017). Aquaculture effluents enter nearby aquatic environments at a rate of approximately 300 million  $\text{m}^3/\text{year}$  (CAW, 2013) and contain hormones found in fish food and any hormones excreted by farmed fish. Androgens have been detected in waters near aquaculture operations in a range of 0.3-3.2 ng/L (Table 1.1). Androgenic substances have been identified in fish feed commonly

used in both freshwater and marine aquaculture (Liu et al., 2015a), with 4-androstene-3,17-dione being the most common, at a concentration of approximately 0.5 ng/g food (Liu et al., 2017). Androgen concentrations in aquaculture food vary depending on farmed species, but research indicates marine aquaculture food contains higher concentrations of androgens compared to freshwater operations (Liu et al., 2015b).

v) Complex effluents

In addition to androgens, many classes of endocrine disrupting chemicals, including estrogens, antiandrogens and antiestrogens, are found in various effluents (Valitalo et al., 2016; Ankley et al., 2018). These chemicals can interact within the effluent and elicit responses in fish not seen during exposure to a single compound/class of compound (Thrupp et al., 2018). Understanding the interactions of various chemicals in complex effluents is a major challenge in toxicology, as chemical composition of effluents can vary due to seasonality (Matamoros and Rodriguez, 2017), industry (Perez et al., 2017) or effluent treatment (Ziajahromi et al., 2016). While the impacts of these chemical classes on teleost steroidogenesis are beyond the scope of this review, it is important to note the presence of these chemicals and the impacts they may have on the bioactivity of androgens present within the same effluent.

Estrogenic effects of effluents have been found in pulp mills (Orrego et al., 2010), sewage treatment effluent (Luo et al., 2019) and animal feedlot runoff (Leet et al., 2015). Effluent estrogenicity can be the result of the presence of specific flavonoids in pulp mill effluent (Orrego et al., 2005; 2006), the use of synthetic estrogens in human pharmaceuticals and cosmetics (Dhodapkar and Ghandi, 2019) or the use of estrogen growth implants in the beef and swine industries (Sellin et al., 2009). Estrogenic EDCs act as estrogen receptor (ER) agonists, binding to both nuclear and membrane ERs (Long et al., 2019) and acting as transcription factors

for estrogen-sensitive genes such as *cyp19a1* (Nikoleris et al., 2016). They may also inhibit genes within the steroidogenic pathway responsible for androgen synthesis, such as *11 $\beta$ hsd* or *17 $\beta$ hsd* (Feswick et al., 2016; Siegenthaler et al., 2017). Exposure to estrogenic EDCs decreases expression of AR in teleosts (Zhang et al., 2014), which may interfere with androgen signalling and responsiveness.

Antiandrogens are commonly used in pesticides (Orton et al., 2011), fungicides (Kojima et al., 2004), sunscreens and cosmetics (Kunz and Fent, 2006), and mechanical coolants (Bonefeld-Jorgensen et al., 2001). These antiandrogens act as androgen receptor antagonists and prevent endogenous androgens from binding to their receptors and initiating signalling cascades (Hotchkiss et al., 2008), or prevent bound androgen receptors from acting as transcription factors for androgen-responsive genes (Siegenthaler et al., 2017). Mummichog (*Fundulus heteroclitus*) exposed to 10 ng/L of the antiandrogen cyproterone acetate for 14 days had depressed ovarian testosterone synthesis (Sharpe et al., 2004), which may occur due to inhibition of positive feedback at the level of the hypothalamus or pituitary through competitive inhibition with testosterone for androgen receptor binding (Namer, 1988). In male smallmouth bass (*Micropterus dolomieu*) exposed to cyproterone acetate there was a decrease in female courtship and nest defense behaviours, which remained absent even in groups co-treated with 11KT (Dey et al., 2010). This demonstrates the efficacy of antiandrogens blocking normal androgen signalling within teleosts.

Antiestrogens are typically found in sewage effluent (Ma et al., 2016), due to their inclusion in anticorrosive agents (Harris et al., 2007), detergents (Preuss et al., 2010) and flame retardants (Hamers et al., 2006). Additionally, chlorine disinfection of waste water can generate antiestrogenic by-products through a reaction between hydrophobic acid precursors and bromide

(Wu et al., 2014). Antiestrogens can act as estrogen receptor antagonists, blocking normal function of estrogens or inhibiting estrogen production (Jordan, 2003; Sharma et al., 2018). These chemicals elicit reproductive changes typically associated with exposure to androgens, such as a reduction in female secondary sexual characteristics (Jesl et al., 2018) and depressed levels of plasma estrogens (Navas and Segner, 1998).

#### **4. Effects of exogenous androgens on fish**

##### 4.1 Receptor-mediated pathways

Exogenous androgens can elicit effects in fish through androgen receptor binding (Frye et al., 2008), altered rates of androgen aromatization to estrogens (Martyniuk and Denslow, 2012), or suppression mechanisms triggered during exposure to high exogenous androgens (Ankley et al., 2003). Receptor-mediated pathways are currently hypothesized as the primary route through which androgenic effluents induce masculinization in fish (Brockmeier et al., 2014). While the androgen receptor is the major target of exogenous androgens, some are also capable of binding to estrogen receptors (ERs). Certain non-aromatizable androgens, such as 5 $\alpha$ -dihydrotestosterone, can act as estrogen receptor agonists at high concentrations (Ornostay et al., 2016). Other androgens, such as 17 $\alpha$ -methyltestosterone, are aromatizable and their metabolites act as estrogens and bind ERs (Pawlowski et al., 2004). Teleosts possess two forms of the enzyme cytochrome P450 19A, one localized in the gonad (CYP19A1A) and one in the brain (CYP19A1B; Tchoudakova and Callard, 1998), that are both capable of altering the structure of androgens to synthesize estrogens, which can lead to endocrine effects typically associated with estrogenic exposure (Zhang et al., 2014) such as the induction of vitellogenin in male fish (Hou

et al., 2018; Lai et al., 2018). Some aromatizable androgens have been identified as by-products of steroid metabolism during manure composting (Zhang et al., 2019).

During exposure to exogenous androgens, negative feedback mechanisms within the HPG axis can result in changes to LH and FSH production and release from the pituitary (Sarter et al., 2006; Hu et al., 2011; Wylie et al., 2018), decreasing the overall gonadal steroidogenesis rate. Expression of *lhβ* and *fshβ*, genes coding for LH-β and FSH-β subunits of LH and FSH respectively, are depressed during androgen exposure (Hellqvist et al., 2008; Shao et al., 2013); however, this depression may only occur at low androgen concentrations as increased expression has been reported in fish exposed to higher ( $\geq 0.5\%$  body weight) androgen concentrations (Shao et al., 2013).

Perturbation of LH and FSH production by exogenous androgens may be a case-by-case phenomenon, as some studies have reported no effect on, or increased production of, LH or FSH during exposure in fish (Huggard et al., 1996; Zucchi et al., 2013; Harding et al., 2016). There may be a compensatory effect in fish species where only one of the gonadotropin hormones is impacted by androgen exposure, as both FSH and LH are capable of binding to the other's receptor (Chauvigne et al., 2012), thereby continuing downstream signalling, even in the absence of the native ligand (Chu et al., 2015). The inconsistent effects that exogenous androgens exhibit on LH and FSH production indicate that other inhibitory loops are present within the steps of the gonadal steroidogenic pathway to account for changes in circulating levels of plasma sex hormones.

#### 4.2 Depression of sex steroids

Androgenic interference of steroid synthesis has been reported from *in vivo* and *in vitro* studies in teleosts (Ankley et al., 2008; Bosker et al., 2010; Dang et al., 2011; Margiotta-Casaluci



and Sumpter, 2011). Excised testis from androgen exposed fish typically have depressed production rates of testosterone (Sharpe et al., 2004), E<sub>2</sub> (Rutherford et al., 2015) and 11KT (Glinka et al., 2015), which may account for the lower circulating levels of these hormones in the blood. During exposure to exogenous androgens, gonadal 11KT production generally has a greater magnitude of change and is depressed for a longer period compared to gonadal T production (Feswick et al., 2014; Bosker et al., 2017; Marjan et al., 2018). This indicates feedback mechanisms for excess androgens may target genes/enzymes responsible for the conversion of testosterone to 11KT (Liu et al., 2014; Rajakumar and Senthilkumaran, 2016).

#### 4.3 Impacts on gonadal steroidogenic enzymes and genes

Investigations of steroidogenic gene expression during androgen exposures have returned variable results (Table 1.3), and gene responses depend on fish sex, age, reproductive status, length of exposure and a variety of environmental variables (Chi et al., 2018; 2019; Guzman et al., 2018; Wang et al., 2019a). Linkages between changes in gene expression and subsequent changes at higher levels of biological organization have not been examined. There is disagreement on the correlative relationship between apical endpoints and gene expression analysis in the literature. This is a critical gap in current research that must be addressed if impacts of androgens on the steroidogenic pathway are to be fully understood.

Some recent work has begun to establish correlations between depression of specific steroidogenic genes and changes in sex steroid synthesis rates (Baroiller et al., 2009; Cowie et al., 2015; Wang et al., 2019b; Rutherford et al., 2019b), with *cyp19a1* being the best studied. A study investigating correlations between changes in gene transcript levels and higher-level endpoints identified *cyp19a1* as strongly correlated to *in vitro* E<sub>2</sub> production (Cowie et al., 2015).

Androgens have variable effects on *cyp19a1* expression, with some studies finding an increase in transcript levels during exposure (Lassiter and Linney, 2007; Mouriec et al., 2009; Li et al., 2013), others finding no impact on expression levels (Andersen et al., 2006; Garcia-Garcia et al., 2017) and still others detecting decreases in expression (Kitano et al., 2000; Raghuveer et al., 2005; Zheng et al., 2016). Regardless of gene transcript response, gonadal E<sub>2</sub> production exhibits a positive correlation with *cyp19a1* expression. The differential response of this gene to various exogenous androgens may be derived from whether the androgen is aromatizable or not (Zhang et al., 2012; Liu et al., 2014). In mammals, androgens severely decrease the enzymatic function of CYP19A1 by binding to an androgen-response element in the promoter region of nuclear receptor subfamily 0, group B, member 1 (DAX-1), which once bound is then recruited into the promoter region of *cyp19a1* containing steroidogenic factor 1 (SF-1) where it acts as a repressor of transcription and suppresses enzymatic activity (Lanzino et al., 2013; Maris et al., 2015). DAX-1 appears to be highly conserved across species and has been identified in numerous teleost fish (Li et al., 2013; Hu et al., 2015; Chen et al., 2016) where it is preferentially expressed in testis tissue (Xia et al., 2018). Androgenic suppression of *cyp19a1* may follow the same DAX-1 mediated path as mammals, but this has not been specifically tested in teleosts.

Fluctuations in *star* gene transcript levels have been linked to changes in plasma hormone levels in male rainbow trout (*Oncorhynchus mykiss*) undergoing spermatogenesis (Kusakabe et al., 2006), and during sexual differentiation in orange grouper (*Epinephelus coioides*) injected with FSH (Huang et al., 2019b). Increased *star* expression correlated to increased levels of plasma E<sub>2</sub> and 11KT in these studies, possibly due to increased cholesterol transport into the gonadal steroidogenic pathway, as indicated by *star* transcript abundance (Huang et al., 2019b).

Recent work with male mummichog injected with model androgens 5 $\alpha$ -dihydrotestosterone (DHT) or 11KT identified strong correlation between periods of plasma sex hormone depression and inhibition of the steroidogenic genes *star*, *11 $\beta$ hsd*, *17 $\beta$ hsd*, *cyp17a1* and *3 $\beta$ hsd* (Rutherford et al., 2019b; 2020). Gene transcripts of *3 $\beta$ hsd* and *cyp17a1* were depressed immediately prior to plasma hormone depression in both studies, and recovery of expression coincided with recovery of plasma hormone levels (Rutherford et al., 2019b; 2020). Depression of *3 $\beta$ hsd* is linked with decreases in overall efficiency of the steroidogenic pathway, with the potential to depress synthesis of both androgens and estrogens (Raghuveer and Senthilkumaran, 2012; Lee et al., 2017; Hou et al., 2018), while *cyp17a1* deficiency causes depressed plasma hormone levels in zebrafish (Zhai et al., 2017). Lower plasma androgen levels have been correlated to lower *cyp17a1* transcripts in fathead minnow (*Pimephales promelas*; Hala et al., 2015), mummichog (Rutherford et al., 2019b) and other common model teleosts (Kusakabe et al., 2006; Zhai et al., 2018).

Other studies show a poor, or completely lacking, correlation between gene expression changes and sex steroid synthesis (Marlatt et al., 2013; Ornostay et al., 2013; Feswick et al., 2014; Fort et al., 2015; Garcia-Garcia et al., 2017). No change in transcript levels of *star*, *11 $\beta$ hsd*, *17 $\beta$ hsd* or *cyp19a1* were found in DHT exposed fathead minnow, despite an increase in gonadal E<sub>2</sub> production (Ornostay et al., 2016). The authors attributed this to pre-existing gene or protein complements within the gonad playing a larger role in E<sub>2</sub> synthesis than *de novo* expression of transcripts. Similarly, mummichog exposed to 50  $\mu$ g/L DHT had depressed gonadal 11KT production, but no change in transcript levels of *star*, *cyp11a1* or *17 $\beta$ hsd* (Feswick et al., 2014).

An excellent example of the variable responses of steroidogenic genes during exogenous androgen exposure can be seen in crucian carp (*Carassius carassius*) exposed to 50 µg/L methyltestosterone for 6 weeks (Zheng et al., 2016). Gonadal levels of 11KT and E<sub>2</sub> were depressed at both 2 and 4 weeks of exposure, but expression of *11βhsd*, *star* and *cyp17a1* were increased at 2 weeks, then decreased at 4 weeks (Zheng et al., 2016). Over the duration of the experiment, both *cyp11a1* and *3βhsd* levels were increased (Zheng et al., 2016).

Differences in experimental approaches or gonadal tissue sampling procedures may also be a source of variation in gene transcript responses. Gene expression responses of tissue frozen immediately during sampling or tissue incubated *in vitro* and frozen following incubation can be vastly different, in both magnitude of response and direction of change (increase or decrease compared to control; Scholz et al., 2012). For example, an increase in transcript abundance for *3βhsd* following 24 h *in vitro* gonadal tissue incubation after sampling was observed in mummichog injected with 1 µg/g body weight 5α-dihydrotestosterone (Rutherford et al., 2019b). Testis sections from the same experiment that were frozen immediately upon excision had decreased transcript abundance for *3βhsd* (Rutherford et al., 2019b), indicating a disparity in gene expression responses from *in vitro* tissue compared to *in vivo* results in androgen exposed fish. This may be due to removal of upper levels of the HPG axis and elimination of feedback responses from *in vitro* incubations (Johnston et al., 2016). Studies investigating temporal gene viability and use of co-culture of tissues for *in vitro* gene expression must be conducted before incorporation of *in vitro* gene expression into predictive risk assessment (Johnston et al., 2016).

Variability in gene expression impacts may indicate that there is a disconnect between gene expression and enzymatic function. There are many steps between gene activation and enzymatic expression, such as ribosomal protein translation (Cao et al., 2019), which may buffer

changes in gene expression from altering enzymatic activity. Currently, the changes in enzymatic activity of various steroidogenic genes during androgenic exposure are understudied. The best researched steroidogenic enzyme is aromatase or CYP19A1, due to its critical role in estrogen synthesis (Yue et al., 2018) and gonad differentiation (Haugen et al., 2012). Other steroidogenic enzymes have few studies investigating their activity levels when exposed to androgens; instead, most studies rely on gene expression analysis as a substitute for enzymatic activity (Ma et al., 2011; Cabas et al., 2013; Di Rosa et al., 2016; Manna et al., 2016). Indeed, there is currently a lack of proof of enzymatic function for the steroidogenic pathway in teleosts, even in the absence of perturbing factors (Tokarz et al., 2015).

It is possible that other factors such as downstream regulation of protein translation (Doyle et al., 2013) or alteration of enzymatic efficiency (Lv et al., 2017) buffer changes in gene expression from impacting higher level endpoints. While a correlation between *cyp19a1* and gonadal E<sub>2</sub> level was found in fathead minnow, there was no correlation between E<sub>2</sub> levels and any other steroidogenic gene (Cowie et al., 2015). In the same study, higher level endpoints such as condition factor, gonadosomatic index or body length had no correlation with gene transcript levels, indicating that gene transcripts may be useful for predicting changes to some, but not all, higher level endpoints.

#### 4.4 Male secondary sexual characteristics and testis differentiation

Exogenous androgens induce development of male secondary sex characteristics in female fish. The masculinizing effects from anthropogenic effluents were first investigated in the Fenholloway River, Florida, USA (Howell et al., 1980). Female mosquitofish (*Gambusia affinis holbrooki*) living downstream of a kraft mill effluent discharge developed elongated anal fins,

typically a male structure used for fertilization (Davis and Bortone, 1992; Cody and Bortone, 1997). Development of male secondary sexual characteristics have been found in female fish living downstream of pulp mill effluent discharge in New Zealand (Ellis et al., 2003), Chile (Orrego et al., 2019) and Sweden (Larsson and Forlin, 2002).

The presence (or absence) and degree of expression of nuptial tubercles in female fish is one of the best quantifiable examples of masculinization from exogenous chemicals (US EPA, 2009). Development of these tubercles in females has been linked to AR agonism (Ankley et al., 2003; LaLone et al., 2013) and has been used as evidence of the masculinizing capability of certain effluents (Ankley and Jensen, 2013).

During gonadal differentiation, exposure to exogenous androgens suppress ovarian development and promote testis development (Pawlowski et al., 2004). Regardless of genetic sex, fish may develop the alternative phenotypic gonad if endogenous hormones are not in the proper balance (Paul-Prasanth et al., 2011). Exogenous androgens have the ability to skew the androgen:estrogen hormone ratio and alter normal gonad development in fish (as reviewed in Shen and Wang, 2018). This can generate monosex cultures of fish (Yamamoto, 1962) and potentially impact future population recruitment (Seki et al., 2004).

## **5. Current knowledge of mammalian vs teleost steroidogenesis**

Steroid hormones, including androgens, have remarkably conserved roles in teleosts and mammals, with most genes and enzymes serving the same function in mammals as they do in fish (as reviewed in Tokarz et al., 2015). Investigation into mammalian reproductive biology is more advanced than that of teleosts. Indeed, proof of function for most steroidogenic enzymes is based on investigations in mammals only, with extrapolation that these enzymes behave in a similar manner in fish based on their homology (Tokarz et al., 2015; Rajakumar and

Senthilkumaran, 2020). The following section outlines some current areas in which teleost reproductive biology is lagging behind the mammalian model.

Various isoforms of steroidogenic genes, including *cyp17* (Zhou et al., 2007), *cyp11a1* (Parajes et al., 2013) and *cyp11b1* (Zhang et al., 2010) have been identified in mammals and teleosts. Comparisons of tissue distribution patterns and substrate specificity between conserved enzymes in mammals and fish show that there are different roles between the species. For example, 17 $\beta$ -hydroxysteroid dehydrogenase 3 is predominantly expressed in mammalian testis tissue (Zhou et al., 2005), but expressed in zebrafish liver, gonad and brain (Mindnich et al. 2005). This enzyme, which converts androstenedione to testosterone in both mammals and fish, also converts 11-ketoandrosterone to 11KT in fish but not in mammals, despite mammals synthesizing 11-ketoandrosterone (Moeller and Adamski, 2009). Similarly, two isoforms of CYP11 (CYP11A1 and CYP11A2) have been identified in Nile tilapia (Kazeto et al., 2000), Japanese medaka (Zhou et al., 2007) and humans (Miller and Auchus, 2010). CYP11A1 functions as both a hydroxylase and lyase in humans, but only possesses lyase activity in medaka and tilapia (Jin et al., 2012). Another isoform, CYP11A2, performs a hydroxylase function in tilapia and medaka (Jin et al., 2012). To further complicate the matter, only one isoform of CYP11A has been identified in Japanese eel (Kazeto et al., 2000), and the role of the various isoforms has not been investigated outside of these three species. Overall, identifying the different roles that isoforms have in both mammals and fish is a current trend in reproductive biology; as noted, research of this type in fish generally lags behind that in mammals.

Transcriptional regulation of steroidogenic genes is better understood in mammals. Regulatory pathways such as the steroidogenic factor-1/nuclear receptor 5A1 (Sf-1/NR5A1), androgen receptor  $\alpha$  and Janus kinase signal transducer and activator of transcription proteins

(JAK-STAT) are well categorized in mammals, but only starting to be investigated in fish (Rasmussen et al., 2013). To date, *cyp19a1* has the best characterized transcription regulation pathways in teleosts (Govoroun et al., 2004), with Forkhead box 12 (Fox12) acting as a co-regulator of gene activation (Wang et al., 2007). There is currently only preliminary work investigating the regulation of other steroidogenic genes such as *cyp11a1* or *17 $\beta$ hsd* in teleosts (as reviewed by Rajakumar and Senthilkumaran, 2020).

Androgen response elements located in promoter regions of steroidogenic genes may play large roles in transcriptional regulation for both mammals and fish (Yazawa et al., 2019). Promoter regions for most steroidogenic genes have been sequenced in mammals (Carelli et al., 2018; Meinsohn et al., 2019), but the same characterization is lacking for fish. With these promoter regions currently unidentified, isolating specific methods of gene regulation or enzyme translation in fish is lagging behind the mammalian counterparts.

Overall understanding how gene regulation is impacted by factors such as environmental conditions, neuroendocrine signals and cross-talk between the HPG and other systems is hampered by a lack of data on isoform behaviour, promoter sequencing, and transcriptional regulation in teleosts. This is complicated by the teleost-specific genome duplication event of approximately 350 million years ago, which increases the probabilities of diverging functions, regulations and expression within steroidogenic genes and enzymes (Goldstone et al., 2010; Glasauer and Neuhauss, 2014). Mammalian studies have progressed further in identifying specific pathways, inhibitors and transcription factors that play roles in normal steroidogenesis and are now focused on better characterizing response elements in promoter regions of genes to further elucidate mechanisms of regulation.



## 6. Summary

There is currently much evidence for, but low technical understanding of, numerous changes in the gonadal steroidogenic pathway during exogenous androgen exposure (Martyniuk and Denslow, 2012; Ankley et al., 2020). Due to the integral role sex steroids play in normal teleost development, growth and reproduction, disruption of normal synthetic ability for these sex steroids can result in negative impacts to individuals or populations of fish. Androgen receptor agonism by exogenous androgens leads to negative impacts on hormone synthesis, gamete production and fecundity, but the causal linkages between molecular changes and apical endpoints are lacking.

Despite experimental design differences, and variable responses between teleost species, certain steroidogenic genes such as *star*, *3 $\beta$ hsd* and *cyp17a1* have been identified as androgen sensitive (Kortner et al., 2009; Lee et al., 2017; Rutherford et al., 2019a; b). Across numerous studies *3 $\beta$ hsd* is the gene with the fastest response rate and largest fold-change in expression after exposure to exogenous androgens (Bluthgen et al., 2013; Liu et al., 2014; Rutherford et al., 2019a). This gene has been suggested as a potential biomarker for androgen exposure (Kim et al., 2007) and may be the main target of androgenic disruption within the steroidogenic pathway (Ankley et al., 2011).

Current research shows that numerous steroidogenic genes are perturbed by exogenous androgens; however, a better linkage between gene transcript changes and fluctuations of enzymatic efficiencies are required to fully understand changes to gonadal steroidogenesis which may lead to reduced plasma sex hormone concentrations. While the steroidogenic pathway in teleosts is generally well understood, better understanding of the relationship between changes in

gene expression and alteration of steroid hormones would reinforce the applicability of using gene expression as a predictive tool in toxicology.

## **7. Thesis structure**

This thesis will use male mummichog to test whether changes in steroidogenic gene transcripts are predictive of changes in steroid production and circulation after exposure to exogenous androgens. Mummichog are a good candidate species to study exposure to exogenous androgens as their small-bodied size and ability to manipulate their reproductive endocrine cycles make them easy to keep in the lab for such studies (MacLatchy et al., 2005); their life stages, reproductive cycles and physiology are well studied (Hsia et al., 1994; Burnett et al., 2007; Bosker et al., 2009); and they have been shown to be responsive to androgenic endocrine disrupting compounds (Sharpe et al., 2004; Feswick et al., 2014; Rutherford et al., 2015). Mummichog are commonly used as a model estuarine teleost and can survive a broad range of environmental salinities (0.0 ppt – 120 ppt) and temperatures (-1.5°C to 36°C; Scott and Scott, 1988) making them an excellent species for studying toxicants in fresh- and saltwater exposures.

The relationship between changes in steroidogenic gene transcripts and alterations of steroid hormone production is not fully characterized, limiting the power of utilizing gene expression as a tool for predictive toxicology. The general null hypothesis for the thesis is that there is no relationship between changes in gene expression and steroidogenesis in mummichog following exogenous androgen exposure. The depression of steroidogenic genes during times of plasma hormone depression is often correlated but not proven to be causal. Due to the variable nature of gene expression responses to exogenous androgens, and the uncertainty that these androgens alter steroidogenic gene expression in mummichog, the following approach was taken:

Chapter 2: Determination of the responses of steroidogenic genes to androgen (DHT and 17 $\alpha$ -methyltestosterone) exposure *in vitro*. This objective was completed through incubation of testis tissue with these model androgens and subsequent determination of steroidogenic gene responses.

Chapter 3: Determination of the short-term responses of gonadal steroidogenic gene expression and plasma reproductive steroids from male mummichog injected with DHT. Single injections of androgens resulted in quantifiable patterns of steroid depression and recovery.

Chapter 4: Determination of the short-term responses of gonadal steroidogenic gene expression and plasma reproductive steroids from male mummichog injected with 11KT. Similar patterns of steroid depression and recovery resulted. Steroid precursors were additionally used in the incubations of gonads from exposed fish to identify points of enzymatic depression in the steroidogenic pathway.

Chapter 5: Discussion of possible future directions based on thesis work.

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Table 1.1 Concentrations of androgens in the aquatic environment downstream of anthropogenic sources.

Androgen Name	Range Detected (ng/L)	Reference	Source
Androstadienedione	0.32-1.8	Liu et al., 2017	Aquaculture
	0.9-1.8	Liu et al., 2015	Aquaculture
	0.96-606.7	Huang et al., 2019	Swine Wastewater Effluent
	2.26-548	Zhang et al., 2018	Sewage Treatment Effluent
Androstenedione	0.28-5.3	Chang et al., 2008b	Sewage Treatment Effluent
	0.6-3.2	Liu et al. 2017	Aquaculture
	0.7-1.6	Liu et al., 2015	Aquaculture
	0.7-220.0	Huang et al., 2019	Swine Wastewater Effluent
	1.1-8	Damkjaer et al., 2018	Wastewater Effluent
	1.89-197	Zhang et al., 2018	Sewage Treatment Effluent
	3.2	Liu et al., 2017	Aquaculture
	5-270	Chang et al., 2011	Sewage Treatment Effluent
	40-700	Jenkins et al. 2003	Pulp Mill Effluent
	98.3	Luo et al., 2019	Sewage Treatment Effluent
	143-155	Sellin et al., 2009	Cattle Feedlot Runoff
	175	Mansell et al., 2011	Cattle Feedlot Runoff
	226-1368	Lui et al., 2012	Sewage Treatment Effluent
	480	Chang et al., 2008	Sewage Treatment Effluent
490-37500	Zhang et al. 2019	Swine and Poultry Biosolids	
997-1260	Hernandez et al., 2013	Pulp Mill Effluent	
Androsterone	14.8-38.2	Zhang et al. 2019	Swine and Poultry Biosolids
	43-1560	Williams et al., 2019	Untreated Wastewater Effluent
	114	Hashmi et al., 2018	Untreated Wastewater Effluent
	2260	Bartelt-Hunt et al., 2012	Cattle Feedlot Runoff
Boldenone	1.2	Liu et al., 2017	Aquaculture

	1.2-1.7	Liu et al., 2015	Aquaculture
	5.53-43.8	Zhang et al., 2018	Sewage Treatment Effluent
	5.84-42.1	Zhang et al. 2019	Swine and Poultry Biosolids
	7.9	Huang et al., 2019	Swine Wastewater Effluent
	14.9-23.2	Zhang et al. 2019	Swine and Poultry Biosolids
	37	Herrara-Melian et al., 2017	Wastewater Effluent
5 $\alpha$ -Dihydrotestosterone	0.1-120	Damkjaer et al., 2018	Wastewater Effluent
	5.8	Hashmi et al., 2018	Untreated Wastewater Effluent
	23-36	Williams et al., 2019	Untreated Wastewater Effluent
Epiandrosterone	1.1-11	Damkjaer et al., 2018	Wastewater Effluent
	119	Hashmi et al., 2018	Untreated Wastewater Effluent
	209-601	Zhang et al. 2019	Swine and Poultry Biosolids
	246-680	Zhang et al., 2018	Sewage Treatment Effluent
Methyltestosterone	0.1-0.38	Zhang et al., 2018	Sewage Treatment Effluent
	3.95-4.45	Zhang et al. 2019	Swine and Poultry Biosolids
Testosterone	0.05-0.89	Liu et al., 2017	Aquaculture
	0.1-0.4	Liu et al., 2015	Aquaculture
	0.2-17.8	Huang et al., 2019	Swine Wastewater Effluent
	0.5-19	Damkjaer et al., 2018	Wastewater Effluent
	2.69	Hashmi et al., 2018	Untreated Wastewater Effluent
	3.2-10	Vymazal et al., 2015	Wastewater Effluent
	7.64-31.9	Zhang et al., 2018	Sewage Treatment Effluent
	7.9-15	Chang et al., 2008b	Sewage Treatment Effluent
	27.6-121	Zhang et al. 2019	Swine and Poultry Biosolids
	33-37	Williams et al., 2019	Untreated Wastewater Effluent
	70.4	Luo et al., 2019	Sewage Treatment Effluent
	113	Herrara-Melian et al., 2017	Wastewater Effluent
17 $\alpha$ -Trenbolone	10.1-23	Khan and Lee, 2012	Cattle Feedlot Runoff

	$\leq 5$ 0.59-0.89	Bartelt-Hunt et al., 2012 Zhang et al., 2018	Cattle Feedlot Runoff Sewage Treatment Effluent
17 $\beta$ -Trenbolone	10-162 270	Khan and Lee 2012 Bartelt-Hunt et al., 2012	Cattle Feedlot Runoff Cattle Feedlot Runoff

Table 1.2. Commonly observed effects in fish from exogenous androgen exposure. Tested concentrations for each study presented as ng/L, unless otherwise noted. Length of exposure may be days post fertilization (dpf), days post hatch (dph), hours (h) or days (d). Life stage of fish noted as juvenile (not sexually mature) or adult (sexually mature); if only one sex used in the experiment this is also noted in this column. Lowest observed effect concentration (LOEC) lists the lowest concentration of androgen at which the listed endpoint was significantly different from the control treatment. Endpoints include gonadosomatic index (GSI), plasma and gonadal levels of testosterone (T), 17 $\beta$ -estradiol (E<sub>2</sub>) and 11-ketotestosterone (11KT), and others.

Endpoint	Androgen	Tested Concentrations (ng/L)	LOEC	Species	Length	Life Stage	Exposure Route	Reference
Male-skewed sex ratio	5 $\alpha$ -Dihydrotestosterone	100, 1,000 ng/g food	1000	<i>Odontesthes bonariensis</i>	77d	Juvenile	Water	Gonzalez et al., 2015
	17 $\beta$ -Trenbolone	4	4	<i>Danio rerio</i>	35d	Juvenile	Water	Boettcher, 2011
	Trenbolone Acetate	9.2, 15.5, 26.2	9.2	<i>Danio rerio</i>	1 dpf to 230 dph	Juvenile	Water	Morthorst et al., 2010
Reduced GSI	17 $\alpha$ -Methyltestosterone	10 mg/kg body wt	10	<i>Epinephelus akaara</i>	28d	Adult, female	Implant	Li et al., 2006
		25, 50, 100	50	<i>Gobiocypris rarus</i>	7d	Adult	Water	Liu et al., 2014
Impaired Gamete Formation	5 $\alpha$ -Dihydrotestosterone	20, 200	200	<i>Pimephalus promelas</i>	45d	Juvenile	Water	Margiotta-Casaluci and Sumpter, 2011

		50, 500, 5,000	50	<i>Fundulus heteroclitus</i>	21d	Adult	Water	Glinka et al., 2015
	17 $\alpha$ -Methyltestosterone	25, 50, 100	25	<i>Gobiocypris rarus</i>	7d	Adult	Water	Liu et al., 2014
Depressed Plasma T	11-Ketotestosterone	20, 200	200	<i>Pimephalus promelas</i>	45d	Juvenile	Water	Margiotta-Casaluci and Sumpter, 2011
	5 $\alpha$ -Dihydrotestosterone	20, 200	20	<i>Pimephalus promelas</i>	45d	Juvenile	Water	Margiotta-Casaluci and Sumpter, 2011
		10,000, 100,000	10,000	<i>Fundulus heteroclitus</i>	14d	Adult, male	Water	Rutherford et al., 2015
		0.001, 1, 1000 /g body wt	1	<i>Fundulus heteroclitus</i>	36h	Adult, male	Injection	Rutherford et al., 2019b
	17 $\alpha$ -Methyltestosterone	1, 10, 100, 250, 1,000	10	<i>Fundulus heteroclitus</i>	14d	Adult	Water	Sharpe et al., 2004
		4.5, 6.6, 8.5, 19.8, 35.9, 62.3	6.6	<i>Danio rerio</i>	7d	Adult, male	Water	Andersen et al., 2006
		0.3, 3, 15, 30 mg/kg	0.3	<i>Centropomus undecimalis</i>	30d	Adult	Implant	Passini et al., 2018
	Testosterone	1000 ng/g body wt	1000	<i>Sparus auratus</i>	21d	Adult, male	Injection	Garcia-Garcia et al., 2017
Depressed Plasma E <sub>2</sub>	Androstenedione	50,000	50,000	<i>Anguilla japonica</i>	45d	Adult, female	Water	Lai et al., 2018

	5 $\alpha$ -Dihydrotestosterone	10,000, 100,000	10,000	<i>Fundulus heteroclitus</i>	14d	Adult, male	Water	Rutherford et al., 2015
	17 $\alpha$ -Methyldihydrotestosterone	100, 700, 4,900	700	<i>Danio rerio</i>	1d	Adult, female	Water	Hoffmann et al., 2008
	17 $\alpha$ -Methyltestosterone	10 mg/kg body wt	10	<i>Epinephelus akaara</i>	28d	Adult, female	Implant	Li et al., 2006
		100, 1,000	1,000	<i>Fundulus heteroclitus</i>	14d	Adult	Water	Rutherford et al., 2015
	Trenbolone	500	500	<i>Oncorhynchus mykiss</i>	7d	Adult, female	Water	Schultz et al., 2013
Depressed Plasma 11KT	5 $\alpha$ -Dihydrotestosterone	0.001, 1, 1,000 ng/g body wt	1	<i>Fundulus heteroclitus</i>	36h	Adult, male	Injected	Rutherford et al., 2019b
	17 $\alpha$ -Methyltestosterone	1, 10, 100, 250, 1,000	10	<i>Fundulus heteroclitus</i>	14d	Adult	Water	Sharpe et al., 2004
		4.5, 6.6, 8.5, 19.8, 35.9, 62.3	8.5	<i>Danio rerio</i>	7d	Adult, male	Water	Andersen et al., 2006
		0.3, 3, 15, 30 mg/kg	3	<i>Centropomus undecimalis</i>	30d	Adult	Implant	Passini et al., 2018
Depressed Gonadal T Production	5 $\alpha$ -Dihydrotestosterone	50, 500, 5000	500	<i>Fundulus heteroclitus</i>	21d	Adult	Water	Glinka et al., 2015



	17 $\alpha$ -Methyltestosterone	1, 10, 100, 250, 1,000	100	<i>Fundulus heteroclitus</i>	14d	Adult	Water	Sharpe et al., 2004
	Trenbolone Acetate	33, 472	33	<i>Pimephalus promelas</i>	8d	Adult	Water	Ekman et al., 2011
Depressed Gonadal E <sub>2</sub> Production	5 $\alpha$ -Dihydrotestosterone	10,000, 100,000	100,000 0	<i>Fundulus heteroclitus</i>	14d	Adult, male	Water	Rutherford et al., 2015
	17 $\alpha$ -Methyltestosterone	100, 1,000	100	<i>Fundulus heteroclitus</i>	14d	Adult	Water	Rutherford et al., 2015
		50,000, 100,000	50,000	<i>Carassius carassius</i>	56d	Juvenile	Water	Zheng et al., 2016
	Trenbolone Acetate	33, 472	33	<i>Pimephalus promelas</i>	8d	Adult	Water	Ekman et al., 2011
Depressed Gonadal 11KT Production	5 $\alpha$ -Dihydrotestosterone	5,000, 50,000	50,000	<i>Fundulus heteroclitus</i>	21d	Adult, male	Water	Feswick et al., 2014
		50, 500, 5000	500	<i>Fundulus heteroclitus</i>	21d	Adult	Water	Glinka et al., 2015
		10,000, 100,000	10,000	<i>Fundulus heteroclitus</i>	14d	Adult, male	Water	Rutherford et al., 2015
	17 $\alpha$ -Methyltestosterone	100, 1,000	100	<i>Fundulus heteroclitus</i>	14d	Adult	Water	Rutherford et al., 2015
		50,000, 100,000	50,000	<i>Carassius carassius</i>	56d	Juvenile	Water	Zheng et al., 2016

Table 1.3. Changes in steroidogenic gene expression in fish exposed to exogenous androgens. Tested androgen concentrations listed in ng/L unless otherwise noted. Lowest observed effect concentration (LOEC) is the lowest tested concentration of androgen at which gene expression is significantly altered compared to control. In studies with multiple sampling times, total exposure duration is noted in one column (days post fertilization (dpf), hours (h) or days (d)) and specific sampling times are noted in another. Concentrations causing changes in gene transcript levels for each specific sampling time are listed. If no observed effect (NOE) was found within a study or specific sampling time, no concentration is listed. Fold change in gene transcript levels are either above (+) or below (-) control treatment levels. Genes include steroidogenic acute regulatory protein (*star*), cytochrome P450 11a1 (*cyp11a1*), 3 $\beta$ -hydroxysteroid dehydrogenase (*3 $\beta$ hsd*), cytochrome P450 17a1 (*cyp17a1*), 17 $\beta$ -hydroxysteroid dehydrogenase (*17 $\beta$ hsd*), cytochrome P450 19a1 (*cyp19a1*) and 11 $\beta$ -hydroxysteroid dehydrogenase (*11 $\beta$ hsd*).

Gene	Androgen	Species	Tested concentrations (ng/L)	Duration of Exposure	Sampling Timepoint	LOEC	Fold Change	Reference	
<i>star</i>	Androstenedione	<i>Danio rerio</i>	4, 45, 487, 980	6 dpf	1d	4	+ 8	Hou et al., 2018	
					2d	487	+ 10		
	5 $\alpha$ -Dihydrotestosterone	<i>Fundulus heteroclitus</i>	5,000, 50,000	21d	21d		NOE	Feswick et al., 2014	
					14d	14d	NOE	Rutherford et al., 2015	
					36h	24h	1	- 5	Rutherford et al., 2019b
						36h	1	NOE	
17 $\alpha$ -Methyltestosterone	<i>Gobiocypris rarus</i>	25, 50, 100	21d	7d	50	- 8			
				14d	100	- 45			

					21d	25	- 3	Liu et al., 2014
		<i>Fundulus heteroclitus</i>	100, 1000	14d	14d		NOE	Rutherford et al., 2015
		<i>Carassius carassius</i>	50,000, 100,000	56d	14d	50,000	+ 5	
					56d	50,000	- 1.5	Zheng et al., 2016
<i>cyp11a1</i>	Androstenedione	<i>Danio rerio</i>	4, 45, 487, 980	6 dpf	1d	45	+ 10	Hou et al., 2018
	5 $\alpha$ -Dihydrotestosterone	<i>Fundulus heteroclitus</i>	5,000, 50,000	21d	21d		NOE	Feswick et al., 2014
		<i>Fundulus heteroclitus</i>	0.001, 1, 1000 ng/ g body wt	36h	24h		NOE	Rutherford et al., 2019b
					36h		NOE	
	17 $\alpha$ -Methyltestosterone	<i>Gobiocypris rarus</i>	25, 50, 100	21d	7d	100	- 57	
					14d	25	- 5	
					21d		NOE	Liu et al., 2014
		<i>Carassius carassius</i>	50,000, 100,000	56d	14d	50,000	+ 14	
					56d	50,000	+ 2.5	Zheng et al., 2016
	Testosterone	<i>Sparus auratus</i>	1000	28d	15d	1000	+ 11	
					21d	1000	- 101	Garcia-Garcia et al., 2017
	Trenbolone Acetate	<i>Pimephalus promelas</i>	33, 472	8d	2d	33	- 1.3	
					4d	33	+ 1	Ekman et al., 2011

<i>3βhsd</i>	Androstenedione	<i>Danio rerio</i>	4, 45, 487, 980	6 dpf	1d	4	+ 12	Hou et al., 2018		
					2d	487	+ 10			
	5α-Dihydrotestosterone	<i>Fundulus heteroclitus</i>	0.001, 1, 1000 ng/ g body wt	36h	24h	1	- 3	Rutherford et al., 2019b		
					36h	1	- 2.5			
	17α-Methyltestosterone	<i>Gobiocypris rarus</i>	25, 50, 100	21d	7d	100	- 8	Liu et al., 2014		
					14d	100	- 4			
					21d		NOE			
					56d	50,000	+ 17.5			
		<i>Carassius carassius</i>	50,000, 100,000	56d	14d	50,000	+ 17.5	Zheng et al., 2016		
					56d	50,000	+ 2			
<i>cyp17a1</i>	Androstenedione	<i>Danio rerio</i>	4, 45, 487, 980	6 dpf	1d	980	+ 15	Hou et al., 2018		
					5d	487	- 10			
	5α-Dihydrotestosterone	<i>Fundulus heteroclitus</i>	0.001, 1, 1000 ng/ g body wt	36h	24h	1	- 2.5	Rutherford et al., 2019b		
					36h		NOE			
	17α-Methyltestosterone	<i>Gobiocypris rarus</i>	25, 50, 100	21d	7d	50	- 6.5	Liu et al., 2014		
					14d	25	- 11.5			
					21d	25	- 4			
					56d	50,000	+ 2.5			
		<i>Carassius carassius</i>	50,000, 100,000	56d	14d	50,000	+ 2.5	Zheng et al., 2016		
					56d	50,000	- 0.5			

<i>17βhsd</i>	Androstenedione	<i>Danio rerio</i>	4, 45, 487, 980	6 dpf	1d	487	- 11	Hou et al., 2018
					5d	487	+ 15	
	5α-Dihydrotestosterone	<i>Fundulus heteroclitus</i>	0.001, 1, 1000 ng/ g body wt	36h	24h	1	- 3	Rutherford et al., 2019b
36h					1	- 3		
	17α- Methyldihydrotestosterone	<i>Danio rerio</i>	100, 700, 4900	7d	24h		NOE	Hoffmann et al., 2008
					168h	4900	+ 4	
<i>cyp19a1</i>	Androstenedione	<i>Danio rerio</i>	4, 45, 487, 980	6 dpf	1d		NOE	Hou et al., 2018
					5d	487	+13	
	5α-Dihydrotestosterone	<i>Odontesthes bonariensis</i>	100, 1000 ng/g food	77d	42d	100	+ 4	Gonzalez et al., 2015
						1000	NOE	
	17α-Methyltestosterone	<i>Fundulus heteroclitus</i>	10,000, 100,000	14d	14d		NOE	Rutherford et al., 2015
							NOE	Andersen et al., 2006
	<i>Danio rerio</i>	4.5, 6.6, 8.5, 19.8, 35.9, 62.3	7d	7d		NOE	Andersen et al., 2006	
						NOE	Li et al., 2006	
	<i>Epinephelus akaara</i>	10 mg/kg bdy wt	28d	28d	10	- 13.5	Li et al., 2006	
	<i>Carassius auratus</i>	100	42d	14d		NOE	Li et al., 2013	
28d					NOE			
42d				100	+ 1.7			

		<i>Gobiocypris rarus</i>	25, 50, 100	21d	7d 14d 21d	50 100	NOE + 6 - 2	Liu et al., 2014
		<i>Fundulus heteroclitus</i>	100, 1000	14d	14d	1000	+ 4.5	Rutherford et al., 2015
		<i>Carassius carassius</i>	50,000, 100,000	56d	14d 56d	50,000 50,000	+ 5 - 2	Zheng et al., 2016
	Testosterone	<i>Sparus auratus</i>	1000	28d	15d 21d		NOE NOE	Garcia- Garcia et al., 2017
<i>11βhsd</i>	Androstenedione	<i>Danio rerio</i>	4, 45, 487, 980	6 dpf	1d 2d	487 45	+ 9 + 10	Hou et al., 2018
	5α-Dihydrotestosterone	<i>Fundulus heteroclitus</i>	5,000, 50,000	21d	21d		NOE	Feswick et al., 2014
		<i>Odontesthes bonariensis</i>	100, 1000 ng/g food	77d	42d 42d	100 1000	- 4 NOE	Gonzalez et al., 2015
		<i>Fundulus heteroclitus</i>	0.001, 1, 1000 ng/g body wt	36h	24h 36h	1 1	- 2.5 - 3	Rutherford et al., 2019b
	17α-Methyltestosterone	<i>Danio rerio</i>	100, 700, 4900	7d	24h	100	+ 6	Hoffmann et al., 2008

Testosterone	<i>Gobiocypris rarus</i>	25, 50, 100	21d	7d		NOE	Liu et al., 2014
				14d	100	- 4	
				21d	50	+ 9	
	<i>Carassius carassius</i>	50,000, 100,000	56d	14d	50,000	+ 28	Zheng et al., 2016
				56d	50,000	+ 45	
	<i>Sparus auratus</i>	1000	28d	15d	1000	+ 70	Garcia- Garcia et al., 2017
21d				1000	- 2		

Figure 1.1. Steroidogenic pathway in teleosts. Red box indicates androgenic compounds within the pathway. 17,20 $\beta$ -P = 17, 20 $\beta$ -dihydroxy-4-pregnen-3-one, 11KT = 11-ketotestosterone, E<sub>2</sub> = 17 $\beta$ -estradiol, *cyp11a1* = cytochrome P450 11a1, *cyp17a1* = cytochrome P450 17a1, *3 $\beta$ hsd* = 3 $\beta$  hydroxysteroid dehydrogenase, *11 $\beta$ hsdb2* = 11 $\beta$  hydroxysteroid dehydrogenase b2, *11 $\beta$ hsdb3* = 11 $\beta$  hydroxysteroid dehydrogenase b3, *17 $\beta$ hsdb1* = 17 $\beta$  hydroxysteroid dehydrogenase b1, *17 $\beta$ hsdb3* = 17 $\beta$  hydroxysteroid dehydrogenase b3, *cyp21a1* = cytochrome P450 21a1, *cyp11b2* = cytochrome P450 11b2, *20 $\beta$ hsd* = 20 $\beta$  hydroxysteroid dehydrogenase.

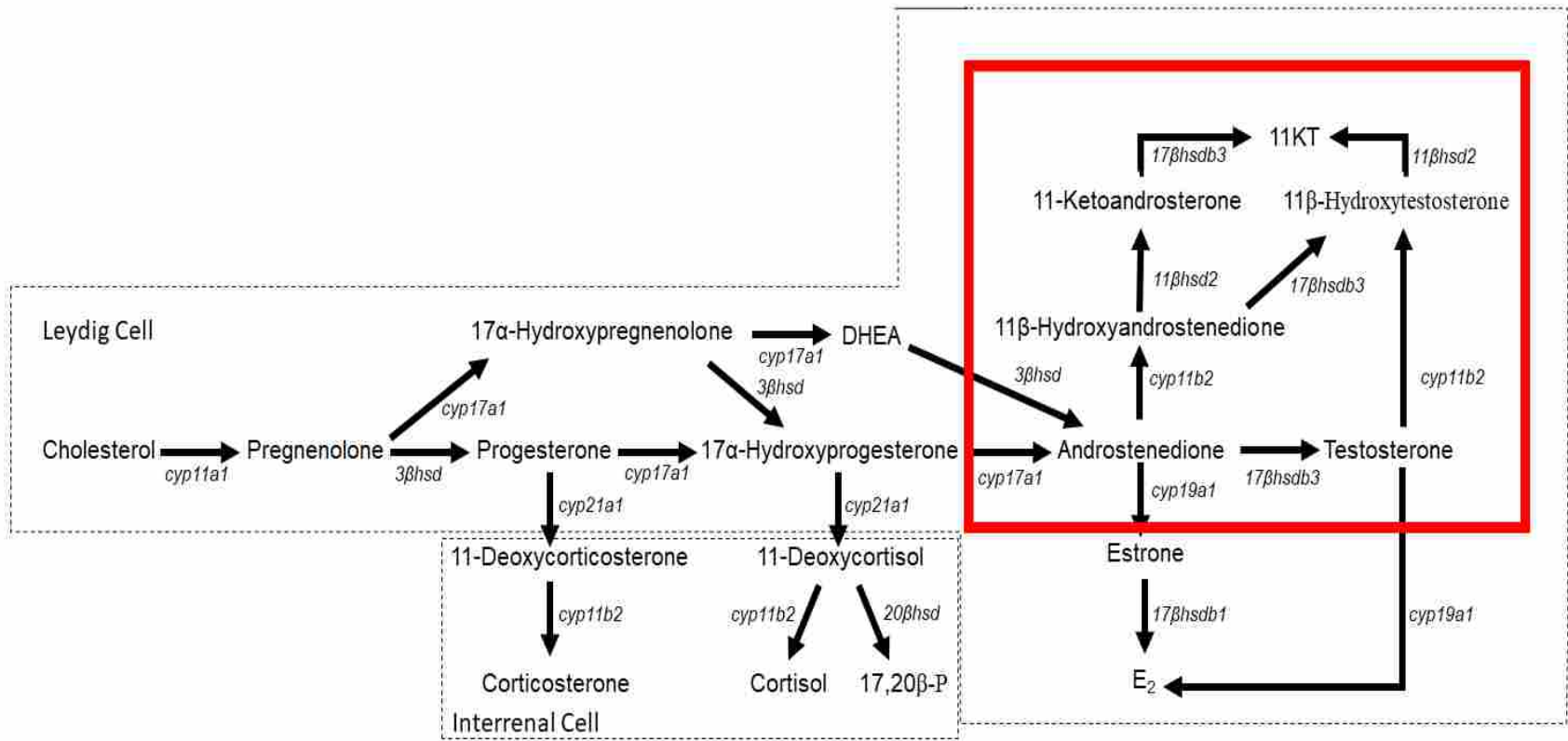
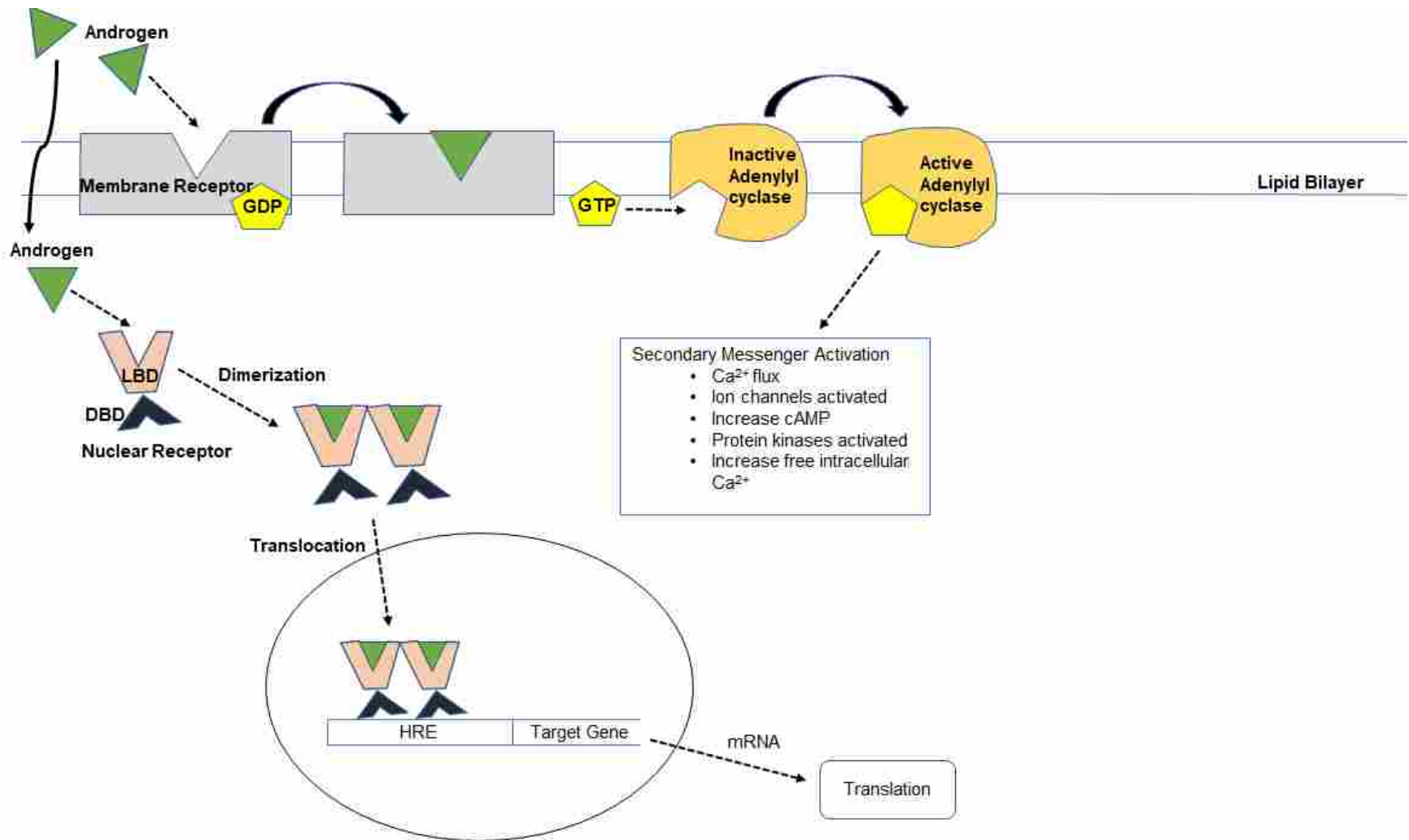




Figure 1.2. Nuclear and membrane androgen receptors and their mechanism of action. Membrane androgen receptors are G protein-coupled receptors that, once bound, convert guanosine diphosphate (GDP) to guanosine triphosphate (GTP). GTP activates adenylyl cyclase, which converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP binds to inactive protein kinase A (Pka), which phosphorylates existing proteins. This messenger system reacts within seconds of androgen binding to membrane receptors. Nuclear androgen receptors bind androgens at the ligand binding domain (LBD) site, which initiates a dimerization facilitated by the DNA binding domain (DBD) site. Once dimerized, receptors are translocated into the nucleus and DBD binds with hormone response elements (HRE) on target genes. Messenger RNA (mRNA) generated from this binding is then translated to protein. Modified from Golshan and Alavi, 2019.



**Chapter Two:**  
**Comparison of steroidogenic gene expression in mummichog (*Fundulus heteroclitus*) testis tissue following exposure to aromatizable or non-aromatizable androgens.**

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## Abstract

Androgens are a recognized class of endocrine disrupting compounds with the ability to impact reproductive status in aquatic organisms. The current study utilized *in vitro* exposure of mummichog (*Fundulus heteroclitus*) testis tissue to either the aromatizable androgen 17 $\alpha$ -methyltestosterone (MT) or the non-aromatizable androgen 5 $\alpha$ -dihydrotestosterone (DHT) over the course of 24 hours to determine if there were differential effects on steroidogenic gene expression. Testis tissue was exposed to androgen concentrations of 10<sup>-12</sup> M, 10<sup>-9</sup> M and 10<sup>-6</sup> M for 6, 12, 18 or 24 hours, after which a suite of steroidogenic genes, including steroidogenic acute regulatory protein, 3 $\beta$ -hydroxysteroid dehydrogenase (*3 $\beta$ hsd*) and cytochrome P450 17A1 (*cyp17a1*), were quantified using real-time polymerase chain reaction. Both androgens affected steroidogenic gene expression, with most alterations occurring at the 24-hour time point. The gene with the highest fold-change, and shortest interval to expression alteration, was *3 $\beta$ hsd* for both androgens. Potential differences between the two model androgens were observed in increased expression of *cyp17a1* and 11 $\beta$ -hydroxysteroid dehydrogenase (*11 $\beta$ hsd*), which were only altered after exposure to DHT and in expression levels of cytochrome P450 11A1 (*cyp11a1*), which was upregulated by MT but not altered by DHT. Results from this study show both androgens interact at the gonadal level of the hypothalamus-pituitary-gonadal axis and may possess some distinct gene expression impacts. These data strengthen the current research initiatives of establishing *in vitro* test systems that allow toxic potential of untested chemicals to be predicted from molecular perturbations.

Keywords: 17 $\alpha$ -methyltestosterone, 5 $\alpha$ -dihydrotestosterone, mummichog, steroidogenic gene expression, temporal comparison, aromatizable and non-aromatizable androgens

## 1. Introduction

Numerous anthropogenic sources such as agricultural runoff (Stoeckel *et al.*, 2012), pulp and paper mill discharge (Scott *et al.*, 2011) and wastewater effluent (Cavallin *et al.*, 2016) contribute androgens, which are a recognized class of endocrine disrupting compounds (Leusch *et al.*, 2017), to both fresh and saltwater environments (Cole *et al.*, 2016). Androgens are commonly used as growth promoters in cattle via injection of a slow release pellet (Montgomery *et al.*, 2001). These pellets may contain over 140 mg of trenbolone acetate, one of the main androgens used in concentrated animal feedlot operations (Kolok and Sellin, 2008; Bartelt-Hunt *et al.*, 2012). These androgens and their metabolites are excreted into the environment (Biswas *et al.*, 2013) and comprise a class of environmental androgens which have the ability to disrupt endocrine function in aquatic organisms (Leet *et al.*, 2015), with some androgenic metabolites detected in higher concentration (Khan and Lee, 2012) and having greater biological activity compared to their parent compounds (Jensen *et al.*, 2006). A study conducted downstream of a concentrated animal feedlot in Ohio, USA measured average concentrations of trenbolone acetate and its metabolites 17 $\alpha$ - and 17 $\beta$ -trenbolone ranged between 5-50 ng/L over a one-year period (Durhan *et al.*, 2006). Dihydrotestosterone equivalents downstream of sewage treatment effluent have been quantified as high as 26.1 ng/L in the Upper Olifants River, South Africa (Truter *et al.*, 2016). In the Nile river the highest level of androgenic activity, as determined by yeast androgen assays, was measured as  $2.2 \pm 0.02$  ng/L methyl dihydrotestosterone equivalents (Osman *et al.*, 2015). Androgenic compounds are also found in sediments downstream of anthropogenic sources (Yost *et al.*, 2014) at concentrations that may be greater than those found in the surrounding surface water (Kolpin *et al.*, 2013). Secondary or tertiary treatment of wastewater effluent can drastically decrease final effluent concentrations of androgens (Volker *et*

*al.*, 2016); however, during wastewater treatment some steroid-like compounds may become unconjugated and thus become biologically active (Gomes *et al.*, 2009). Several species of bacteria found downstream from wastewater treatment plants are capable of converting conjugated steroids to free forms (Fahrbach *et al.*, 2010), resulting in effluents with the potential to impact endocrine function in fish living in the receiving waters (Kirk *et al.*, 2002).

The gonadal steroidogenic pathway (Figure 2.1) is responsible for the synthesis of plasma sex steroids such as 11-ketotestosterone (11KT) and 17 $\beta$ -estradiol (E<sub>2</sub>). These end products are a result of a series of metabolic conversions from cholesterol, facilitated by a series of proteins and enzymes including steroidogenic acute regulatory protein (*star*), 3 $\beta$ -hydroxysteroid dehydrogenase (*3 $\beta$ hsd*) and cytochrome P450 17A1 (*cyp17a1*; Figure 2.1; Leusch and MacLatchy, 2003). Androgens are capable of altering gene expression of these specific enzymes in fish (Doyle *et al.*, 2013; Feswick *et al.*, 2014). Androgenic impacts on gene expression are variable depending on species, age at exposure, sex and exposure length (Leet *et al.*, 2011). The linkage between effects on steroidogenic genes from androgen exposure to higher levels of biological organization is lacking (Leet *et al.*, 2011) and previous work on androgen perturbation of the steroidogenic pathway has mainly focused on females.

Fish exposed to androgens have shown impacts at all levels of biological organization (Glinka *et al.*, 2015). Some of these effects include masculinization of females (Wartman *et al.*, 2009), steroid hormone depression (Sharpe *et al.*, 2004), male-skewed sex ratios (Gonzalez *et al.*, 2015) and smaller clutch sizes (Glinka *et al.*, 2015). Androgens can illicit both estrogenic and androgenic responses in fish, depending on their ability to be aromatized (Hornung *et al.*, 2004). Teleosts possess two cytochrome P450 19A1 (*cyp19a1*) genes, encoding for the ovarian (*cyp19a1a*) or brain (*cyp19a1b*) form of aromatase (Tchoudakova and Callard, 1998). These

isoforms have been shown to have their own distinct physiological relevance and regulatory mechanisms (Callard *et al.*, 2001). Non-aromatizable androgens, such as 5 $\alpha$ -dihydrotestosterone, exhibit impacts in aquatic organisms associated with traditional androgenic or masculinizing effects (Cripe *et al.*, 2010). Some androgens, such as 17 $\alpha$ -methyltestosterone, can be aromatized into estrogenic equivalents capable of estrogen receptor agonism (Pawlowski *et al.*, 2004). The estrogenic responses observed during exposure to MT can be attributed to the aromatization of MT to 17 $\alpha$ -methyleneestradiol (ME<sub>2</sub>; Simpson *et al.*, 1994), which occurs soon after initial androgen exposure (Hornung *et al.*, 2004). Estrogenic responses linked to aromatization of androgens include decreased expression of androgen receptor (*ar*; Zhang *et al.*, 2014), decreased *cyp19a1a* expression (Park *et al.*, 2009) and induction of vitellogenin in male fish (Filby *et al.*, 2007). Androgenic impact on *cyp19a1a* expression may be highly variable, as other studies have found increased *cyp19a1a* expression (Zheng *et al.*, 2016) or no effect on expression (Kortner *et al.*, 2009) during androgen exposure. Underlying mechanistic causes of these alterations in gene expression are not fully understood (Martyniuk *et al.*, 2014), but effects on gene expression may be the molecular initiating events (MIEs) that lead to the higher-level biological perturbations observed during androgen exposure (Munn *et al.*, 2015).

Reductions in plasma sex hormones have been observed in many fish species during androgen exposure (Sharpe *et al.*, 2004; Morthorst *et al.*, 2010; Rutherford *et al.*, 2015; Zheng *et al.*, 2016). In a study exposing male zebrafish (*Danio rerio*) to low levels of MT for 7 days, decreases in testosterone (T) and 11KT were observed at 6.4 ng/L and higher concentrations; however, no gene expression endpoints were analyzed (Andersen *et al.*, 2006). In a more recent study, male mummichog (*Fundulus heteroclitus*) exposed to 50  $\mu$ g/L DHT for 21 days had no impact on *star*, 11 $\beta$ -hydroxysteroid dehydrogenase (*11 $\beta$ hsd*) or cytochrome P450 11A1

(*cyp11a1*) expression, despite a reduction in plasma 11KT (Feswick *et al.*, 2014). The lack of gene expression effects in the previous study does not match with other studies analyzing gene expression (Ankley *et al.*, 2011; Martyniuk *et al.*, 2012; Cowie *et al.*, 2015) that found downregulation in many steroidogenic genes. Investigation of androgenic effects on gene expression has historically been done *in vivo* utilizing complex chemical mixtures such as wastewater effluent (Leet *et al.*, 2011) or model compounds (Doyle *et al.*, 2014; Rutherford *et al.*, 2015). Mechanistic studies utilizing only *in vitro* exposure are lacking in the literature, and where present usually focus on tissue from females (Nendza *et al.*, 2016).

To address the limited and contradictory mechanistic information related to the effects on testicular gene expression, the current study exposed mummichog testis tissue *in vitro* to DHT and MT to characterize the magnitude and temporal changes in expression of a suite of steroidogenic genes. By testing a broad range of concentrations from environmentally relevant ( $10^{-12}$  M) to pharmaceutical ( $10^{-6}$  M), the current study seeks to determine the potential for these two androgen classes (aromatizable/non-aromatizable) to act differently within the steroidogenic pathway as indicated by variations in gene expression responses. Identification of changes in gene expression and potential differences between androgens can serve as biomarkers for identifying androgenic effects from complex effluents in environmental exposures which may result in adverse endocrine-mediated outcomes at higher levels of biological organization.

## **2. Materials and Methods**

### **2.1 Fish Collection and Holding**

Wild mummichog were caught in June 2015 by seine netting (1/4" mesh size) at an estuary near Shemogue Harbour, NB, Canada (N 46°10, W 64°08). Fish were transferred to



Wilfrid Laurier University, Waterloo, ON, Canada in aerated totes and housed in 380L recirculating stock tanks until use. Stock tanks contained 18 ppt seawater consisting of dechlorinated City of Waterloo water with Crystal Sea Salts (Marine Enterprise International Inc., Baltimore, MD, USA). Fish were fed to satiation daily with commercial trout pellets (2mm pellet size, EWOS Pacific, Vancouver, BC, Canada) and maintained at a photoperiod of 16L:8D. Routine water quality testing maintained stock tanks at 0-0.25 mg/L ammonia, 1-1.25 mg/L nitrite, 0-5 mg/L nitrate,  $7.5 \pm 0.1$  pH, 20-22°C temperature, >80% dissolved oxygen (DO) and 17.5-18.5 ppt salinity. Minimal mortalities occurred in stock tanks (< 5%). Fish collection, housing and *in vitro* exposures were conducted according to Canadian Animal Care protocols as approved by the Wilfrid Laurier University Animal Care Committee.

## 2.2 Model Androgen Concentrations

17 $\alpha$ -methyltestosterone (CAS #58-18-4) and 5 $\alpha$ -dihydrotestosterone (CAS #521-18-6) were purchased from Steraloids (Newport, RI, USA). Stock concentrations of 1 mg androgen per mL ethanol were diluted with ethanol to deliver 5  $\mu$ L androgen aliquots to 995  $\mu$ L medium, that when added achieved the final *in vitro* treatment concentrations. Stock solutions were stored at -20°C and made at the beginning of *in vitro* testing for use in all experiments. Treatments for every *in vitro* consisted of control (5  $\mu$ L ethanol),  $10^{-12}$  M (290.4 pg/L DHT or 302.5 pg/L MT),  $10^{-9}$  M (290.4 ng/L DHT or 302.5 ng/L MT) and  $10^{-6}$  M (290.4  $\mu$ g/L DHT or 302.5  $\mu$ g/L MT) DHT or MT in 1 mL incubations.

## 2.3 Male Mummichog Morphometrics

Condition factor (CF) and gonadosomatic index (GSI) were calculated for each fish. CF was calculated as  $CF = (\text{total weight (g)} / \text{length (cm)}^3) * 100$ . GSI was calculated as  $GSI = (\text{tissue}$

weight (g)/[total weight (g) - tissue weight (g)]\*100. There were no differences ( $p > 0.05$ ) in selected fish for length (9.80-10.73 cm), body weight (12.31-14.17 g), GSI (1.72-1.87%) or CF (1.18-1.31%). For statistical comparison of GSI and CF, fish were grouped by incubation length. Comparison of gonad weight (as GSI) was done using analysis of covariance (ANCOVA), with total body weight as the co-variate. For condition (as CF), ANCOVA was conducted with length as the co-variate.

#### 2.4 *In Vitro* Exposures

Prior to *in vitro* treatment, Medium 199 (Sigma Aldrich, Oakville, ON) was made according to McMaster *et al.* (1995) without the addition of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, and placed on ice. Male mummichog were anaesthetized with 0.05% buffered tricaine methane sulfonate (Sigma Aldrich), weighed (g), lengthed (mm) and killed by spinal severance. The testes were excised from each fish and weighed (g) before being minced with a scalpel while submerged in cold Medium 199. Two gonad pieces (20-25 mg total) from the same fish were placed in a 5 mL borosilicate glass tube (16 X 125mm) containing 1 mL of chilled Medium 199. A total of 4 borosilicate glass tubes were created per fish, regardless of remaining tissue weight. These four tubes were separated into different treatment groups (control,  $10^{-12}$  M,  $10^{-9}$  M and  $10^{-6}$  M DHT or MT). For each timepoint, a total of 12 fish were sampled with each fish having testis tissue used in all four treatments. Immediately prior to timing commencement, media were removed and 995  $\mu$ L fresh Medium 199 added, followed by 5  $\mu$ L of treatment with mixing by pipetting. Samples were then covered and incubated at 18°C for 6, 12, 18 or 24 hours on an orbital shaker. Following incubation, tissue and medium were separated, snap frozen and stored at -80°C until gene expression analysis.

## 2.5 Gene Expression Analysis

Total RNA from testis tissue was extracted according to manufacturer's instructions with minor modifications (e.g., 25 mg tissue added to 800  $\mu$ L TRIzol) using TRIzol solution (Invitrogen, Carlsbad, CA, USA). The RNA pellet was air dried and reconstituted in 20-30  $\mu$ L molecular grade water (Invitrogen) depending on pellet size. Samples were heated for 10 min at 55°C to dissolve RNA pellet into solution. RNA was quantified on a NanoDrop-8000 spectrophotometer (Agilent Technologies, Mississauga, ON) at an absorbance of 260 nm and 280 nm. Samples were diluted to 1  $\mu$ g/ $\mu$ L in molecular grade distilled water. Random samples (~20% of all samples) were checked for RNA quality using the Experion Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA). All checked RNA was intact (RNA Quality Index  $\geq$  8.7).

Samples were treated with 1  $\mu$ L DNase enzyme (AMP-D1 kit; Sigma Aldrich) and 1  $\mu$ L 10X reaction buffer (AMP-D1 kit) according to manufacturer's instructions prior to reverse transcription. Reverse transcription of total RNA (2  $\mu$ g/2  $\mu$ L) was completed using 4  $\mu$ L iScript RT Supermix (Bio-Rad Laboratories), containing 5X RT Supermix, reverse transcriptase, RNase inhibitor, dNTPs, random primers, MgCl<sub>2</sub> and stabilizers. Manufacturer's instructions were followed and included 5 min at 25°C for priming, 30 min at 42°C for reverse transcription, 5 min at 85°C for inactivation and cooling to 4°C before storage in -80°C until quantitative PCR (qPCR) analysis. The relative standard curve/SsoFast Evagreen method was used with primer pair efficiency being determined through creation of a standard curve by serial dilution of RT product. To demonstrate primer specificity and amplification of a single product, melting curve analyses were applied to all reactions. Selected primer pairs generated a single peak during melt-curve analysis, had R<sup>2</sup>  $\geq$  0.94 and efficiencies between 90-110% (Table 2.1).

mRNA expression levels for gonadal steroidogenic acute regulatory protein, cytochrome P450 17A1, cytochrome P450 11A1, cytochrome P450 19A1, 11 $\beta$ -hydroxysteroid dehydrogenase, 17 $\beta$ -hydroxysteroid dehydrogenase (*17 $\beta$ hsd*), 3 $\beta$ -hydroxysteroid dehydrogenase, androgen receptor, beta-actin ( *$\beta$ actin*), 18s ribosomal RNA (*18s*) and elongation factor 1 $\alpha$  (*ef1*) were measured from each post-incubation sample. Primer Express software v 3.0 (Applied Biosystems, Forster City, CA) was used to design primers. Accession numbers and primer sequences are shown in Table 2.1.

Potential reference genes analyzed were *ef1*, *18s* and  *$\beta$ -actin*. All three genes increased transcript levels between 12 and 18 hours incubation time but were not affected by androgen treatment within any time period. Changing expression of reference genes over time has been reported in many studies, and in this study the reference gene that was most stable within each time period was chosen as the final reference gene. Genes of interest were normalized against expression levels of *ef1*, as this gene had no significant changes between treatment groups within the same time period for any of the incubation lengths tested.

Samples were run in duplicate through qPCR using the CF96X Real-time System and C1000 touch thermocycler (Bio-Rad Laboratories). Each reaction received 2.5  $\mu$ L cDNA template (diluted 200X in UltraPure distilled water), 1.25  $\mu$ L of both forward and reverse primers and 5  $\mu$ L SsoFast EvaGreen Supermix (Bio-Rad Laboratories). The qPCR cycling conditions were 3 min at 95°C, followed by 30 sec at 55°C, then 40 cycles of 10 sec at 95°C, 36 sec at 65°C, finishing with 60 cycles of 5 sec at 65°C. A standard sample (pooled mummichog testis cDNA) was run on each plate of every assay in duplicate to estimate coefficients of variation among runs. Inter-assay variability was  $\leq 9.3\%$  for every gene. During analysis, any sample with an intra-sample variation of  $\geq 0.5$  Cq between the raw data duplicates was rerun to

assess the variation. Any sample that was retested resulted in an acceptable ( $< 0.5$  Cq) level of intra-sample variation.

## 2.6 Statistical Analysis

Statistical comparison between time periods was not possible, as expression of three potential reference genes increased over the 24 hours of incubation. Statistical analyses were conducted within each time period, on genes of interest normalized to *efl $\alpha$*  expression. Sigmaplot 11.0 (Systat Software INC, Chicago, IL, USA) and IBM SPSS Statistics v 22 (IBM, Armonk, NY, USA) were used to perform statistical analyses. Assumptions of normality and homogeneity of variance were tested with Shapiro-Wilk and Levene's tests, respectively, prior to parametric analysis. All data were normally distributed. A one-way analysis of variance (ANOVA;  $p \leq 0.05$ ) was used to test for significant differences among treatments within an androgen (DHT or MT). If applicable, a Tukey's post-hoc test was conducted to determine treatment differences.

## 3. Results

### 3.1 Six-hour *In Vitros*

Methyltestosterone did not alter expression of *star* at the six-hour timepoint in any treatment ( $p= 0.554$ , Figure 2.2). Expression of *cyp17a1* in the  $10^{-9}$  M treatment was upregulated compared to the  $10^{-12}$  M treatment ( $p= 0.047$ , Figure 2.3), while  $10^{-12}$  M MT depressed *11 $\beta$ hsd* expression compared to the other two androgen treatments ( $p= 0.019$ , Figure 2.4), and  $10^{-9}$  M MT significantly increased *ar* expression compared to the other two androgen treatments ( $p= 0.010$ , Figure 2.5).  $10^{-6}$  M MT increased *3 $\beta$ hsd* expression by 1.35 times over control ( $p= 0.004$ , Figure 2.6). An increase in *17 $\beta$ hsd* transcript abundance was found in the  $10^{-9}$  M treatment ( $p=$

0.020, Figure 2.7). Both  $10^{-12}$  M and  $10^{-6}$  M MT treatments decreased expression of *cyp11a1*, while  $10^{-9}$  M had no impact on expression level ( $p= 0.033$ , Figure 2.8).

Dihydrotestosterone had no impact on *cyp11a1* ( $p= 0.335$ , Figure 2.8), *cyp17a1* ( $p= 0.561$ , Figure 2.3), or *11 $\beta$ hsd* ( $p= 0.776$ , Figure 2.4) expression at any tested concentration in the six-hour *in vitro*. *Star* expression was increased by 1.56 times over control in the  $10^{-6}$  M DHT treatment ( $p= 0.036$ , Figure 2.2). The largest induction of any gene at any timepoint was found in the  $10^{-12}$  M treatment for *3 $\beta$ hsd*, with the  $10^{-6}$  M DHT treatment also increasing expression ( $p= 0.012$ , Figure 2.6). There was depression of *17 $\beta$ hsd* in the  $10^{-12}$  M and  $10^{-9}$  M DHT treatments, but there was no alteration in expression in the  $10^{-6}$  M treatment ( $p= 0.026$ , Figure 2.7). A 1.79X increase in *ar* expression occurred in the  $10^{-9}$  M treatment and a 1.71X increase was found in the  $10^{-6}$  M treatment ( $p= 0.009$ , Figure 2.5).

### 3.2 Twelve-hour *In Vitros*

There was no change in expression for *cyp17a1* ( $p= 0.125$ , Figure 2.3) or *17 $\beta$ hsd*, ( $p= 0.114$ , Figure 2.7) in the MT exposure at the twelve-hour timepoint. While  $10^{-12}$  M MT decreased *ar* expression compared to the other two treatments, none of these were significant changes from control expression ( $p= 0.029$ , Figure 2.5). All three MT treatments depressed *star* expression at the twelve-hour timepoint ( $p = 0.028$ , Figure 2.2). An increase over control expression was observed in *cyp11a1* from the  $10^{-12}$  M treatment, but not in either of the other two androgen treatments ( $p= 0.041$ , Figure 2.8). A similar induction from the same treatment was found in *3 $\beta$ hsd* expression and did not occur in the higher MT treatments ( $p= 0.042$ , Figure 2.6). Depression of *11 $\beta$ hsd* occurred in the  $10^{-6}$  M treatment ( $p= 0.025$ , Figure 2.4). While

expression of *11βhsd* was different between  $10^{-12}$  M MT and the other two androgen treatments, this observed increase in expression was not significant compared to control.

Neither *cyp11a1* ( $p=0.846$ , Figure 2.8) nor *cyp17a1* ( $p=0.881$ , Figure 2.3) were impacted by DHT exposure at the twelve-hour timepoint. Both *star* and *ar* expression were downregulated compared to control in the  $10^{-6}$  M treatment ( $p=0.033$ , Figure 2.2;  $p=0.045$ , Figure 2.5, respectively). *11βhsd* expression was depressed by both  $10^{-12}$  M and  $10^{-9}$  M DHT, while not altered by the  $10^{-6}$  M treatment ( $p=0.015$ , Figure 2.4). Increased expression of *3βhsd* occurred in both the  $10^{-12}$  M and  $10^{-9}$  M treatments compared to  $10^{-6}$  M and control treatments ( $p=0.021$ , Figure 2.6). A 1.70-fold increase in *17βhsd* expression occurred in the  $10^{-12}$  M treatment, and a 1.93-fold increase occurred in the  $10^{-6}$  M DHT treatment. There was no observed change in expression between control and  $10^{-9}$  M treatments for the same gene ( $p=0.029$ , Figure 2.7).

### 3.3 Eighteen-hour *In Vitros*

No changes in expression were found in *cyp17a1* ( $p=0.446$ , Figure 2.3) or *17βhsd* ( $p=0.248$ , Figure 2.7) in any MT concentration at the eighteen-hour timepoint. Expression of *ar* in all three treatments were not different from control, however;  $10^{-9}$  M MT increased *ar* expression compared to  $10^{-12}$  M and  $10^{-6}$  M treatments ( $p=0.035$ , Figure 2.5).  $10^{-6}$  M MT decreased expression of *star* ( $p=0.039$ , Figure 2.2) and both  $10^{-6}$  M and  $10^{-12}$  M MT depressed *11βhsd* transcript levels ( $p=0.034$ , Figure 2.4). An increase in expression was found in *cyp11a1* from the  $10^{-9}$  M MT ( $p=0.043$ , Figure 2.8) and in *3βhsd* from the  $10^{-12}$  M MT treatment ( $p=0.034$ , Figure 2.6).

DHT did not alter expression of *cyp11a1* ( $p=0.517$ , Figure 2.8), *star* ( $p=0.718$ , Figure 2.2) or *17 $\beta$ hsd* ( $p=0.248$ , Figure 2.7) at the eighteen-hour timepoint in any treatment. While not different from control transcript levels, *cyp17a1* was increased in the  $10^{-9}$  M treatment compared to the other two MT concentrations ( $p=0.048$ , Figure 2.3). All three DHT treatments decreased *11 $\beta$ hsd* expression ( $p=0.014$ , Figure 2.4), while only  $10^{-12}$  M and  $10^{-9}$  M depressed expression of *3 $\beta$ hsd* compared to control ( $p=0.007$ , Figure 2.6). The only observed increase in transcript abundance after eighteen hours of exposure to DHT was in *ar* expression, with a 2.19-fold increase in  $10^{-12}$  M, a 2.93-fold increase in  $10^{-9}$  M and a 1.78-fold increase in  $10^{-6}$  M ( $p=0.028$ , Figure 2.5).

### 3.4 Twenty-four-hour *In Vitros*

MT did not alter *cyp17a1* expression at the 24-hour timepoint, like all other tested timepoints ( $p=0.859$ , Figure 2.3). Expression of *11 $\beta$ hsd* and *17 $\beta$ hsd* were increased by  $10^{-6}$  M MT only ( $p=0.019$ , Figure 2.4;  $p=0.020$ , Figure 2.7, respectively). While  $10^{-6}$  M MT increased *cyp11a1* expression, a larger increase was observed in the  $10^{-12}$  M treatment (1.52 times increase vs 1.94 times increase, respectfully;  $p=0.027$ , Figure 2.8). All three treatments increased expression of *star* ( $p=0.018$ , Figure 2.2). An increase in transcript abundance was found in both  $10^{-12}$  M and  $10^{-6}$  M treatments for *3 $\beta$ hsd* ( $p=0.016$ , Figure 2.6). A decrease in *ar* expression was observed for all three treatments, the only depression from MT exposure at this timepoint ( $p=0.037$ , Figure 2.5).

DHT did not impact expression of *cyp11a1* ( $p=0.337$ , Figure 2.8), *17 $\beta$ hsd* ( $p=0.221$ , Figure 2.7) or *ar* ( $p=0.339$ , Figure 2.5) at the 24-hour timepoint. All three DHT treatments increased transcript levels of both *11 $\beta$ hsd* ( $p=0.007$ , Figure 2.4) and *star* ( $p=0.027$ , Figure 2.2).



All three treatments also increased *3βhsd* expression (p= 0.018, Figure 2.6). No effect on *cyp17a1* expression occurred in the 10<sup>-6</sup> M treatment, while both 10<sup>-12</sup> M and 10<sup>-9</sup> M treatments increased transcript abundance (p= 0.019, Figure 2.7).

#### 4. Discussion

This study is the first to compare the effects of a non-aromatizable androgen, DHT, and an aromatizable androgen, MT, on steroidogenic gene expression on an hourly scale. This mechanistic study tested concentrations ranging from environmentally relevant (10<sup>-12</sup> M) to pharmaceutical (10<sup>-6</sup> M), while remaining within a concentration range where androgen receptor binding between the two androgens have similar induction factors (Bain *et al.*, 2015). While many steroidogenic genes were similarly affected by both androgens, there are some notable differences between MT and DHT. At the 24-hour timepoint MT increased expression of *cyp11a1*, there was no effect from DHT exposure (Figure 2.8). This finding agrees with previous work where mummichog exposed to 5 or 50 µg/L DHT for 21 days had no change in *cyp11a1* expression (Feswick *et al.*, 2014), whereas crucian carp (*Carassius carassius*) exposed to 50 or 100 µg/L MT for 4 weeks had increased expression of *cyp11a1* (Zheng *et al.*, 2016). Also in the 24-hour timepoint, DHT increased expression of *cyp17a1* at the two higher treatments and *11βhsd* in all treatments, whereas MT had no impact on *cyp17a1* expression and only increased *11βhsd* expression at the highest concentration in the current study. *Cyp17a1* is integral to T synthesis in teleost gonads (Kumar *et al.*, 2000) and plays a role in gonadal development of the Korean rockfish (*Sebastes schlegelii*) through formation of 11KT (Mu *et al.*, 2013). If MT is aromatized to 17α-methylestradiol (ME<sub>2</sub>; Simpson *et al.*, 1994) the ability to alter *cyp17a1* expression would be less than that of non-aromatizable DHT, resulting in this difference between the two androgens. DHT has been shown to have a similar androgenic potency as 11KT in male

juvenile fathead minnow (Margiotti-Casaluci *et al.*, 2011). This may account for the current study's finding of DHT affecting *cyp17a1* and *11 $\beta$ hsd* while MT does not.

Aromatase is a product of the *cyp19a* gene (Simpson *et al.*, 2004), which catalyzes estrogen biosynthesis from androgens (Zhang *et al.*, 2014). Aromatase gene structure is highly conserved among teleosts (Zhang *et al.*, 2014). Expression of *cyp19a1a* is down-regulated during testis differentiation in teleosts (Guiguen *et al.*, 2010), which may account for increased levels of circulating T in male fish compared to females. The current study was unable to successfully quantify *cyp19a1a*. Expression of this gene was below detection limits in the current study and steps were taken to test whether this was a biological finding or artefact of the testing regime. Various methods were employed to increase amplification of cDNA during sample preparation including different concentrations of RNA synthesized to cDNA, testing of annealing temperature gradients and using various inhibitor-tolerant supermixes during qPCR, with no improvement in product amplification. Different primer pairs, including those from other studies that successfully quantified *cyp19a1a* (Doyle *et al.*, 2013) were tested with no success on testis tissue. When the original primer pair, which yielded non-detectable amplification in testes, was tested on mummichog ovarian cDNA, successful amplification was found. Therefore, it was not possible in the current study to determine if there were effects on expression levels of *cyp19a1a* by the two model androgens.

Although *cyp19a1a* was not successfully quantified, depression of *ar* expression indicates that MT was aromatized. The decrease in *ar* expression from MT is evidence of aromatization, as this is typically an estrogenic response (Zhang *et al.*, 2014). Depression of *ar* mRNA expression has been found in human cell lines (Diel *et al.*, 2002), Italian wall lizard (*Podarcis sicula*; Cardone *et al.*, 1998) and zebrafish (*Danio rerio*; Santos *et al.*, 2014) exposed

to various estrogenic compounds. Expression of estrogen receptor genes have been shown to be autoinducted by increasing levels of plasma estrogens (Bagamasbad and Denver, 2011), which in turn exerts a negative cross-regulation effect on *ar* gene expression, causing a decrease in transcript abundance (Bagamasbad and Denver, 2011). This cross-regulation helps coordinate cell responses to fluctuating hormone concentrations (Bagamasbad and Denver, 2011). In the current study exposure to MT decreased *ar* expression at the 24-hour timepoint at all concentrations, and in the  $10^{-12}$  M treatment at 12 hours (Figure 2.5). DHT exposure in the current study caused *ar* depression in the  $10^{-6}$  M treatment at 12 hours only, with a return to control level expression at the 24-hour timepoint (Figure 2.5). MT can be aromatized ME<sub>2</sub>, which can act as an exogenous estrogen (Simpson *et al.*, 1994). This conversion may then decrease the expression of the *ar* gene. The current study attempted to quantify estradiol levels in the exposure media at each timepoint as a way of indirectly analyzing *cyp19a1* impacts. Media was incubated with only stock androgen solutions and analyzed for cross-reactivity between the androgens and the enzyme-linked immunoassay. In control samples at all time periods there was no detection of E<sub>2</sub>, however; at all timepoints in all androgen treatments the cross-reactivity was too great to successfully determine if any differences in aromatization of T to E<sub>2</sub> occurred (data not shown). In a study on fathead minnow (*Pimephales promelas*) exposed to 50 µg/L MT for 3 weeks, a significant increase in plasma ME<sub>2</sub> was found in both male and female fish (Pawlowski *et al.*, 2004). ME<sub>2</sub> has a relative binding affinity of 68.3% of E<sub>2</sub> to the estrogen receptor (Hornung *et al.*, 2004), and was as estrogenic as E<sub>2</sub> in a fathead minnow ER binding assay (Pawlowski *et al.*, 2004). Treatment of fathead minnow with both MT and fadrazole, an aromatase inhibitor, blocked the induction of vitellogenin in male fish usually accompanying MT

exposure (Zerulla *et al.*, 2002), thus providing evidence that the estrogenic responses from MT exposure are due to aromatization (Pawlowski *et al.*, 2004).

Expression of androgen receptor varied between MT and DHT exposed tissue in the current study. DHT exposed tissue had an increase in *ar* expression at 18 hours in all three treatments, followed by a decrease back to control-level expression at the 24-hour timepoint. This indicates that DHT is not aromatized to an estrogenic equivalent which alters *ar* gene expression.

Another difference between DHT and MT was the observation of a non-monotonic response in testis tissue exposed to MT. The high ( $10^{-6}$  M) and low ( $10^{-12}$  M) MT treatments induced gene expression in *3βhsd* and *cyp11a1* at 24 hours of exposure (Figures 2.6 and 2.8, respectively). The mid-range dose of MT ( $10^{-9}$  M) had no impact on expression in either of these genes. This non-monotonic response has not been reported before in MT dose-response *in vitro* exposures (Bado-Nilles *et al.*, 2014; Liu *et al.*, 2014); however, in a study conducted with pejerrey (*Odontesthes bonariensis*) exposed to DHT via supplemented food at concentrations of 0.1 µg/g and 10 µg/g, a non-monotonic response was seen where the low dose impaired *11βhsd* expression but the high dose did not (Gonzalez *et al.*, 2015). The assumption in regulatory toxicology that the dose-response curve is monotonic is challenged by non-monotonic results (Vandenburg *et al.*, 2012) including the current study, as well as many others across multiple vertebrate taxa (Cai and Arnsten, 1997; Villeneuve *et al.*, 2007a; Ribeiro *et al.*, 2009; Lemos *et al.*, 2010; Rochester *et al.*, 2010). In cases where non-monotonic responses are well represented in the literature, high-dose testing regimes cannot be used to assess the safety of low doses (Vandenburg *et al.*, 2012), which must be considered when incorporating mechanistic data into predictive models for perturbation at higher levels of biological organization. Before

assumptions of non-monotonicity can be established for MT, a limit of the current study must be addressed. Only excised gonadal tissue was analyzed in the current study, therefore, complete feedback loops incorporating the hypothalamus and pituitary were not evaluated, nor were clearance mechanisms (e.g., due to hepatic processes). A study investigating the applicability of *in vitro* extrapolation to *in vivo* effects found that co-cultured *in vitro* incubations consisting of brain, pituitary and gonad tissues from fathead minnow reacted closer to observed *in vivo* responses after exposure to trenbolone (TB; Johnston *et al.*, 2016). In the co-cultured wells, tissues had the ability to establish feedback loops normally present in whole-fish exposures, representing an important linkage between traditional *in vitro* testing and *in vivo* organismal effects (Johnston *et al.*, 2016). A challenge to this current study, and many others, may be the inclusion of only one tissue type in the *in vitro* wells, which makes extrapolation to higher levels of biological order more challenging. Only through incorporating *in vivo* responses to MT can non-monotonicity be more robustly established.

While some differences were observed between the two model androgens, there were similarities in gene expression effects observed as well. There was an upregulation of many genes, including *star*, *11 $\beta$ hsd* and *3 $\beta$ hsd* at the 24-hour timepoint for both MT and DHT treatments. This upregulation may indicate a disconnect between gene expression changes and plasma hormone impacts, as gene upregulation should hypothetically lead to an increase in gonadal sex steroid production. A limitation of the current study is the inability to quantify gonadal production of T, E<sub>2</sub>, or 11KT, due to high cross-reactivity between the androgens used for exposure and these terminal steroids in the enzyme-linked immunoassays (ELISAs). An upregulation of steroidogenic genes after exposure to model androgens has been found in previous studies (Filby *et al.*, 2007; Ekman *et al.*, 2011; Gonzalez *et al.*, 2015; Rutherford *et al.*,

2015). Linking upregulation of specific genes to higher level biological perturbation may be seen in a study examining production of E<sub>2</sub> from fathead minnow ovarian tissue exposed to DHT for 6, 9 or 12 hours (Ornostay *et al.*, 2016). Excised ovarian tissue was exposed to 10<sup>-6</sup> M, 10<sup>-7</sup> M and 10<sup>-8</sup> M DHT and E<sub>2</sub> was quantified by ELISA (Ornostay *et al.*, 2016). The resulting increase in E<sub>2</sub> production, in a dose-response manner, could not be attributed to fluctuations in *star*, *cyp19a1a*, *11βhsd* or *17βhsd*, expression and the authors concluded that the resulting E<sub>2</sub> increase was dependant on pre-existing gene complements rather than gene expression alteration (Ornostay *et al.*, 2016). The authors hypothesized that the binding of DHT to estrogen receptors at high concentrations may result in E<sub>2</sub> induction, or that a compensatory mechanism within the fathead minnow ovary may counteract high concentrations of androgen (Ornostay *et al.*, 2016). The current study did observe increases in *star*, *11βhsd* and *17βhsd*; however, these were detected at timepoints past that which Ornostay *et al.* tested. In another study, fathead minnow exposed to 300 µg/L of the *3βhsd* inhibitor trilostane for up to 8 days had increased expression of follicle stimulating hormone receptor (*fshr*) and *cyp19a1a* (Ankley *et al.*, 2011). The authors concluded that this was a compensatory mechanism by the fish to counteract the inhibition of *3βhsd*, and accounted for the quick recovery back to the control level of plasma sex steroids after cessation of trilostane exposure (Ankley *et al.*, 2011). The current results may indicate that mummichog are capable of upregulation of steroidogenic genes to counteract androgenic perturbation. Species-specific mechanisms of action may also account for the differences in observed effects on gene expression, as concentrations of DHT were similar in both studies.

*3βhsd* has been suggested as a biomarker for androgen action as it is impacted by many androgens (Kim *et al.*, 2007), with the current study showing that both MT and DHT alter expression during *in vitro* exposure (Figure 2.6). The enzyme 3βHSD is responsible for

producing  $\Delta^4$ -3-ketosteroids from  $\Delta^5$ -3 $\beta$ -hydroxysteroids (Norris, 2007). It is prevalent throughout the steroidogenic pathway and its products include progesterone, androstenedione and 17 $\alpha$ -hydroxyprogesterone (Norris, 2007). Both MT and DHT altered *3 $\beta$ hsd* expression in at least one treatment at all time points sampled. The largest fold-changes among all genes were observed in the *3 $\beta$ hsd* treatments of 10<sup>-6</sup> M MT at 24 hours (3.41-fold increase over control) and 10<sup>-12</sup> M DHT at 6 hours (3.72-fold increase over control). In a study analyzing appearance of gonadotrophs through gonadal stages, *3 $\beta$ hsd* was detected in juvenile mummichog as early as two weeks after hatch (Shimzu *et al.*, 2008). In that study, prior to *3 $\beta$ hsd* detection via immunocytochemistry, all gonads of mummichog fry were undifferentiated. As gonads developed, a differential expression pattern of *3 $\beta$ hsd* was observed between male and female gonads, with males having stronger staining for *3 $\beta$ hsd* (Shimzu *et al.*, 2008). Expression of *3 $\beta$ hsd* is detected early during ontogeny in air-breathing catfish (*Clarias gariepinus*), in both undifferentiated and differentiated stages (Raghuveer and Senthilkumaran, 2012). Stimulation of gonadal slices with human chorionic gonadotropin (hCG), an analogue for luteinizing hormone, resulted in upregulation of *3 $\beta$ hsd*, indicating it plays a role in spermatogenesis and oogenesis (Raghuveer and Senthilkumaran, 2012). Previously, *3 $\beta$ hsd* was observed to be downregulated during androgen exposure (Prisco *et al.*, 2008; Bluthgen *et al.*, 2013; Liu *et al.*, 2014); however, it is upregulated in the current study. This may be because previous studies focused on freshwater fish species, or on *in vivo* androgen exposure followed by *in vitro* analysis, with potential feedback loops not present in *in vitro* exposures. Whole fish studies completed in mummichog have not quantified *3 $\beta$ hsd* expression, instead focusing on *11 $\beta$ hsd* (Feswick *et al.*, 2014). Future whole fish exposures to model androgens should analyze *3 $\beta$ hsd* expression comparison of whole animal responses to the current *in vitro* responses.

Both MT and DHT increased expression of *star* in all treatments at 24 hours of exposure (Figure 2.2). Rather than a direct effect of DHT or MT (which cannot be ruled out), this may be a compensatory mechanism to increase the rate-limiting step of cholesterol movement across the mitochondrial membrane (Schroeder *et al.*, 2017). If gene expression reflects protein translation, the increased capacity of *star* could counteract any inhibitory effects of exogenous androgens on the steroidogenic pathway (Villeneuve *et al.*, 2007a). *Star* expression has been positively correlated to GSI in fathead minnow (Villeneuve *et al.*, 2007b) but this was not a factor in the current study, as GSI of selected fish did not differ among treatments.

Time to gene expression perturbation is variable, and gene-specific, as identified in the current study. Effects in sensitive genes, such as *3 $\beta$ hsd*, can be seen as quickly as six hours post-exposure, whereas other genes, such as *cyp17a1*, require 18 hours before altered expression is observed. In the current study, most changes in gene expression were observed at the 18- and 24-hour timepoints. Other than *ar* in the MT treatments, all significant effects on gene expression at 24 hours were upregulation. Studies of temporal effects of chemicals on the hypothalamus-pituitary-gonadal (HPG) axis are rare (Ankley *et al.*, 2011), with most temporal studies conducted on a daily, instead of an hourly, scale (Ekman *et al.*, 2011). There are three possible methods of interaction between an exogenous androgen and the HPG axis: 1) androgens may interfere with signalling within the HPG axis, 2) androgens may alter synthesis or metabolism of hormones, and 3) androgens may interact with the hormone receptors and act as an agonist/antagonist (Castaneda-Cortes *et al.*, 2014). As the current study did not incorporate the hypothalamus and pituitary, only *in vitro* gonadal exposure, the results show that androgens are capable of interacting at the level of the gonad and can alter steroidogenic gene expression. The inability to quantify plasma sex steroids from the *in vitro* medium, as addressed previously,



limits the applicability of the second method of interaction for this study. Data from the current study support the third mode of action, as a majority of impacts on gene expression were found at the 18- and 24-hour timepoints. In a previous study, Japanese medaka (*Oryzias latipes*) exposed to the *cyp19a1a* inhibitor fadrozole had rapid (8-hour) effects on plasma E<sub>2</sub> levels, whereas medaka exposed to the androgen 17β-trenbolone had no gene expression alteration until 32 hours post-exposure (Zhang *et al.*, 2008). The authors attributed this difference in response rate to the fact that fadrozole directly inhibits *cyp19a1a*, resulting in a direct effect on E<sub>2</sub> synthesis, while TB acts via effects on the luteinizing hormone receptor (*lhr*) gene and requires additional time to bind to and activate the signalling cascade which alters gonadal steroidogenic gene expression levels (Zhang *et al.*, 2008). While *lhr* and *fshr* were not quantified in the current study, the temporal findings support the interaction of both DHT and MT via receptor genes compared to direct impacts on specific genes.

Challenges to using *in vitro* exposures to identify general mechanisms of action include differences in species sensitivities that result in variations in dose-dependant inductions of molecular initiating events (Zhou, 2015). Mummichog have traditionally been established to have higher tolerances for many endocrine disruptors, including estrogenic (Doyle *et al.*, 2013; Gillio Meina *et al.*, 2013; Bosker *et al.*, 2017) and androgenic (Sharpe *et al.*, 2004; Glinka *et al.*, 2015) chemicals. Work with sheepshead minnow (*Cyprinodon variegatus*) and inland silverside (*Menidia beryllina*), both estuarine species, have suggested higher concentrations of known endocrine-active chemicals are required to initiate adverse effects compared to freshwater counterparts (Cripe *et al.*, 2010; Cole *et al.*, 2015). This may be due to species-specific mechanisms of action, such as high metabolism and elimination seen in mummichog exposed to

ethinylestradiol (Blewitt *et al.*, 2014) or the potential buffering effects of salinity in an estuarine environment (Glinka *et al.*, 2015).

The current study identifies *3 $\beta$ hsd* gene expression as the gene most affected by both aromatizable and non-aromatizable androgens. A comparison of these two androgen types shows some shared steroidogenic gene targets, such as *star*, and some distinct effects (e.g. MT increasing expression of *cyp11a1*) that may help classify androgenic responses in the future. This study shows that both MT and DHT are capable of interacting with the HPG axis at the gonadal level, and the results from the temporal comparison strengthen the hypothesis that this interaction is via receptor genes. Future studies should include whole-fish bioassays to compare responses elicited from *in vitro* exposure to those where feedback loops may differentially alter steroidogenic gene expression. *In vivo* exposures will also aid in investigating the potentially non-monotonic responses seen in MT *in vitro*. Linkage of short-term exposure to long-term effects is paramount in predictive modelling such as the adverse outcome pathway and this study serves as a basis for steroidogenic pathway effects in a model estuarine species exposed to aromatizable and nonaromatizable androgens. The data from this study can strengthen the current research initiative of establishing *in vitro* test systems, based on biological plausibility, essentiality of key events and consistency of supporting data, that allows prediction of toxic potential of untested chemicals from early key events (Munn *et al.*, 2015).

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Table 2.1. List of primer sequences used in real-time polymerase chain reaction to quantify changes in relative mRNA expression in testes of mummichog (*Fundulus heteroclitus*) exposed to 0 M (control),  $10^{-12}$  M,  $10^{-9}$  M, or  $10^{-6}$  M  $17\alpha$ -methyltestosterone or  $5\alpha$ -dihydrotestosterone *in vitro* for 6, 12, 18 or 24 hours. Standard curve efficiencies (%) and R2 values also given. Primer sequences without accession numbers derived from Doyle *et al.*, 2013.

<b>Gene</b>	<b>Accession no. or Reference</b>	<b>F1 (5' - 3')</b>	<b>R1 (5' - 3')</b>	<b>R<sup>2</sup></b>	<b>Efficiency (%)</b>
<i>star</i>	Doyle <i>et al.</i> 2013	CCT TCA AAA GAT CGG ACA GG	TTT CTG CTC AGG CAT TTG TG	0.967	101.2
<i>cyp11a1</i>	AB471800	GAG GCT TGG GAT GGG ATA TT	TCA TAC AGC GTC CAC AGG GAG	0.982	99.64
<i>cyp17a1</i>	XM01289547	CTG CTT CAA CTC CTC CTA TTC	CCT TGT GCT CGT TGT ACT T	0.991	99.13
<i>3βhsd</i>	XM01288063	CCA CTC TCA AGT TCA GCT ACA	TCT GGT AGA GAC ATC CGA AAG	0.975	91.37
<i>11βhsd</i>	Doyle <i>et al.</i> 2013	TCT GCC TTG AAC GGA TTC TT	TCG TCG CTC CTG TTA TGA TG	0.937	92.85
<i>17βhsd</i>	AB353263	GTG GTG GAG GAG ATA AG	CTA TGA ATA AGG TCC CAA TC	0.976	98.11
<i>ar</i>	XM012869900	TCC CAC TGC TGT CTC TAT CT	AGA GGT TTG TCC GCA GTA TTT	0.937	94.51
<i>ef1</i>	DN957027	TCA CCA TCA GCT TTT ATA AAG	GAC GCT CCA TTG CCT ACT GTC	0.979	102.9
<i>18s</i>	M91180	TTCAGCCACACGAGATTGAG	ATCAACGCGAGCTTATGACC	0.977	97.2
<i>βactin</i>	AF397165	CCTCCAAGACACCCAACAAC	TAACGCCTCCTTCATCGTTC	0.985	98.92

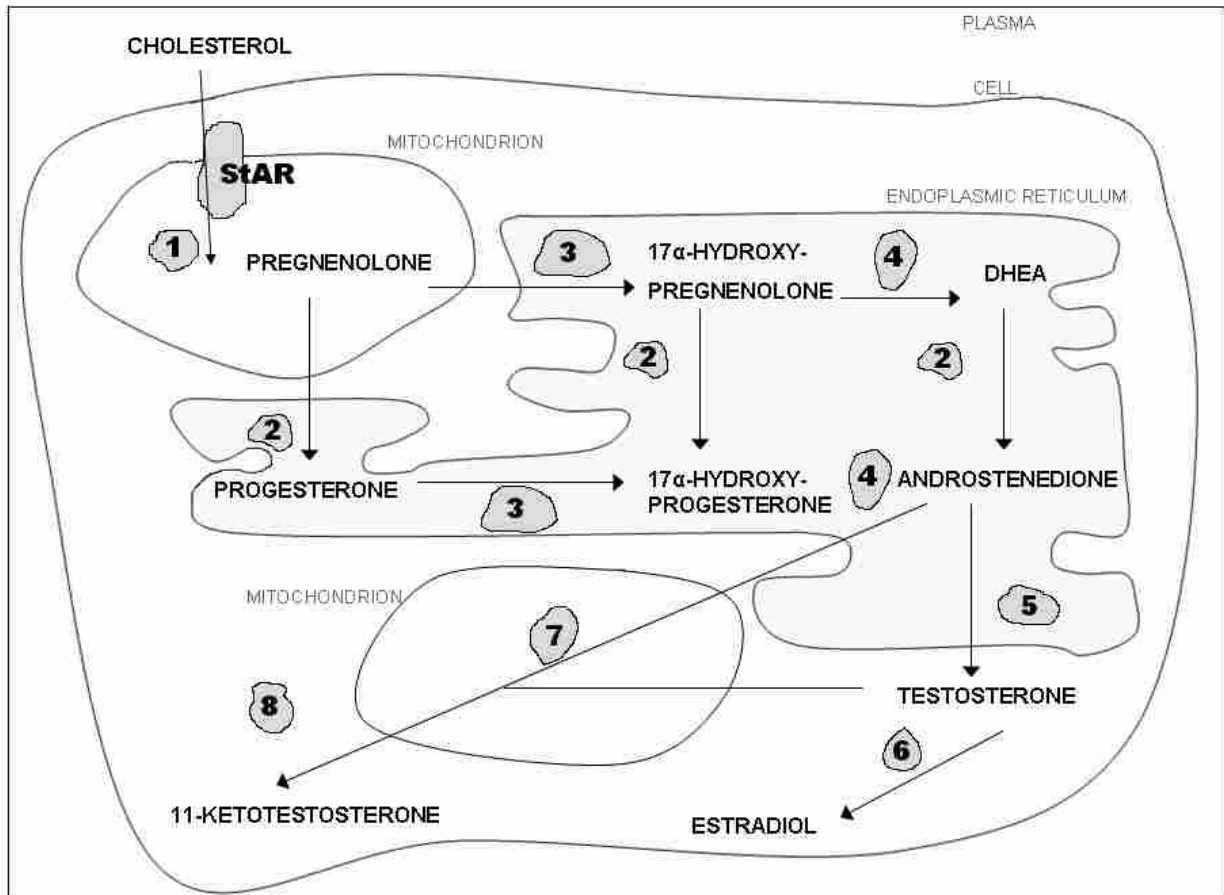


Figure 2.1. The steroidogenic pathway within gonadal cells (modified from Nagahama, 1994; Leusch and MacLatchy, 2003). *StAR* = steroidogenic acute regulatory protein; 1 = cytochrome P450 11A1; 2 = 3 $\beta$ -hydroxysteroid dehydrogenase; 3 = 17 $\alpha$ -hydroxylase; 4 = C17,20-lyase; 5 = 17 $\beta$ -hydroxysteroid dehydrogenase; 6 = cytochrome P450 19a1; 7/8 = 11 $\beta$ -hydroxysteroid dehydrogenase.

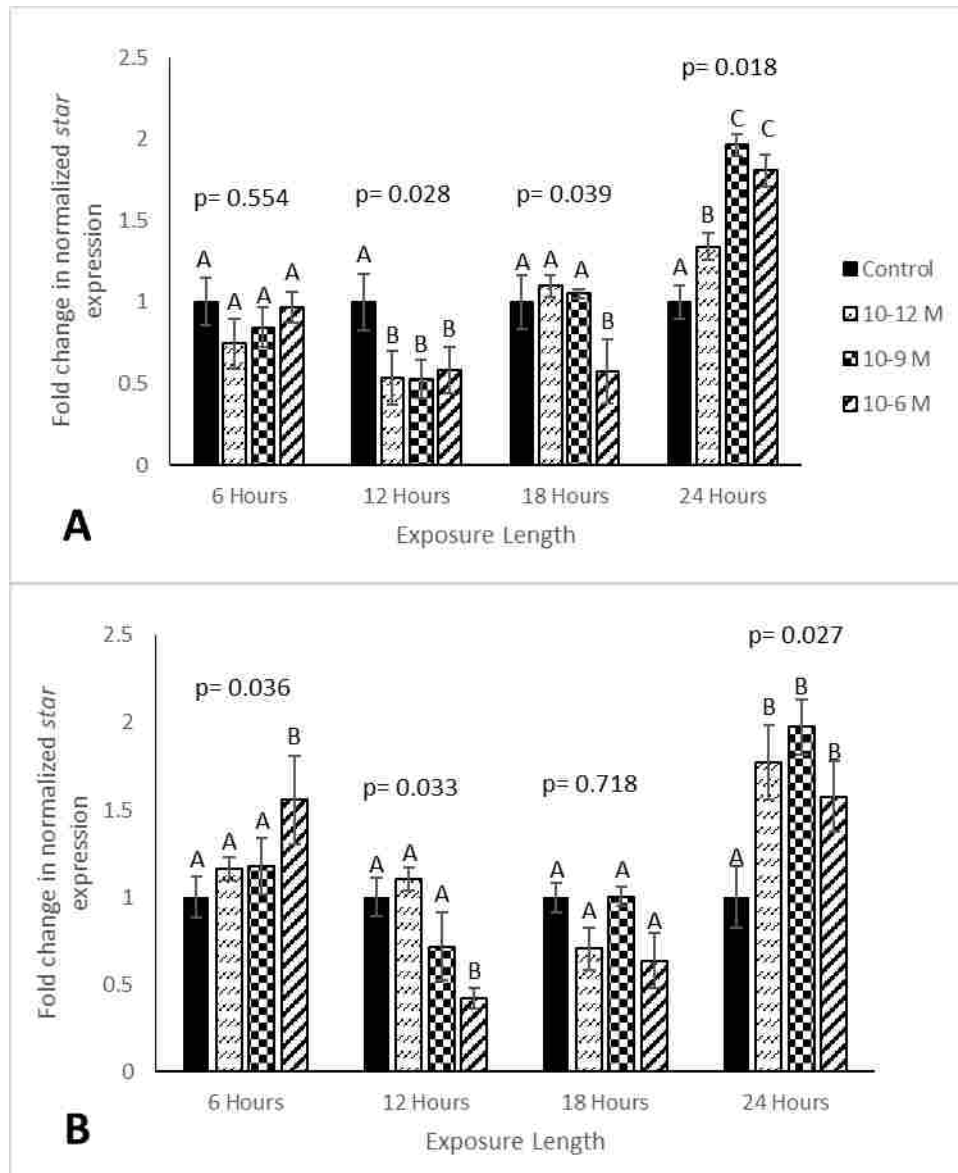


Figure 2.2. Gonadal expression ( $\pm 1$  SEM) of steroidogenic acute regulatory protein (*star*) in mummichog testis tissue exposed *in vitro* to control (ethanol),  $10^{-12}$  M,  $10^{-9}$  M or  $10^{-6}$  M  $17\alpha$ -methyltestosterone (A) or  $5\alpha$ -dihydrotestosterone (B) and incubated for various times at  $18^{\circ}\text{C}$  ( $n = 12$  per treatment). Expression levels were normalized to elongation factor *1a*. Significant ( $p < 0.05$ ) differences between treatments denoted by different capital lettering, derived from Tukey post-hoc analysis. Statistical analysis compares responses within each time period only.

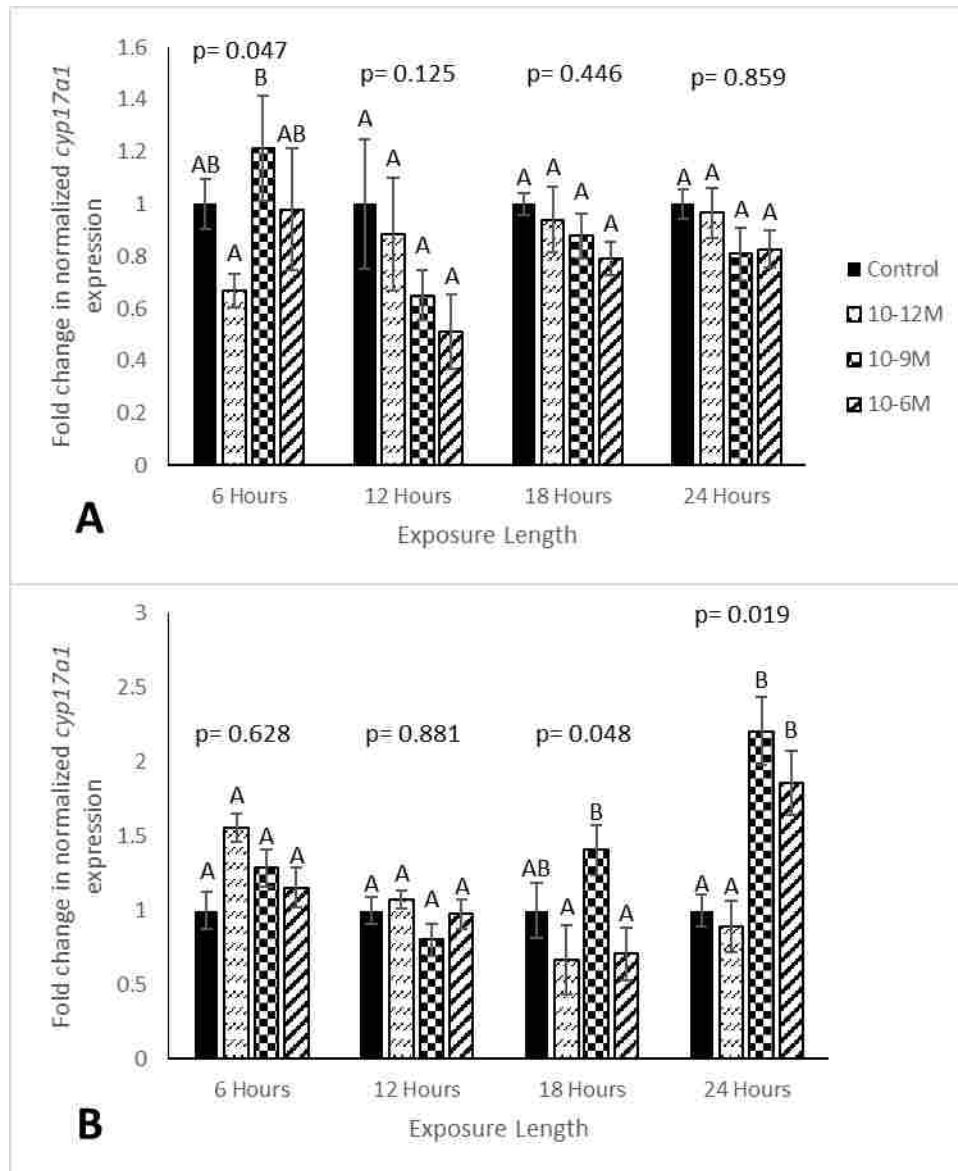


Figure 2.3. Gonadal expression ( $\pm 1$  SEM) of cytochrome P450 17A1 (*cyp17a1*) in mummichog testis tissue exposed *in vitro* to control (ethanol),  $10^{-12}$  M,  $10^{-9}$  M or  $10^{-6}$  M  $17\alpha$ -methyltestosterone (A) or  $5\alpha$ -dihydrotestosterone (B) and incubated for various times at  $18^{\circ}\text{C}$  ( $n = 12$  per treatment). Expression levels were normalized to elongation factor  $1\alpha$ . Significant ( $p < 0.05$ ) differences between treatments denoted by different capital lettering, derived from Tukey post-hoc analysis. Statistical analysis compares responses within each time period only.



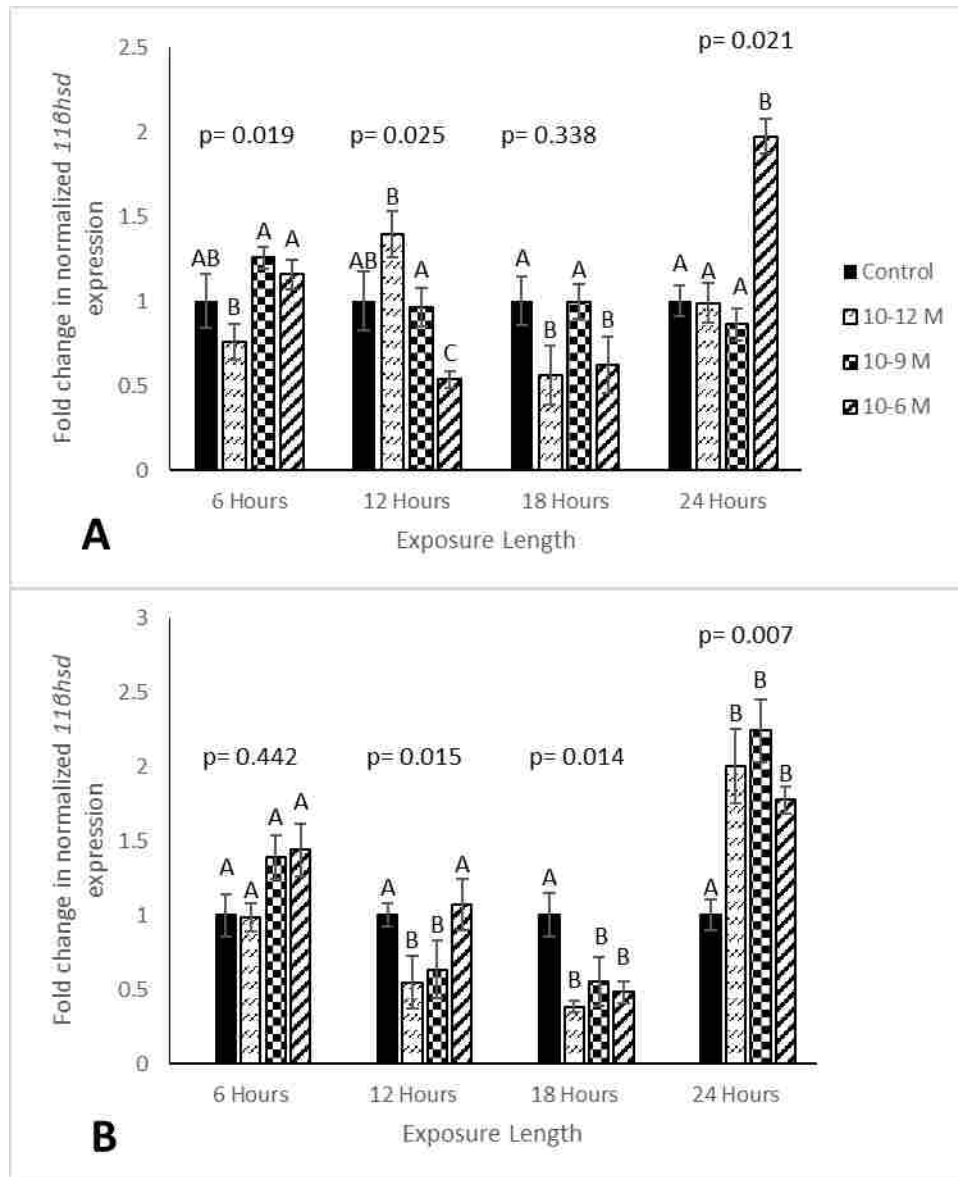


Figure 2.4. Gonadal expression ( $\pm 1$  SEM) of 11 $\beta$ -hydroxysteroid dehydrogenase (*11 $\beta$ hsd*) in mummichog testis tissue exposed *in vitro* to control (ethanol), 10<sup>-12</sup> M, 10<sup>-9</sup> M or 10<sup>-6</sup> M 17 $\alpha$ -methyltestosterone (A) or 5 $\alpha$ -dihydrotestosterone (B) and incubated for various times at 18 $^{\circ}$ C (n = 12 per treatment). Expression levels were normalized to elongation factor 1 $\alpha$ . Significant ( $p < 0.05$ ) differences between treatments denoted by different capital lettering, derived from Tukey post-hoc analysis. Statistical analysis compares responses within each time period only.

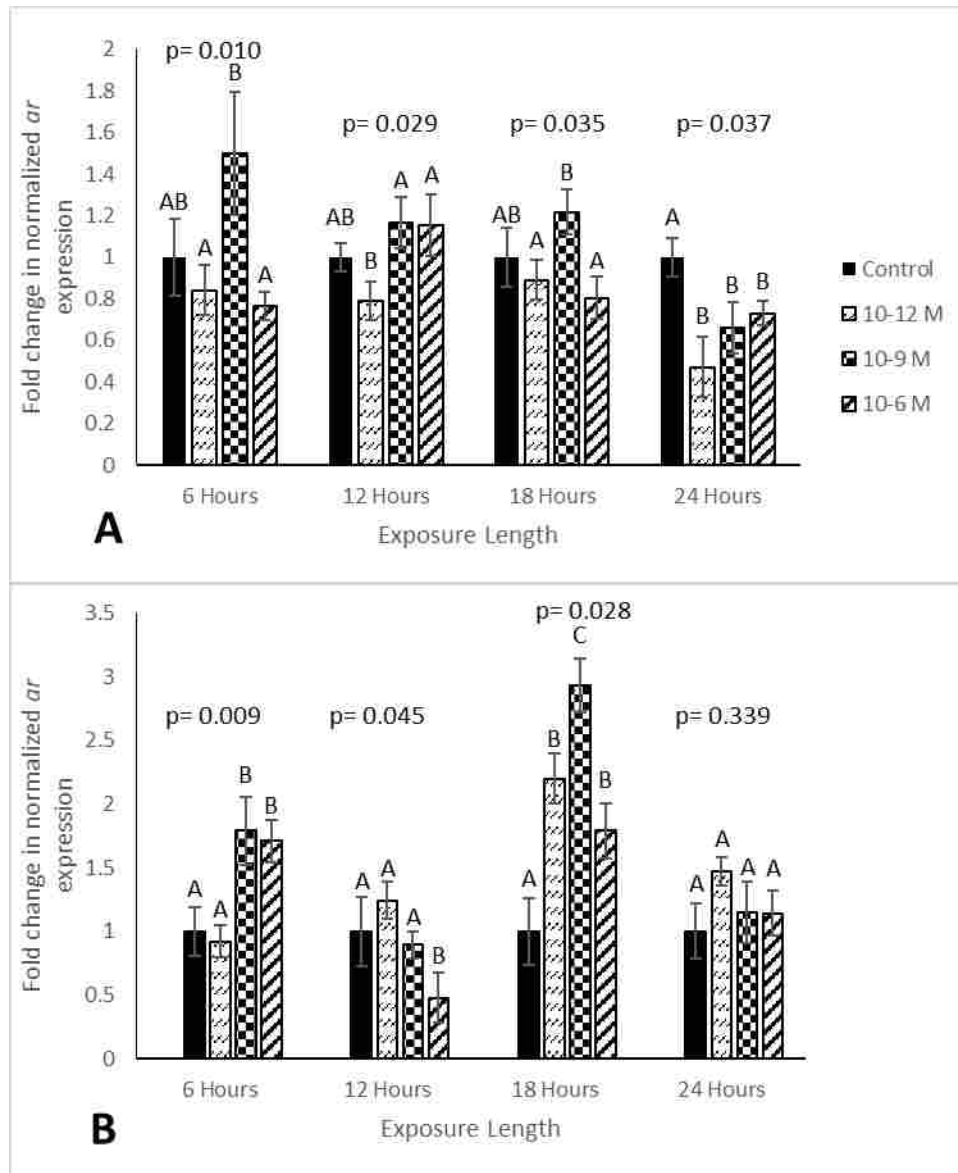


Figure 2.5. Gonadal expression ( $\pm 1$  SEM) of androgen receptor (*ar*) in mummichog testis tissue exposed *in vitro* to control (ethanol),  $10^{-12}$  M,  $10^{-9}$  M or  $10^{-6}$  M  $17\alpha$ -methyltestosterone (A) or  $5\alpha$ -dihydrotestosterone (B) and incubated for various times at  $18^{\circ}\text{C}$  ( $n = 12$  per treatment). Expression levels were normalized to elongation factor  $1\alpha$ . Significant ( $p < 0.05$ ) differences between treatments denoted by different capital lettering, derived from Tukey post-hoc analysis. Statistical analysis compares responses within each time period only.

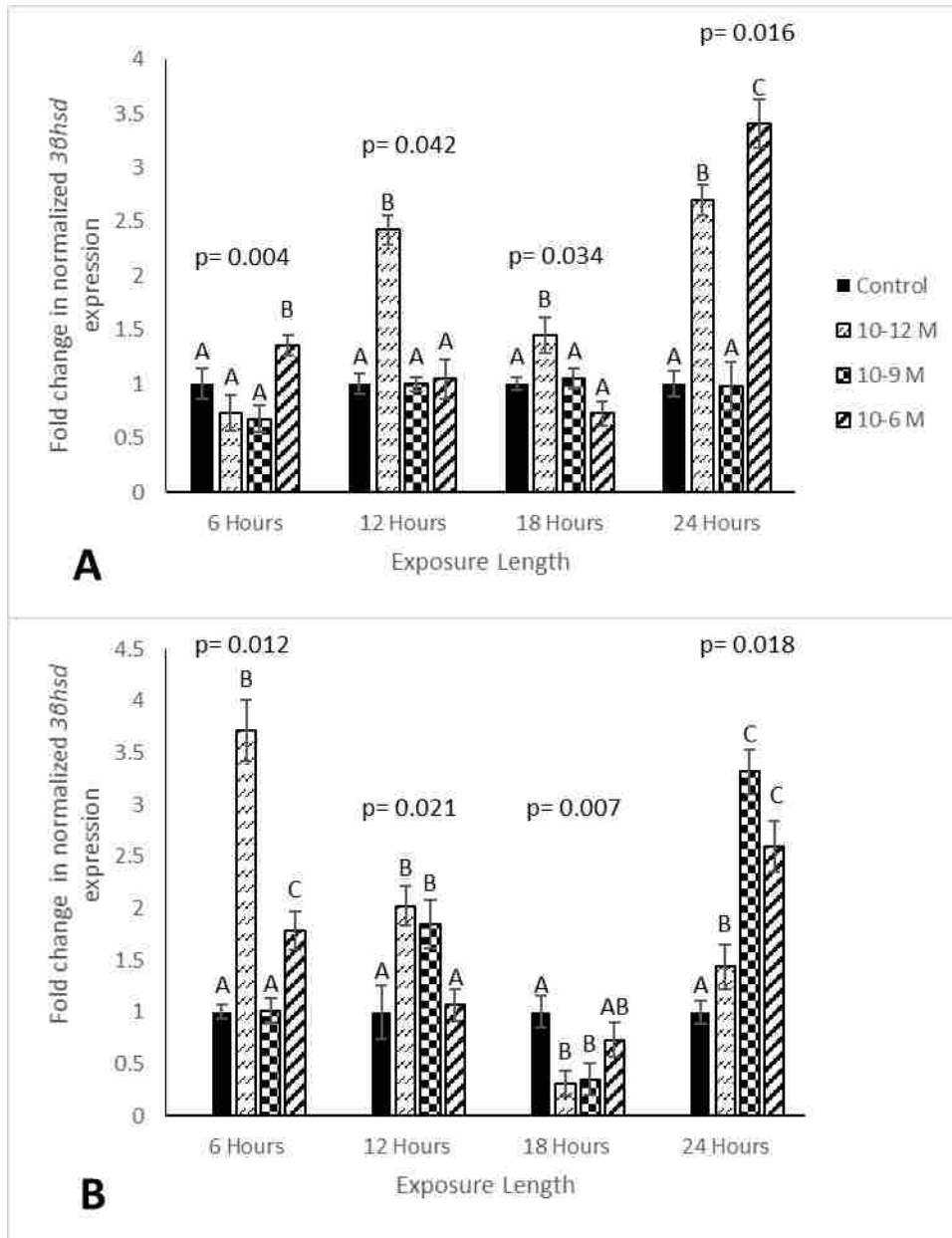


Figure 2.6. Gonadal expression ( $\pm 1$  SEM) of  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ hsd) in mummichog testis tissue exposed *in vitro* to control (ethanol),  $10^{-12}$  M,  $10^{-9}$  M or  $10^{-6}$  M  $17\alpha$ -methyltestosterone (A) or  $5\alpha$ -dihydrotestosterone (B) and incubated for various times at  $18^{\circ}\text{C}$  ( $n = 12$  per treatment). Expression levels were normalized to elongation factor  $1\alpha$ . Significant ( $p < 0.05$ ) differences between treatments denoted by different capital lettering, derived from Tukey post-hoc analysis. Statistical analysis compares responses within each time period only.

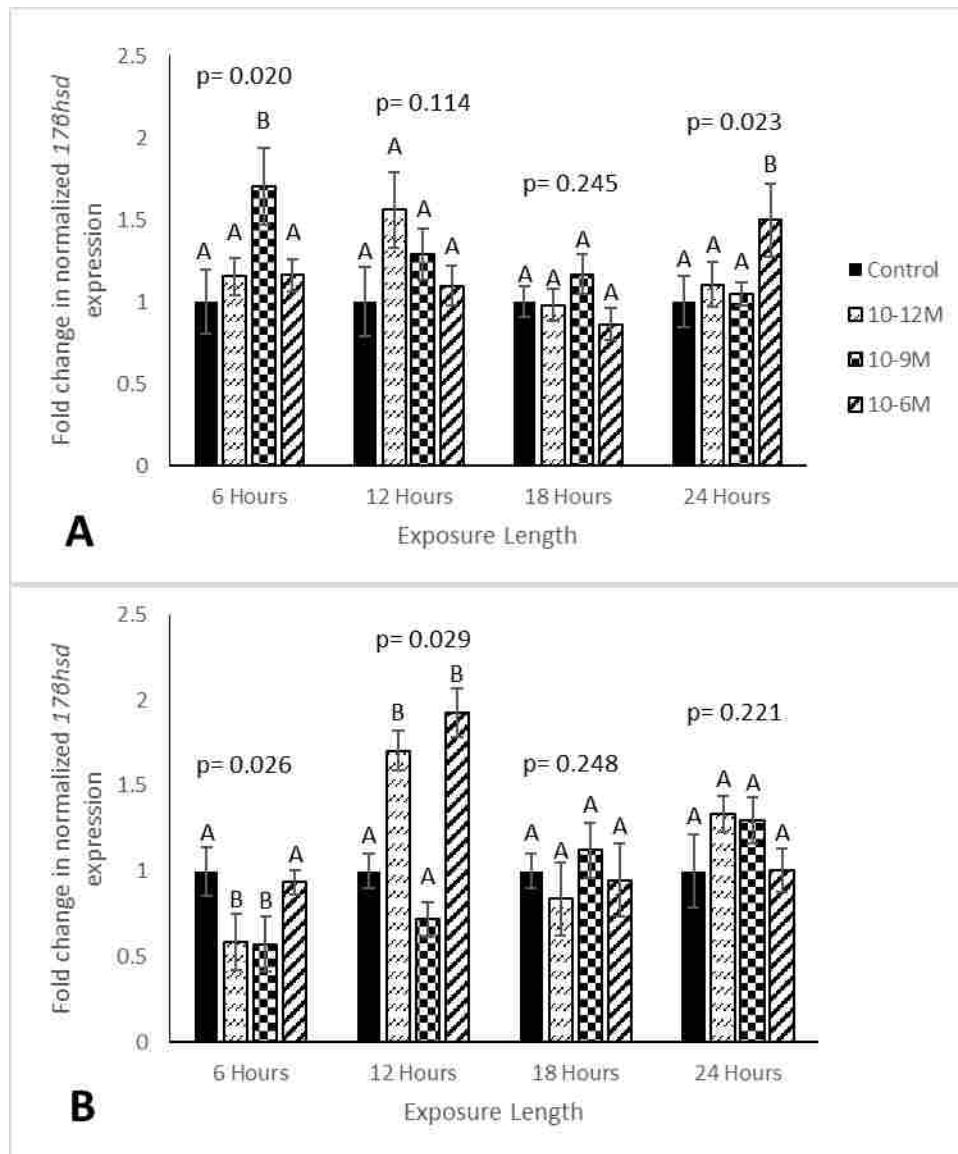


Figure 2.7. Gonadal expression ( $\pm 1$  SEM) of 17 $\beta$ -hydroxysteroid dehydrogenase (*17\beta*hsd) in mummichog testis tissue exposed *in vitro* to control (ethanol), 10<sup>-12</sup> M, 10<sup>-9</sup> M or 10<sup>-6</sup> M 17 $\alpha$ -methyltestosterone (A) or 5 $\alpha$ -dihydrotestosterone (B) and incubated for various times at 18°C (n = 12 per treatment). Expression levels were normalized to elongation factor 1 $\alpha$ . Significant ( $p < 0.05$ ) differences between treatments denoted by different capital lettering, derived from Tukey post-hoc analysis. Statistical analysis compares responses within each time period only.

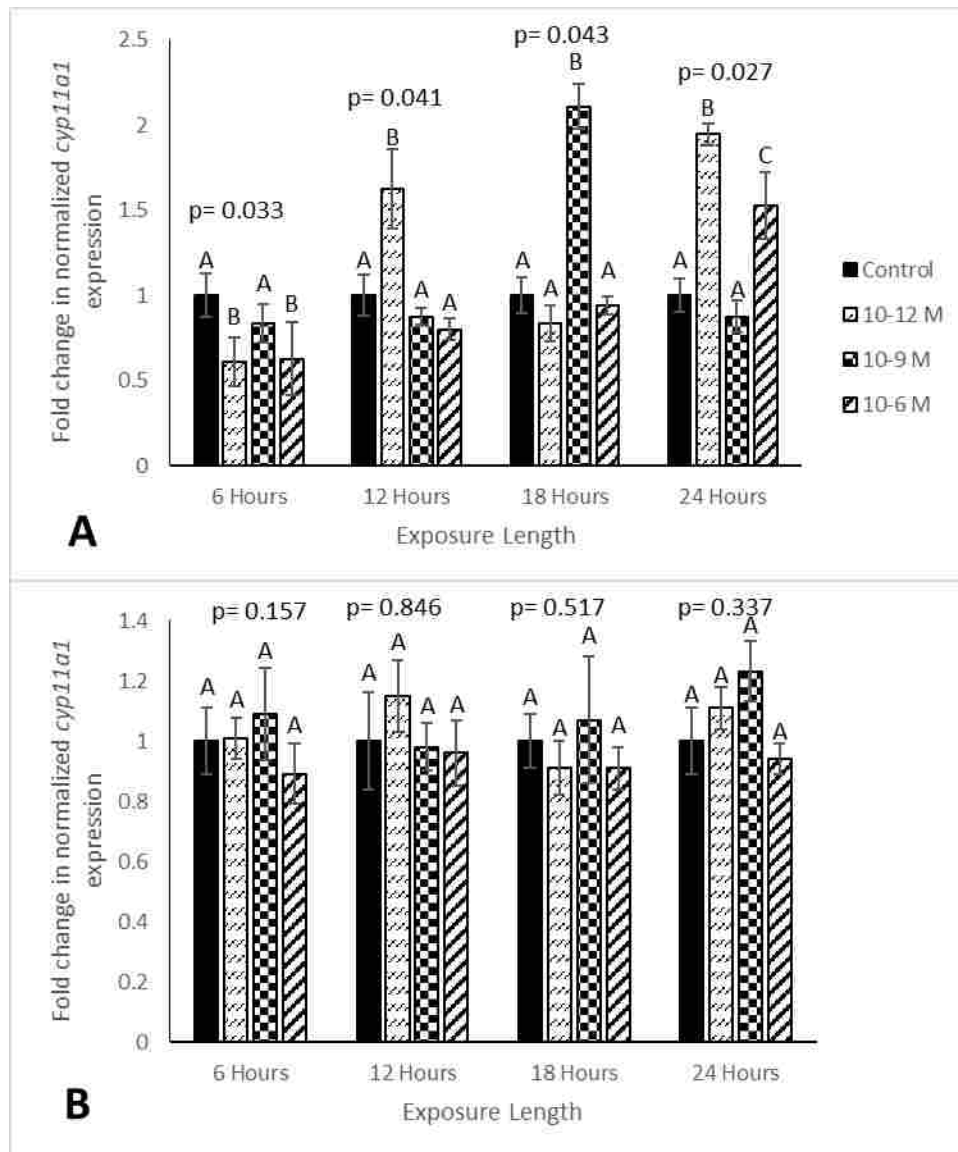


Figure 2.8. Gonadal expression ( $\pm 1$  SEM) of cytochrome P450 11A1 (*cyp11a1*) in mummichog testis tissue exposed *in vitro* to control (ethanol),  $10^{-12}$  M,  $10^{-9}$  M or  $10^{-6}$  M  $17\alpha$ -methyltestosterone (A) or  $5\alpha$ -dihydrotestosterone (B) and incubated for various times at  $18^{\circ}\text{C}$  ( $n = 12$  per treatment). Expression levels were normalized to elongation factor  $1\alpha$ . Significant ( $p < 0.05$ ) differences between treatments denoted by different capital lettering, derived from Tukey post-hoc analysis. Statistical analysis compares responses within each time period only.

**Chapter Three:**  
**Physiological effects of 5 $\alpha$ -dihydrotestosterone in male mummichog (*Fundulus heteroclitus*) are dose and time dependent**

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## Abstract

Numerous anthropogenic sources, such as pulp mill and sewage treatment effluents, contain androgenic endocrine disrupting compounds that alter the reproductive status of aquatic organisms. The current study injected adult male mummichog (*Fundulus heteroclitus*) with 0 (control), 1 pg/g, 1 ng/g or 1 µg/g body weight of the model androgen 5 $\alpha$ -dihydrotestosterone (DHT) with the intent to induce a period of plasma sex hormone depression, a previously-observed effect of DHT in fish. A suite of gonadal steroidogenic genes were assessed during sex hormone depression and recovery. Fish were sampled 6, 12, 16, 18, 24, 30 and 36h post-injection, and sections of testis tissue were either snap frozen immediately or incubated for 24h at 18°C to determine *in vitro* gonadal hormone production and then frozen. Plasma testosterone (T) and 11-ketotestosterone (11KT) were depressed beginning 24h post-injection. At 36h post-injection plasma T remained depressed while plasma 11KT had recovered. In snap frozen tissue there was a correlation between plasma sex hormone depression and downregulation of key steroidogenic genes including steroidogenic acute regulatory protein (*star*), cytochrome P450 17a1 (*cyp17a1*), 3 $\beta$ -hydroxysteroid dehydrogenase (*3 $\beta$ hsd*), 11 $\beta$ -hydroxysteroid dehydrogenase (*11 $\beta$ hsd*) and 17 $\beta$ -hydroxysteroid dehydrogenase (*17 $\beta$ hsd*). Similar to previous studies, *3 $\beta$ hsd* was the first and most responsive gene during DHT exposure. Gene responses from *in vitro* tissue were more variable and included the upregulation of *3 $\beta$ hsd*, *11 $\beta$ hsd* and *star* during the period of hormone depression. The differential expression of steroidogenic genes from the *in vitro* testes compared to the snap frozen tissues may be due to the lack of regulators from the hypothalamo-pituitary-gonadal axis present in whole-animal systems. Due to these findings it is recommended to use snap frozen tissue, not post-incubation tissue from *in vitro* analysis, for gonadal steroidogenic gene expression to more accurately reflect *in vivo* responses.

Keywords: 5 $\alpha$ -dihydrotestosterone, mummichog, steroidogenic gene expression, temporal comparison, *in vivo* vs *in vitro* responses



## 1. Introduction

Endocrine disrupting compounds (EDCs) are introduced into the environment through a variety of natural and anthropogenic sources (as reviewed in Leet *et al.*, 2011). These EDCs may disrupt sensitive hormone pathways in exposed organisms, resulting in negative effects at the individual and population levels (Mihaich *et al.*, 2018). Androgens, a recognized class of EDCs, have been found in numerous anthropogenic sources, including pulp and paper mill discharge (Milestone *et al.*, 2012), sewage treatment plant effluent (Leusch *et al.*, 2017; Park and Lee, 2017), and agricultural runoff (Bertram *et al.*, 2018). As many effluents are discharged into estuarine and saltwater environments (Mearns *et al.*, 2018), it is important to understand the impact these EDCs can have on fish living in these vital ecosystems.

Reproductive endocrine function in teleosts can be altered at very low concentrations of androgenic EDCs (Sharpe *et al.*, 2004; Bosker *et al.*, 2017). Fish exposed to androgenic EDCs will have different responses depending on life-stage (Bogers *et al.*, 2006; DeCourten *et al.*, 2019), sex (Margiotta-Casaluci and Sumpter, 2011) and exposure duration (Cripe *et al.*, 2010). Typical responses in fish exposed to androgens include male-skewed sex ratios (Ankley *et al.*, 2018), impaired immune function (Massart *et al.*, 2015), altered production of luteinizing hormone and follicle stimulating hormone (Shao *et al.*, 2013), decreased plasma testosterone (T) and 11-ketotestosterone (11KT) levels (Rutherford *et al.*, 2015), and reduced gonadal sex steroid production (Feswick *et al.*, 2014). Plasma sex hormone reductions after androgenic EDC exposure have been reported in numerous fish species (Anderson *et al.*, 2006; Schultz *et al.*, 2013; Rutherford *et al.*, 2015; Wu *et al.*, 2015; Passini *et al.*, 2018; Yue *et al.*, 2018). The quantification of plasma or gonadal sex steroids provides powerful indicators for detection of EDC perturbations across reproductive stages (McMaster *et al.*, 2001; Bosker *et al.*, 2010) and as

predictors of higher levels of biological and ecological function (Arcand-Hoy and Bensen, 1998; Scott *et al.*, 2006).

Previous work with the mummichog (*Fundulus heteroclitus*), a model estuarine species inhabiting the eastern seaboard of North America (Bigelow and Schroeder, 1953), has shown it to be responsive to androgenic endocrine disruption (Feswick *et al.*, 2014; Glinka *et al.*, 2015). Reductions in plasma sex hormones are established in mummichog during exposure to androgenic EDCs (Sharpe *et al.*, 2004; Rutherford *et al.*, 2015); however, underlying physiological changes accounting for these depressions are not known. A recent study exposed mummichog testis tissue *in vitro* to varying concentrations of the model androgen 5 $\alpha$ -dihydrotestosterone (DHT) for 6h, 12h, 18h or 24h (Rutherford *et al.*, 2019) in an attempt to determine if steroidogenic gene expression was directly impacted. Steroidogenic gene expression was analyzed as a potential link between androgenic exposure and plasma sex hormone depression; however, gene transcripts were either non-responsive or increased in abundance over the DHT exposure period (Rutherford *et al.*, 2019). This increase in transcript abundance is counterintuitive to depressions in plasma T and 11KT following DHT exposure (Margiotta-Casaluci and Sumpter, 2011; Glinka *et al.*, 2015), and may indicate that steroidogenic gene expression is not strongly correlated with plasma sex hormone depressions in male mummichog and/or that *in vitro* gonadal incubations lack required factors from the hypothalamo-pituitary-gonadal (HPG) and clearance axes to mimic whole-organism responses (Rutherford *et al.*, 2019). A drawback to the study was the inability to quantify T or 11KT production from the *in vitro* media due to cross-reactivity between DHT and T/11KT in the enzyme immunoassay (EIA), which made linkage between steroidogenic gene expression changes and sex hormone levels impossible (Rutherford *et al.*, 2019).

To build on the previous study and strengthen our understanding of steroidogenic gene expression during plasma sex hormone depression, the current study injected male mummichog with 1 pg/g, 1 ng/g or 1 µg/g DHT and sampled fish over a cycle of plasma depression (immediately prior to, during and at the beginning of recovery). A pilot study was conducted to establish the timing between DHT injection and plasma hormone depression, where plasma reductions in T and 11KT occurred approximately 18h after injection. A subsequent study sampled fish 16h, 18h, 24h, 30h and 36h post-injection to capture timepoints throughout the plasma hormone depression cycle. Through use of an *in vivo* exposure protocol, changes in gonadal steroidogenic gene expression can be measured alongside plasma hormone levels and gonadal steroid production. A comparison between responses elicited in the current study to the gene expression responses in Rutherford *et al.* (2019) confirmed whole organism responses differ from *in vitro* responses; this may provide guidance for future gene expression research linking tissue and whole-organism responses. This study provides novel information on gene expression levels during the onset of plasma steroid depression in fish, as well as the time course for plasma steroid and gene expression recovery following DHT exposure in male mummichog.

## **2. Materials and Methods**

### **2.1 Mummichog collection and housing**

In September 2017, wild mummichog were caught by seine netting (1/4" mesh size) in an uncontaminated estuary near Shemogue Harbour, NB, Canada (N 40°10, W 64°08). Fish were transported to Wilfrid Laurier University, Waterloo, ON, Canada in aerated totes and housed in 380L recirculating stock tanks. Stock tanks were maintained with  $18 \pm 0.5$  ppt seawater (dechlorinated City of Waterloo water with Crystal Sea Salts; Marine Enterprise International

Inc, Baltimore, MD, USA), and fish were fed to satiation daily with commercial trout pellets (2mm pellets; EWOS Pacific, Vancouver, BC, Canada). Photoperiod was maintained at 16 L: 8 D, dissolved oxygen (DO) at  $\geq 80\%$  and water temperature at  $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . Routine testing of water quality parameters ensured a range within stock tanks of 0-0.25 mg/L ammonia, 0-1.25 mg/L nitrite, 0-5 mg/L nitrate and  $7.5 \pm 0.1$  pH. Minimal mortalities occurred in stock tanks (<5%). Mummichog collection, transportation, housing and experimentation were conducted according to Canadian Council of Animal Care protocols as approved by the Wilfrid Laurier University Animal Care Committee.

## 2.2 DHT stock solutions

5 $\alpha$ -dihydrotestosterone (CAS # 521-18-6) was purchased from Steraloids (Newport, RI, USA). An initial stock of 1 mg/ml was created by dissolving DHT in ethanol and then diluted with sterile filtered (0.1  $\mu\text{m}$ ) phosphate buffered saline (GE Healthcare Life Sciences, UT, USA) to either 0.25  $\mu\text{g}/\mu\text{l}$ , 0.25  $\text{ng}/\mu\text{l}$  or 0.25  $\text{pg}/\mu\text{l}$ . Injection volume was calculated for each fish using:

$$\text{fish weight (g)} / 0.25 = \text{injection vol } (\mu\text{l})$$

Stock concentrations were chosen to limit volume injected to  $\leq 75 \mu\text{l}$ . Stock solutions were created immediately before use and stored on ice prior to injection. Treatments consisted of 0  $\text{pg}/\text{g}$  (control; saline solution only), 1  $\text{pg}/\text{g}$  body weight (first experiment only), 1  $\text{ng}/\text{g}$  body weight and 1  $\mu\text{g}/\text{g}$  body weight.

## 2.3 DHT injection studies

### 2.3.1 First study

Male mummichog were randomly selected from stock tanks and placed into sixteen 40 L glass aquaria (n= 6 fish per tank). Each aquarium was aerated (DO  $\geq$  80%), contained 36 L of 18 ppt water and was  $19^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . Fish were allowed to acclimate for 5d and were fed to satiation daily. Every 24 hours, 75% of aquarium water was replaced to control ammonia levels (0-0.1 mg/L). Immediately prior to injection, fish were removed individually from each aquarium by netting and placed in a glass beaker containing aerated, 18 ppt water with 1 mg/L 0.05% buffered tricaine methane sulfonate (TMS; Sigma Aldrich, Oakville, ON). Once equilibrium was lost, fish were removed from the beaker, patted dry, weighed to the nearest 0.01 g and injected intraperitoneally with appropriate volumes of saline solution (without DHT for control, containing DHT at appropriate concentrations for treatments) using a 0.5ml syringe with 27G needle (Beckton-Dickinson, Franklin Lakes, NJ, USA). Treatments for experiment one consisted of control (saline only), 1 pg/g, 1 ng/g and 1  $\mu\text{g/g}$  body weight DHT. Each fish was then placed in an aerated recovery aquarium, until regular movements returned, when it was transferred to its original glass aquarium for the duration of the exposure. There were no mortalities during the course of the exposure, and all mummichog resumed normal swimming behavior within five minutes of placement in the recovery aquarium. Fish were sampled 6h, 12h, 18h and 24h post-injection. For each treatment, an entire aquarium was sampled at each timepoint (n= 6 fish per timepoint, per treatment). At the time of sampling, mummichog were anaesthetized with TMS, lengthed (nearest 0.1 cm), weighed (nearest 0.01 g) and blood collected from caudal vasculature using heparinized 26G, 1mL tuberculin syringes (Beckton-Dickinson). Blood was centrifuged

(12 min, 4°C at 3200 g) to isolate plasma, which was stored at -20°C until ether extraction and EIA analysis for plasma steroid concentrations.

### 2.3.2 Second study

Male mummichog were randomly selected from stock tanks and placed into fifteen 40 L glass aquaria (n= 10 per tank). Acclimation period and water quality parameters were identical to the preliminary study. Based on findings from the first study, fish were injected using the same protocol with either 0 ng/g (control; saline only), 1 ng/g or 1 µg/g DHT and sampled 16h, 18h, 24h, 30h or 36h post-injection. For each treatment, an entire aquarium was sampled at each timepoint (n= 10 fish per timepoint, per treatment) and there were no mortalities prior to sampling. Prior to commencement of sampling, Medium 199 (Sigma Aldrich) was made according to McMaster *et al.* (1995) without the addition of 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor. Fish were anaesthetized and blood collected according to the protocol above, and fish were then killed by spinal severance. Liver and testes were excised and weighed (nearest 0.001 g) for determination of somatic indices. Condition factor (*CF*) was calculated as  $CF = (\text{total weight (g)} / \text{length(cm)}^3) * 100$ , gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated as  $GSI \text{ or } HSI = (\text{tissue weight (g)} / [\text{total weight (g)} - \text{tissue weight (g)}]) * 100$ . Testis tissue from each fish was sectioned, with one portion allocated for *in vitro* analysis, and the remaining tissue snap frozen and stored at -80°C until gene expression analysis.

### 2.4 *In vitro* protocol

The *in vitro* gonadal protocol optimized by MacLatchy *et al.* (2003) was followed. Briefly, once excised from the fish, testis sections allocated for *in vitro* analysis were placed in 1

ml of Medium 199, on ice. Testis tissue were then removed from Medium 199, blotted dry, and weighed (nearest 0.001 g). Testis tissue (20-30 mg) was then minced with a scalpel in a petri dish while submerged in Medium 199. Once minced, tissue was placed in a 5ml borosilicate glass tube (16 X 125 mm) already containing 1 ml of Medium 199. All steps with Medium 199 were done over ice. Immediately prior to the start of incubation, media were replaced with new Medium 199 (1 ml) and samples were covered to protect from light. Samples were incubated at 18°C for 24h on an orbital shaker with gentle mixing. After incubation, tissue was removed from media, snap frozen, and stored at -80°C until gene expression analysis. Media were also snap frozen and stored at -80°C for EIA analysis of sex hormone production.

## 2.5 Enzyme immunoassay for hormone determination

Extraction of hormones from plasma samples was done in triplicate, according to the protocol developed by McMaster *et al.* (1992). Extracted steroids were reconstituted in 1 ml EIA buffer (Cayman Chemical, Ann Arbor, MI, USA) and stored at -80°C prior to EIA analysis. Steroid levels in the *in vitro* media were measured directly from the media. EIA kits were purchased from Cayman Chemical and used to measure plasma and *in vitro* T and 11KT according to manufacturer's instructions. Standards were analyzed in triplicate and samples were analyzed in duplicate. Plates were read at 420 nm after 60, 90 and 120 min incubation for T and 90 and 105 min incubation for 11KT. All inter- and intra-assay variations were within acceptable limits for all assays (< 15% for interassay and < 10% for intra-assay).

## 2.6 Gene expression analysis

Testis tissue was processed according to Rutherford *et al.* (2019). Briefly, TRIzol solution (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from testis tissue,

according to manufacturer's instructions with minor modifications (*e.g.*, 20-30 mg tissue added to 800  $\mu$ l TRIzol). The resulting RNA pellet was air dried and reconstituted in 30-40  $\mu$ l molecular grade water (Invitrogen) depending on pellet size and quantified on a NanoDrop-8000 spectrophotometer (Agilent Technologies, Mississauga, ON) at an absorbance of 260 nm and 280 nm. Samples were diluted to 1  $\mu$ g/ $\mu$ l in molecular grade water, and all samples requantified to ensure concentration of 1  $\mu$ g/ $\mu$ l  $\pm$  0.1  $\mu$ g. RNA quality was assessed on random samples (~25% of samples) using the Experion Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, CA). All checked RNA was intact (RNA Quality Index  $\geq$ 8.9).

Samples were treated with 1  $\mu$ l DNase enzyme (AMP-D1 kit; Sigma Aldrich) and 1  $\mu$ l 10X reaction buffer (AMP-D1 kit) prior to reverse transcription. Reverse transcription steps are described in Rutherford *et al.* (2019). Samples were then stored at -80°C until quantitative polymerase chain reaction (qPCR) analysis. Primer pair efficiency was determined through the relative standard curve/SsoFast Evagreen method, with a standard curve of serially diluted RT product. Melting curve analysis was applied to all reactions to demonstrate primer specificity and amplification of a single product. Selected primer pairs generated a single peak during melt-curve analysis, had  $R^2 > 0.95$  and efficiencies between 90 and 105%.

Primer sequences for gonadal steroidogenic acute regulatory protein (*star*), cytochrome P450 17A1 (*cyp17a1*), cytochrome P450 11A1 (*cyp11a1*), cytochrome P450 19A1 (*cyp19a1*), 11 $\beta$ -hydroxysteroid dehydrogenase (*11 $\beta$ hsd*), 3 $\beta$ -hydroxysteroid dehydrogenase (*3 $\beta$ hsd*), 17  $\beta$ -hydroxysteroid dehydrogenase (*17 $\beta$ hsd*) and beta-actin ( *$\beta$ actin*) were based on Rutherford *et al.* (2019). mRNA expression levels of each gene were measured from tissue that was snap frozen directly following sampling and from each post-incubation *in vitro* sample. Accession numbers and primer sequences are reported in Rutherford *et al.* (2019). Genes of interest were normalized



to *βactin*, as transcript levels of this gene were not impacted by androgen treatment, nor by sample processing (snap frozen or *in vitro*).

Samples were run in duplicate through qPCR using the CF96X Real-time System and C1000 touch thermocycler (Bio-Rad Laboratories). Reaction volumes and qPCR cycling conditions are identical to Rutherford *et al.* (2019). Coefficients of variations among runs were assessed with a standard sample (pooled mummichog testis cDNA) run in duplicate on each plate. Interassay variability was  $\leq 9.7\%$  for each gene. During analysis, any sample with an intrasample variation  $\geq 0.5$  Cq between raw data duplicates was rerun to assess the variation. Any sample that was retested resulted in an acceptable ( $< 0.5$  Cq) level of intrasample variation.

## 2.7 Statistical analysis

Statistical analysis was completed using Sigmaplot 11.0 (Systat Software INC, Chicago, IL, USA) and IBM SPSS Statistics v 22 (IBM, Armonk, NY, USA). Assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk or Levene's test, respectively, prior to parametric analysis. Somatic measurements were normally distributed and had equal variance. An analysis of co-variance (ANCOVA) was used to analyze liver weight or gonad weight, with total fish weight as the co-variate. To evaluate condition, an ANCOVA for total body weight was conducted with standard length as the co-variate. Steroid levels were normally distributed and had equal variances. A one-way analysis of variance (ANOVA;  $\alpha = 0.05$ ) was conducted to test for differences in plasma concentrations, or *in vitro* production, of T and 11KT. Normal distribution and equal variance were also found for gene expression data. A two-way ANOVA with DHT treatment (factor 1) and sample processing (factor 2; snap frozen vs *in vitro*)

was used to measure differences in gene expression data. Where applicable, a Tukey's post-hoc test was used to determine differences among timepoints.

### **3. Results**

#### **3.1 First study**

There were no significant differences in length (97-122 mm), weight (10.19-14.63 g), GSI (1.22-1.57%), HSI (3.14-3.79%) or CF (1.26-2.02%) among treatments ( $p > 0.05$ ). Plasma T levels did not change in the 6h or 12h timepoints for any treatment, but were depressed at 18h and 24h timepoints by 1 ng/g and 1  $\mu$ g/g DHT treatments ( $p = 0.027$ , Figure 3.1A). Plasma 11KT levels did not change in the 6h or 12h timepoints for any treatment, but were depressed at the 18h and 24h timepoints ( $p = 0.033$ , Figure 3.1B), a similar response to plasma T. Injection with 1 pg/g DHT did not elicit any significant responses in plasma hormone levels at any timepoint.

#### **3.2 Second study**

##### **3.2.1 Mummichog morphometrics and plasma hormones**

There were no significant differences in length (102-131 mm), weight (9.88-14.13 g), GSI (1.38-2.11%), HSI (3.28-4.16%) or CF (1.41-2.36%) among treatments ( $p > 0.05$ ). Plasma T was depressed at 24h, 30h and 36h timepoints, but not at 16h or 18h timepoints by both DHT treatments ( $p = 0.008$ ; Figure 3.2A). Plasma 11KT levels were not altered at the 16h or 18h timepoints, were depressed at the 24h timepoint, but had recovered to control level expression at the 36h timepoint in both DHT treatments ( $p = 0.011$ ; Figure 3.2B).

### 3.2.2 Steroidogenic gene expression

As the goal of this study was to compare steroidogenic gene expression to periods of plasma androgen depression and recovery, the 16h timepoint was not analyzed for gene expression due to its similarity to the 18h timepoint in plasma hormone levels, and the 30h timepoint was not analyzed for gene expression because of its similarity in plasma androgen response to the 24h timepoint. Gene expression results for the 1  $\mu\text{g/g}$  DHT treatment are discussed below. Gene expression results from the 1  $\text{ng/g}$  DHT treatment are included in the supplementary material, as responses were similar to the 1  $\mu\text{g/g}$  DHT treatment. There was no interaction effect between DHT treatment and sample processing in any genes.

### 3.2.3 *In vivo* gene expression

Expression of *star* was depressed at the 24h timepoint, but not at either the 18h or 36h timepoints ( $p = 0.017$ ; Figure 3.3A). There were no changes in *cyp11a1* expression at any timepoint ( $p = 0.104$ ; data not shown). *Cyp17a1* expression was depressed at 18h and 24h timepoints ( $p = 0.021$ ; Figure 3.3B). Expression levels of *11 $\beta$ hsd* were decreased at 24h and 36h timepoints ( $p = 0.034$ ; Figure 3.3C). Transcript levels of *3 $\beta$ hsd* were depressed at all timepoints, and had the highest fold changes of any genes ( $p = 0.029$ ; Figure 3.3D). At both the 24h and 36h timepoints, depression of *17 $\beta$ hsd* occurred in both treatments ( $p = 0.019$ ; Figure 3.3E).

### 3.2.4 *In vitro* gene expression

Expression of *star* was increased at the 18h, 24h and 36h timepoints in the 1  $\mu\text{g/g}$  treatment ( $p = 0.031$ ; Figure 3.4C). There were no changes in *cyp11a1* expression at any timepoint ( $p = 0.216$ ; data not shown). Increased *cyp17a1* expression occurred at the 24h

timepoint only ( $p = 0.026$ ; Figure 3.4D). At the 24h timepoint, 1  $\mu\text{g/g}$  DHT increased expression of *11 $\beta$ hsd* while the same gene was depressed at the 36h timepoint ( $p = 0.015$ ; Figure 3.4E). There was an increase in *3 $\beta$ hsd* expression at both the 24h and 36h timepoints ( $p = 0.003$ ; Figure 3.4F). *17 $\beta$ hsd* transcript abundance was increased at the 24h and 36h timepoints ( $p = 0.029$ ; Figure 3.4G).

### 3.2.5 *In vitro* T and 11KT production

Gonadal T production was not affected by DHT treatment over the course of the study ( $p = 0.41$ ; Figure 3.4A). Production of 11KT was not altered at the 18h timepoint, but was depressed at the 24h and 36h timepoints ( $p = 0.022$ ; Figure 3.4B).

## 4. Discussion

The current study found depressed plasma T and 11KT in male mummichog 24h post-injection with 1  $\mu\text{g/g}$  DHT. Prior to this time point, plasma T and 11KT were not depressed compared to control fish. There is discrepancy between the first experiment, which found plasma hormone depression at 18h post-injection and the second study. The difference in timing to plasma hormone depression may be a natural response, as the stock of mummichog used for both experiments were at a later stage in the reproductive cycle during the second experiment and therefore producing higher levels of testosterone (Biran and Levavi-Sivan, 2018), resulting in a lag between exogenous androgen exposure and depression of circulating hormone levels. Depression of plasma sex hormones can lead to adverse effects on apical endpoints such as reduced fertility (Tang *et al.*, 2018), and are indicative of the perturbation of the hypothalamus-pituitary-gonadal axis in teleosts (Ankley *et al.*, 2009). Plasma hormone changes can occur rapidly after exposure to exogenous chemicals, as evidenced by a decrease in plasma 17 $\beta$ -

estradiol (E<sub>2</sub>) in fathead minnow (*Pimephales promelas*) exposed for 2h to 1500 µg/L trilostane (Ankley *et al.*, 2011). Depression of sex hormones in teleosts exposed to androgenic EDCs are well documented in the literature (Andersen *et al.*, 2006; Morthorst *et al.*, 2010; Rutherford *et al.*, 2015; Zheng *et al.*, 2016), and the current study supports the findings that androgens are capable of altering concentrations of circulating plasma sex hormones T and 11KT within hours of exposure.

T and 11KT are primarily synthesized in the gonads of teleosts (Stocco, 2001). In the current study, there was no change in *in vitro* gonadal T production at any timepoint, regardless of treatment (Figure 3.4A). Gonadal T production in teleosts may not be responsive to androgenic exposure (Bosker *et al.*, 2017); however, measuring 11KT production has been suggested as a sensitive endpoint (Marjan *et al.*, 2018). In the current study, gonadal 11KT production was decreased at 24h and 36h (Figure 3.4B). This is similar to other studies that have found a decrease in 11KT production, but no change in gonadal T production, in male mummichog exposed to model androgens (Feswick *et al.*, 2014; Glinka *et al.*, 2015; Rutherford *et al.*, 2015).

With the current evidence, the correlation between *in vitro* 11KT production and depression of plasma 11KT is stronger than the correlation between *in vitro* T production and plasma T concentrations. The continued depression of gonadal 11KT synthesis at 36h, even though plasma concentrations had recovered, may indicate that 11KT is synthesized in locations other than the gonad, and may compensate for lower gonadal production after a certain length of time (Matsubara *et al.*, 2003). The increase in plasma 11KT levels may also be linked to decreased steroid clearance, but there is little evidence that androgens alter steroid clearance rates in teleosts (Martin-Skilton *et al.*, 2006; Linderoth *et al.*, 2007; Ornostay *et al.*, 2016).

A key finding in this study is the correlation between plasma sex hormone depression and gonadal steroidogenic gene expression levels measured in snap frozen testis tissue (Figure 3.3). Two genes, *cyp17a1* and *3 $\beta$ hsd*, were depressed prior to plasma hormone depression (Figure 3.3B and 3.3D). These genes are the first to respond to androgenic exposure, and their resultant change in expression could alter the capacity of the steroidogenic pathway to produce plasma sex hormones by limiting the synthesis of progesterone or 17-OH-progesterone (Zhai *et al.*, 2017).

The depression of *3 $\beta$ hsd* in snap frozen tissue at all timepoints indicates this gene is sensitive to androgenic exposure. An *in vitro* exposure of mummichog testis tissue to DHT found *3 $\beta$ hsd* was the first gene to respond to DHT exposure, and had the largest fold-change in expression over the 24 hours tested (Rutherford *et al.*, 2019). While the fold-change observed in the current study is not as large as Rutherford *et al.* (2019), the alteration in *3 $\beta$ hsd* transcript levels are among the greatest in all quantified steroidogenic genes, supporting the suggestion to use *3 $\beta$ hsd* as a biomarker for androgen exposure (Kim *et al.*, 2007). The downregulation of *3 $\beta$ hsd* observed in this study is similar to the downregulation seen in other studies (Bluthgen *et al.*, 2013; Liu *et al.*, 2014), and has been hypothesized to be an environmentally relevant pathway for androgenic disruption of the hypothalamus-pituitary-gonadal axis (Ankley *et al.*, 2011).

In addition to *3 $\beta$ hsd* depression, lower transcript levels of *cyp17a1* have been linked with impaired androgen synthesis (Rajakumar and Senthilkumaran, 2014; Zhai *et al.*, 2018). A reduction in plasma T and 11KT was reported in *cyp17a1*-deficient zebrafish, indicating this enzyme is a key regulator of androgen synthesis (Zhai *et al.*, 2018). The current study supports this evidence, as plasma androgens were depressed shortly after *cyp17a1* gene transcripts decreased. The recovery of *cyp17a1* expression coincides with the recovery of plasma T levels, a

linkage that should be explored in future research to determine if this is a main component of changes in plasma T levels.

During the period of plasma hormone depression, gene transcripts for *star*, *cyp17a1*, *3 $\beta$ hsd*, *11 $\beta$ hsd* and *17 $\beta$ hsd* were all depressed (Figure 3.3). The downregulation of all genes in the steroidogenic pathway, with the exception of *cyp11a1*, indicates a decreased capacity for T and 11KT synthesis at multiple points in the pathway (Aspden *et al.*, 1998; Tian *et al.*, 2017). Changes in plasma hormone levels have been linked to *star* expression (Stocco *et al.*, 2000; Kusakabe *et al.*, 2006), as this represents the first step of cholesterol entering the steroidogenic pathway (Muth-Kohne *et al.*, 2016). While *star* was depressed in the current study, the correlation between it and plasma hormone levels is weaker than other steroidogenic genes.

Plasma 11KT recovered faster than plasma T, with normal levels resuming between 30h and 36h post-injection, while plasma T was depressed at 36h post-injection. Gene expression levels during this time had returned to control-level values in all genes except *11 $\beta$ hsd*, *3 $\beta$ hsd* and *17 $\beta$ hsd*, which were still depressed (Figure 3.3C-E). The downregulation of these three genes correlated to the persistent depression of plasma T. As *17 $\beta$ hsd* represents the step converting androstenedione to testosterone (Tang *et al.*, 2018), this gene may be a strong indicator of the occurrence of inhibition of plasma T. The recovery of plasma 11KT levels does not correlate with the continued depression of *11 $\beta$ hsd*, which converts T to 11KT. It has been suggested that serum T levels can regulate the kinetics of 11KT synthesis (via *11 $\beta$ hsd*) in teleosts (González *et al.*, 2015; Garcia-Garcia *et al.*, 2017), and the correlation between depressed plasma T and reduced *11 $\beta$ hsd* expression in the current study supports this. It is also possible that a compensatory mechanism, such as an increase in 11 $\beta$ -hydroxylase activity (Schiffer *et al.*, 2017),

which was not measured in the current study, may counteract the depression of *11βhsd* and account for the recovery of plasma 11KT concentrations.

Gene expression analysis of testis tissue following *in vitro* incubation found an increase in transcript abundance of *star*, *cyp17a1*, *3βhsd*, *11βhsd* and *17βhsd*. This is an opposite response in transcript abundance compared to snap frozen tissue (Figure 3.3). This difference appears to be a biological effect, as there was no interaction between DHT treatment and tissue handling (snap frozen vs *in vitro*), there were no observed changes in mRNA levels as a result of tissue incubation, and RNA quality was not impacted by incubation at 18°C for 24h. In a study profiling potential differences in gene expression between incubated testis tissue and snap frozen tissue in mummichog, there were no differences in *star* or *11βhsd* mRNA levels between the tissue types; however, there was a general decrease in mRNA levels in *cyp11a1*, regardless of treatment (Feswick *et al.*, 2014). This was not a factor in the current study, as comparison between snap frozen and *in vitro* control samples showed no decrease in any steroidogenic gene. The upregulation observed from *in vitro* tissue in the current study is similar to other studies that have found increased mRNA transcript levels in steroidogenic genes after androgen exposure (Filby *et al.*, 2007; Ekman *et al.*, 2011; Rutherford *et al.*, 2019).

Another discrepancy between snap frozen and *in vitro* gene responses is the amplitude of change in gene expression. *In vitro* samples had a larger change in gene transcript levels compared to snap frozen samples. It is actually counterintuitive that gene expression is increased *in vitro* in testes exposed to DHT (via whole organism (this study) or directly in excised tissue (Rutherford *et al.*, 2019)) when plasma steroid levels are depressed by DHT. This phenomena has been observed in mammalian studies (Heise *et al.*, 2012; Bergamin *et al.*, 2019) and may be due to the removal of negative feedback loops associated with the hypothalamus and pituitary



from *in vitro* incubations (Wong *et al.*, 2006). A recent study found a co-culture of pieces of all tissues involved in the HPG axis (hypothalamus, pituitary, gonad and liver) generated responses in hormone production similar to *in vivo* responses when exposed to the androgenic compound trenbolone (Johnston *et al.*, 2016). Mono-culture *in vitro* samples had variable responses to the same androgen, and the authors cautioned that extrapolation from *in vitro* results to *in vivo* effects may be difficult for mono-culture studies (Johnston *et al.*, 2016). Use of only gonadal tissue for *in vitro* incubations may lessen EDC impact on steroidogenic gene expression by inadvertent removal of one of the main areas of interaction (hypothalamus or pituitary) between exogenous androgens and the HPG pathway. From the current study, it can be concluded that measuring gene expression in gonadal tissue following *in vitro* incubation is not representative of gene expression in snap frozen tissue sampled directly from the exposed organism and gene expression of *in vitro* incubations should not be used as a surrogate for *in vivo* biological responses; rather, the snap frozen methodology described here is preferred.

The linkage between gonadal steroidogenic gene expression and enzymatic activity is currently lacking (Wang, 2008). While all steroidogenic genes are identified in teleosts, studies linking changes in transcript abundance to altered enzymatic activity are sparse for most steroidogenic genes (Fernandino *et al.*, 2012; Muth-Kohne *et al.*, 2016). An exception to this is the well-established correlation between expression levels of cytochrome P450 19a1 (*cyp19a1*) and the activity of its corresponding enzyme, aromatase (Villeneuve, 2016), with a decrease in *cyp19a1* transcript abundance correlating with decreased E<sub>2</sub> levels in the plasma (Schroeder *et al.*, 2017). When a strong correlation is established between gene expression levels and enzymatic activity, gene expression can be used as a surrogate for enzymatic activity (Ohtsuki *et al.*, 2012); however, the correlative strength for most steroidogenic genes is not as strong as

*cyp19a1*. The presence of biochemical regulatory networks in the steps between gene expression and enzyme activity may account for a lack of correlation between gene expression and enzymatic products (Brüggemann *et al.*, 2018). The current study establishes correlations between plasma sex hormone depression and steroidogenic gene expression. The next step is to compare gene expression levels to enzymatic activity during a period of plasma hormone depression to strengthen the causative relationship between gene expression perturbation and plasma sex hormone depression.

In conclusion, the key finding of this study is the correlation between plasma sex hormone depression and recovery and downregulation of most steroidogenic genes from snap frozen testis tissue of male mummichog injected with DHT. Depression of *cyp17a1* and *3 $\beta$ hsd* are predictive of plasma T depression, and *3 $\beta$ hsd* is the gene most impacted by androgenic exposure. Additionally, future studies should use snap frozen gonadal tissue for steroidogenic gene expression evaluation. It is not recommended to use *in vitro* tissue post-incubation to measure gene expression as responses are not representative of snap frozen gonadal responses taken from exposed fish. Feedback loops not present in *in vitro* incubations may account for the differences noted between the two tissue groups. This study adds to the knowledge of biochemical changes that underpin adverse outcomes at higher levels of biological organization, such as plasma sex hormone depression.

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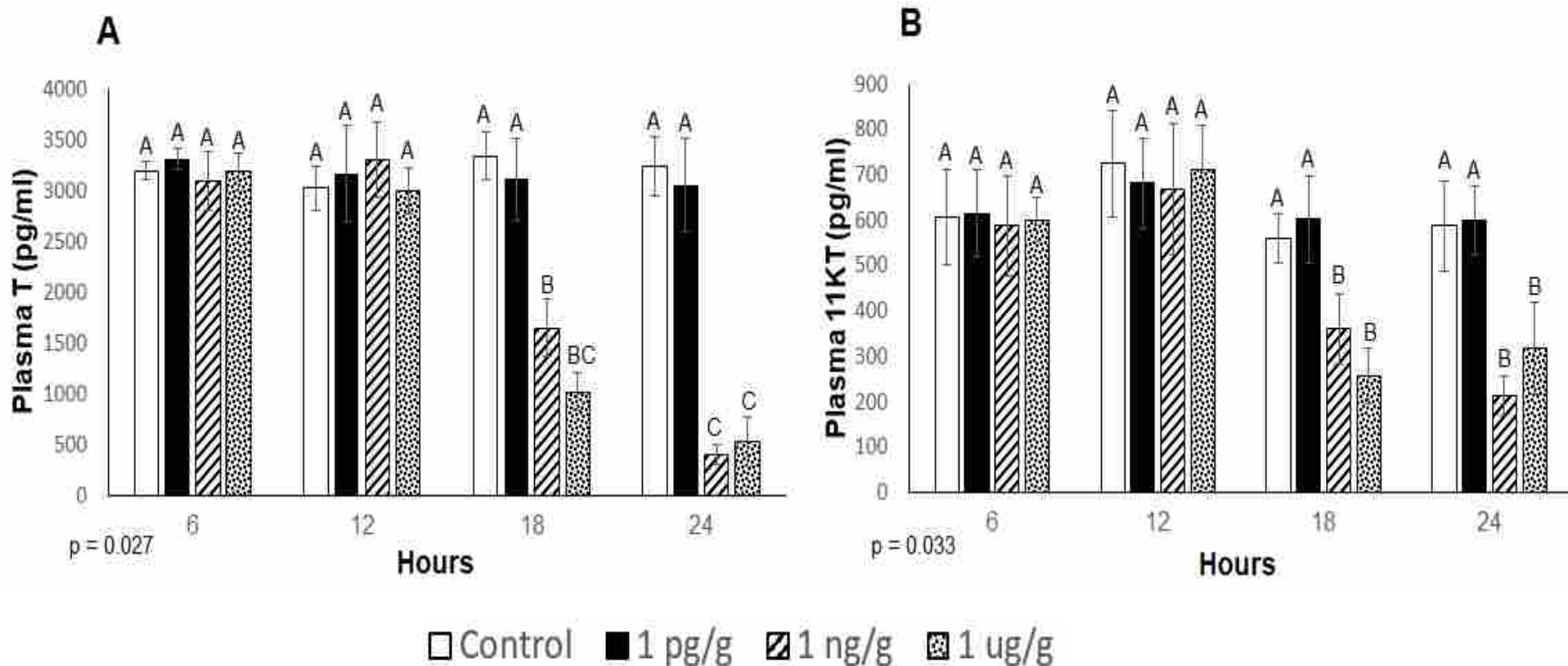


Figure 3.1. Experiment one. Mean ( $\pm 1$ SEM) plasma testosterone (T; graph A) or 11-ketotestosterone (11KT; graph B) levels (pg/ml) in male mummichog (*Fundulus heteroclitus*) injected with 0 (control), 1 pg/g 5 $\alpha$ -dihydrotestosterone (DHT), 1 ng/g DHT or 1  $\mu$ g/g DHT and sampled 6 hours, 12 hours, 18 hours or 24 hours post-injection (n= 6 per treatment, per timepoint). Significant ( $p < 0.05$ ) differences denoted by differing letters, derived from Tukey post-hoc analysis.

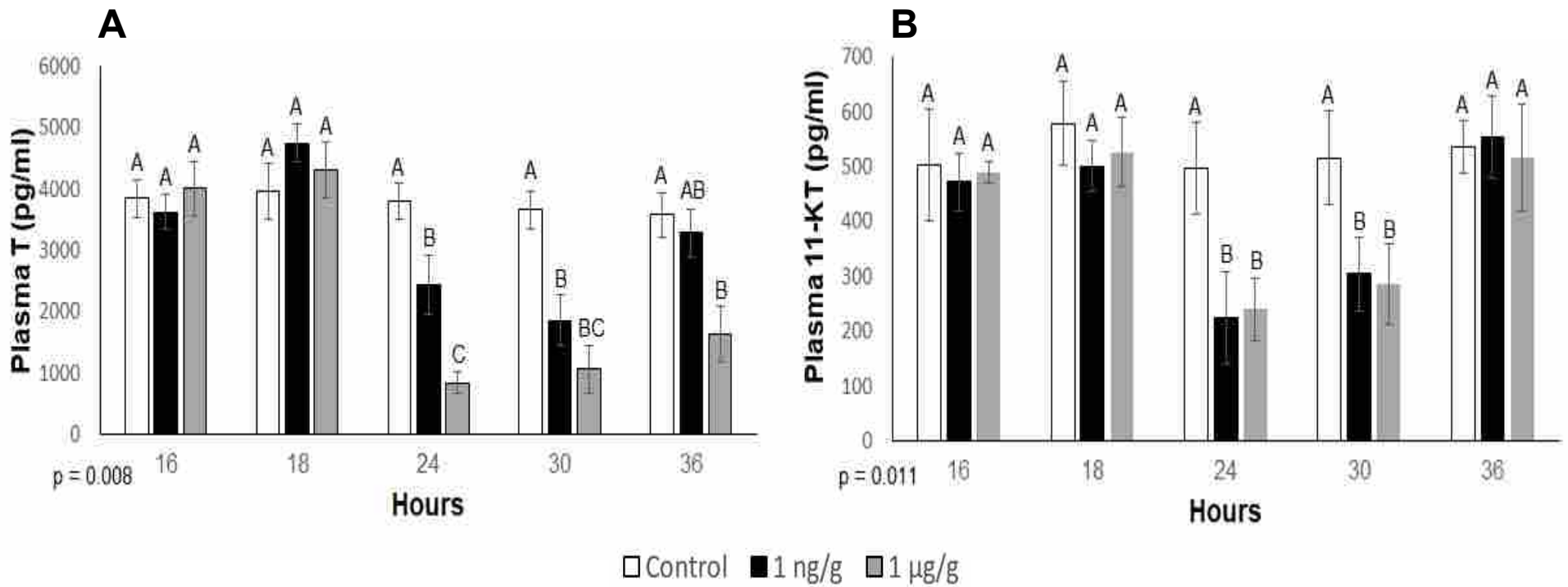


Figure 3.2. Experiment two. Mean ( $\pm 1$ SEM) plasma testosterone (T; graph A) or 11-ketotestosterone (11KT; graph B) levels (pg/ml) in male mummichog (*Fundulus heteroclitus*) injected with 0 (control), 1 ng/g 5 $\alpha$ -dihydrotestosterone (DHT) or 1  $\mu$ g/g DHT and sampled 16 hours, 18 hours, 24 hours, 30 hours or 36 hours post-injection (n= 10 per treatment, per timepoint). Significant ( $p < 0.05$ ) differences denoted by differing letters, derived from Tukey post-hoc analysis.

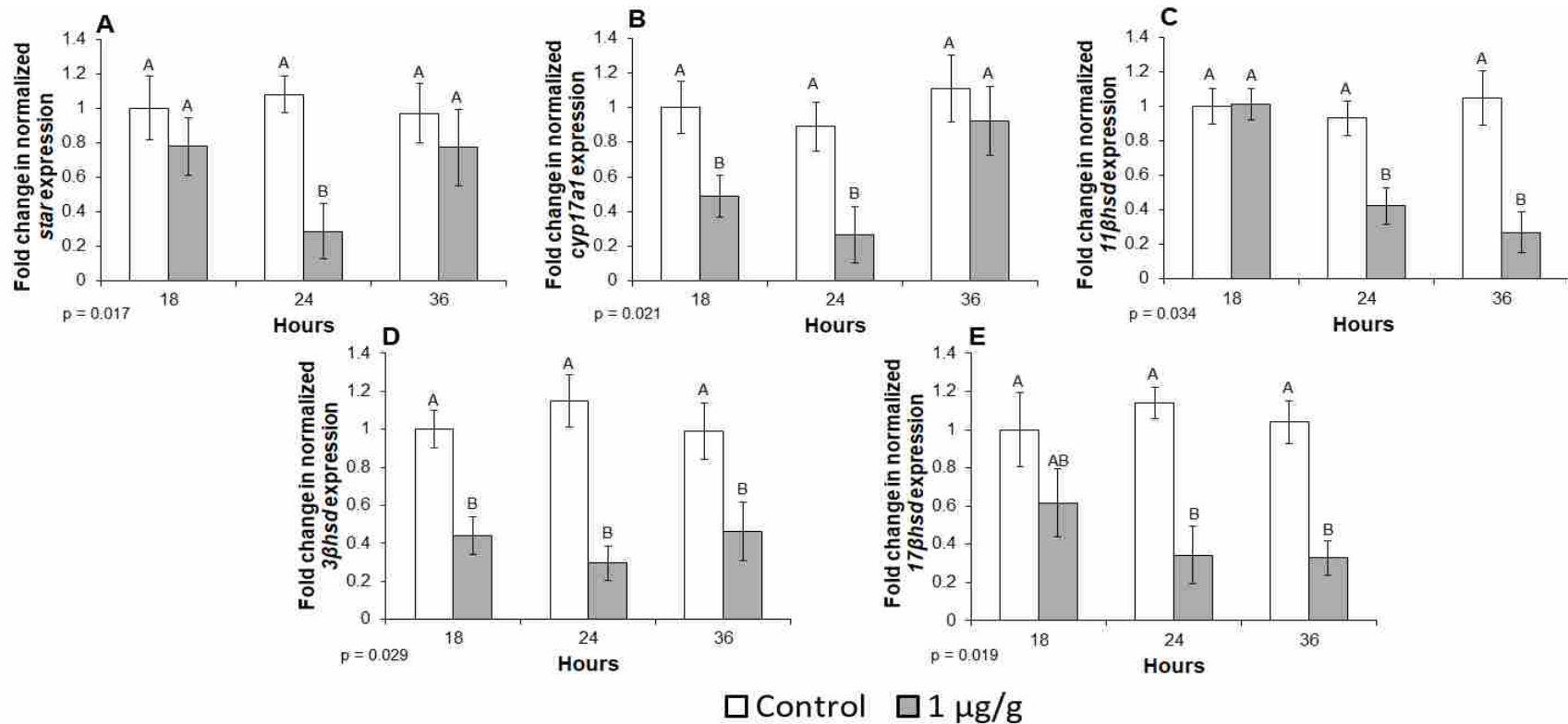
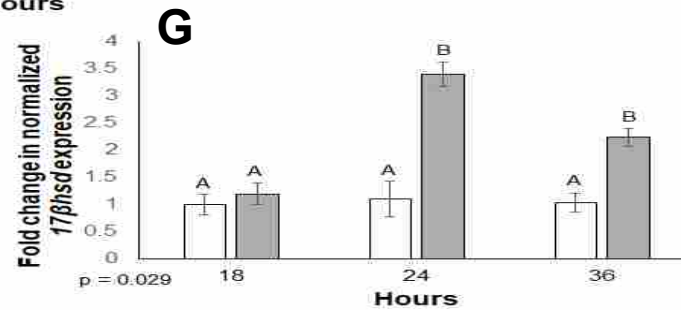
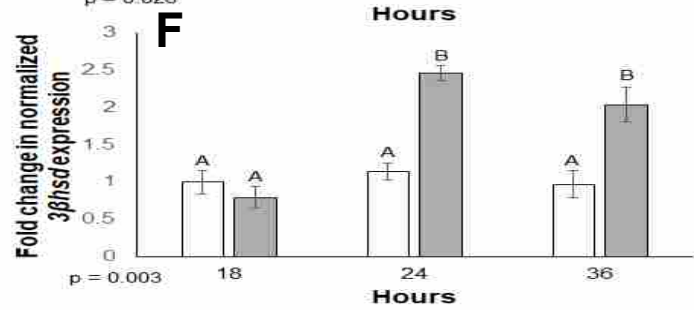
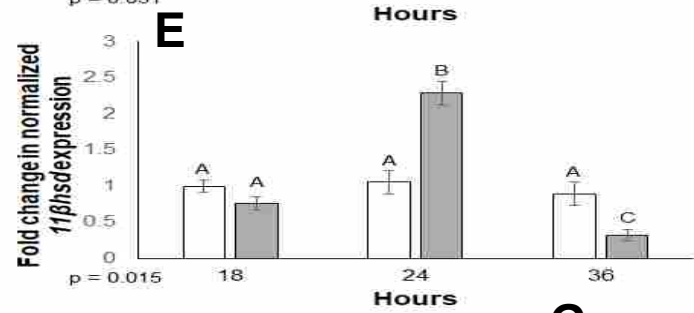
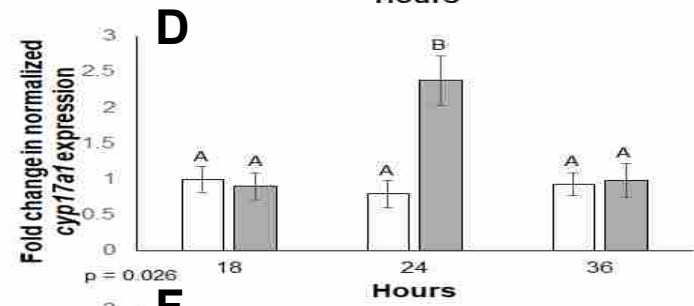
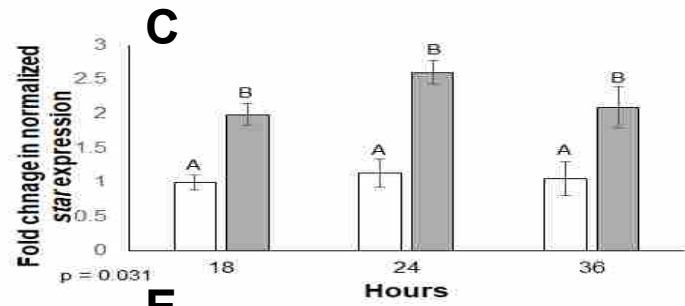
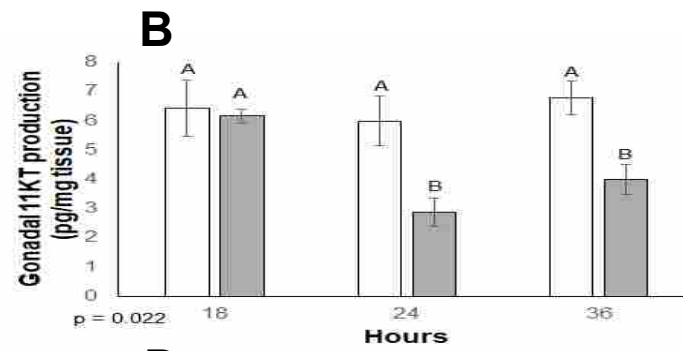
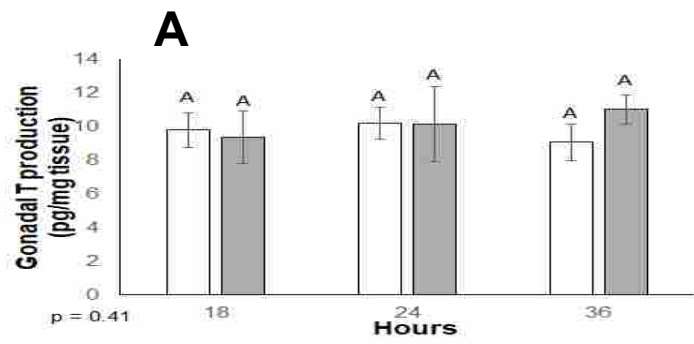


Figure 3.3. Gonadal expression ( $\pm 1$  SEM) of steroidogenic acute regulatory protein (*star*; A), cytochrome P450 17A1 (*cyp17a1*; B), 11 $\beta$ -hydroxysteroid dehydrogenase (*11βhsd*; C), 3 $\beta$ -hydroxysteroid dehydrogenase (*3βhsd*; D) or 17 $\beta$ -hydroxysteroid dehydrogenase (*17βhsd*; E) in male mummichog injected with 0 (control) or 1  $\mu$ g/g 5 $\alpha$ -dihydrotestosterone and sampled 18 hours (prior to plasma hormone depression), 24 hours (during plasma hormone depression) or 36 hours (during recovery of plasma hormone levels) post-injection (n= 10 per treatment, per timepoint). Expression levels normalized to  $\beta$ actin. Significant ( $p < 0.05$ ) differences among treatments denoted by different letters, derived from Tukey post-hoc analysis.

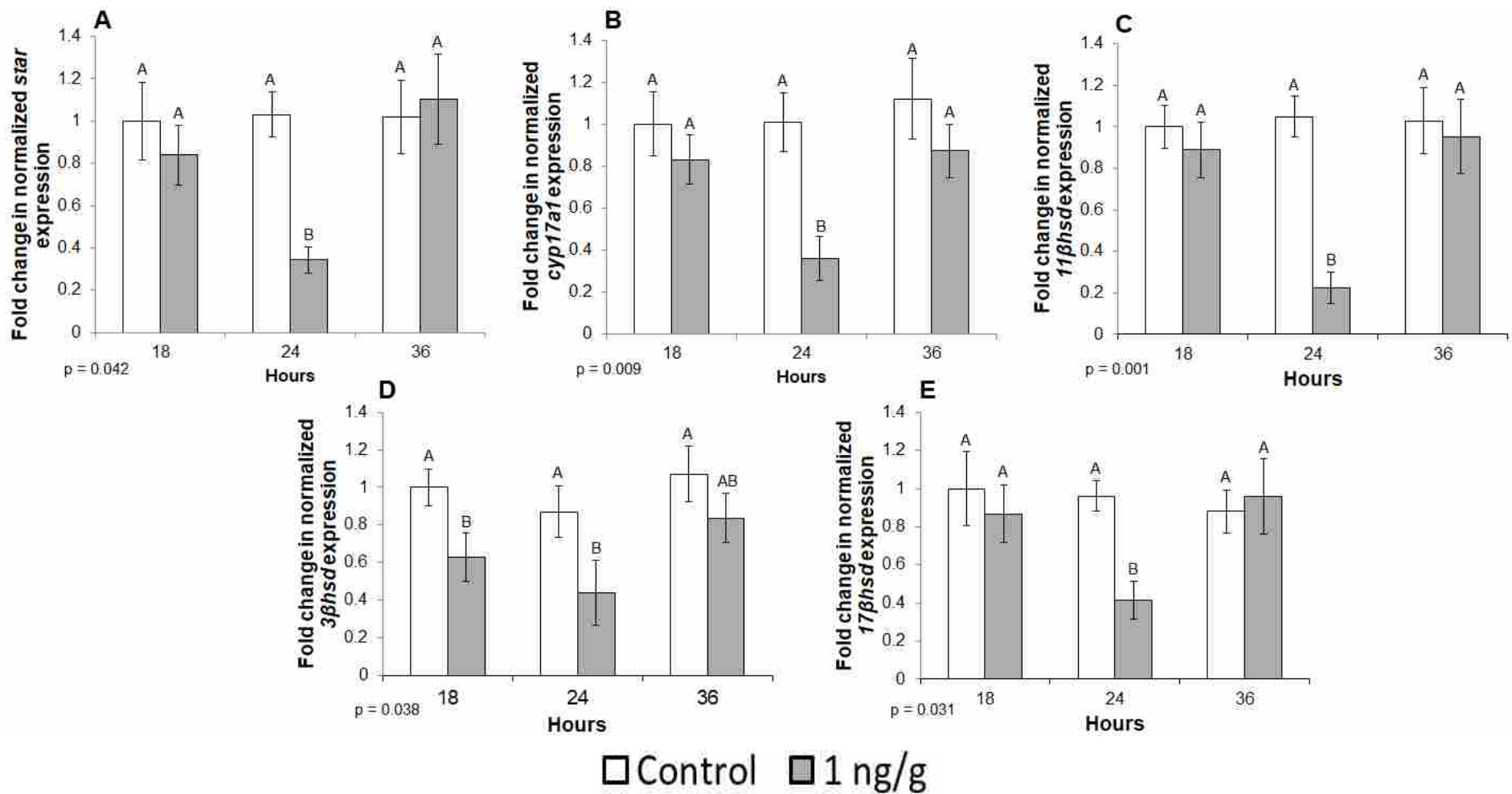


□ Control    ■ 1 µg/g

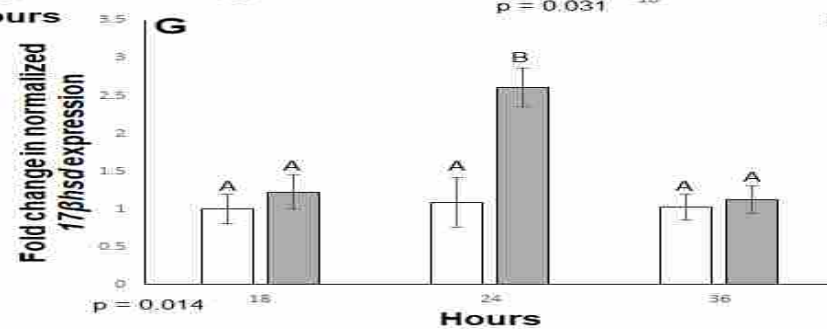
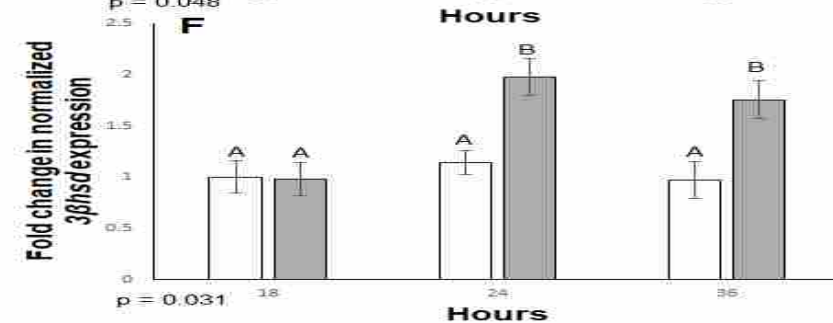
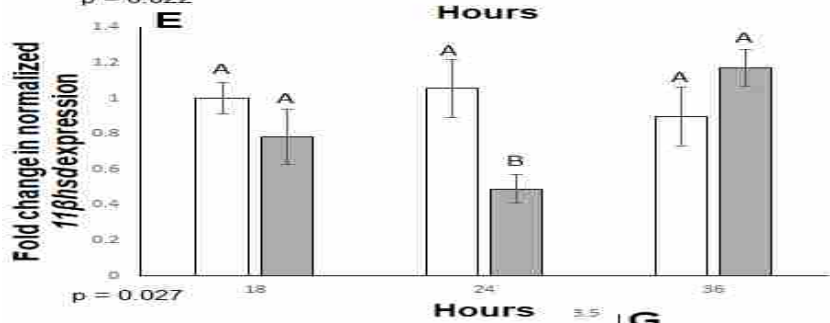
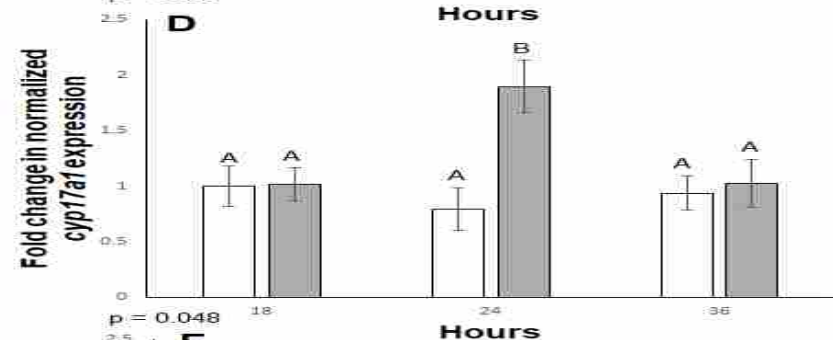
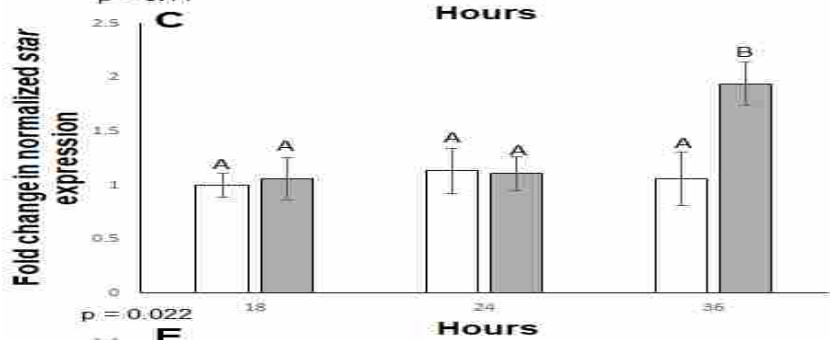
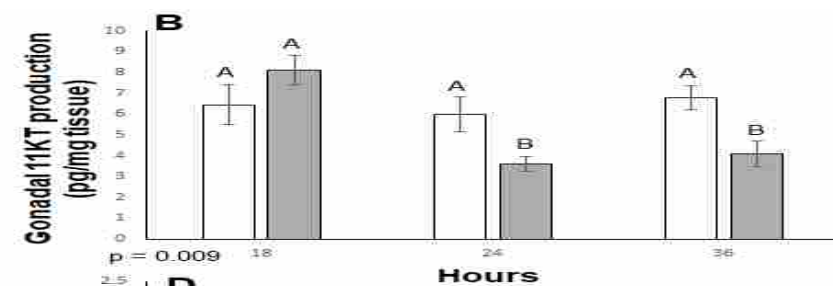
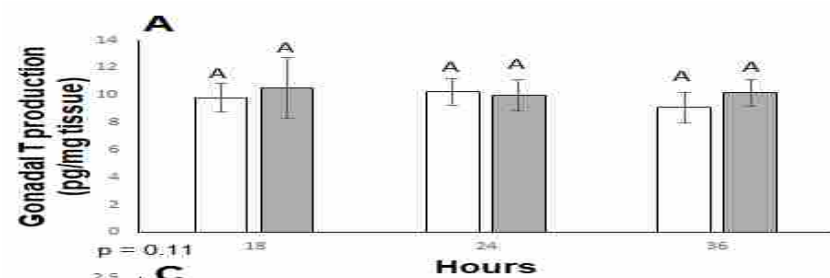
Figure 3.4. Mean ( $\pm 1$ SEM) gonadal *in vitro* production (pg/mg tissue) of testosterone (T; A) and 11-ketotestosterone (11KT; B), or gonadal expression ( $\pm 1$  SEM) of steroidogenic acute regulatory protein (*star*; C), cytochrome P450 17A1 (*cyp17a1*; D), 11 $\beta$ -hydroxysteroid dehydrogenase (*11bhsd*; E), 3 $\beta$ -hydroxysteroid dehydrogenase (*3bhsd*; F) or 17 $\beta$ -hydroxysteroid dehydrogenase (*17bhsd*; G) in male mummichog injected with 0 (control) or 1  $\mu$ g/g 5 $\alpha$ -dihydrotestosterone and sampled 18, 24 or 36 hours post-injection (n= 10 per treatment, per timepoint). Gonadal tissue was then incubated at 18°C for 24 hours before gene expression analysis. Expression levels normalized to  $\beta$ actin. Significant ( $p < 0.05$ ) differences among treatments denoted by different letters, derived from Tukey post-hoc analysis.



## **Supplementary Material**



Supplementary Figure 3.1. Gonadal expression ( $\pm$  1 SEM) of steroidogenic acute regulatory protein (*star*; A), cytochrome P450 17A1 (*cyp17a1*; B), 11 $\beta$ -hydroxysteroid dehydrogenase (*11βhsd*; C), 3 $\beta$ -hydroxysteroid dehydrogenase (*3βhsd*; D) or 17 $\beta$ -hydroxysteroid dehydrogenase (*17βhsd*; E) in male mummichog injected with 0 (control) or 1 ng/g 5 $\alpha$ -dihydrotestosterone and sampled 18 hours (prior to plasma hormone depression), 24 hours (during plasma hormone depression) or 36 hours (during recovery of plasma hormone levels) post-injection (n= 10 per treatment, per timepoint). Expression levels normalized to  $\beta$ actin. Significant ( $p < 0.05$ ) differences among treatments denoted by different letters, derived from Tukey post-hoc analysis.



□ Control    ■ 1 ng/g

Supplementary Figure 3.2. Mean ( $\pm 1$ SEM) gonadal *in vitro* production (pg/mg tissue) of testosterone (T; A) and 11-ketotestosterone (11KT; B), or gonadal expression ( $\pm 1$  SEM) of steroidogenic acute regulatory protein (*star*; C), cytochrome P450 17A1 (*cyp17a1*; D), 11 $\beta$ -hydroxysteroid dehydrogenase (*11bhsd*; E), 3 $\beta$ -hydroxysteroid dehydrogenase (*3bhsd*; F) or 17 $\beta$ -hydroxysteroid dehydrogenase (*17bhsd*; G) in male mummichog injected with 0 (control) or 1 ng/g 5 $\alpha$ -dihydrotestosterone and sampled 18, 24 or 36 hours post-injection (n= 10 per treatment, per timepoint). Gonadal tissue was then incubated at 18°C for 24 hours before gene expression analysis. Expression levels normalized to  $\beta$ actin. Significant ( $p < 0.05$ ) differences among treatments denoted by different letters, derived from Tukey post-hoc analysis.

**Chapter Four:**

**Exogenous 11-ketotestosterone impacts sex hormone synthesis in male mummichog (*Fundulus heteroclitus*) by altering 3 $\beta$ -hydroxysteroid dehydrogenase and cytochrome P450 17A1**

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## Abstract

Exogenous androgens enter aquatic ecosystems from numerous anthropogenic sources. While typically introduced in low concentrations, their potency allows them to interact with the hypothalamus-pituitary-gonadal (HPG) axis in fish and elicit negative responses. A well-established response in teleosts of exposure to exogenous androgens is the depression of plasma sex hormones. While these depressions are speculated to occur due to negative feedback within the HPG axis, the specific points of inhibition are currently unknown. Previous work with mummichog (*Fundulus heteroclitus*) has indicated negative feedback is occurring within the gonadal steroidogenic pathway, with inhibition of gene transcripts for cytochrome P450 17A1 (*cyp17a1*) and 3 $\beta$ -hydroxysteroid dehydrogenase (*3 $\beta$ hsd*) immediately preceding plasma hormone depression. In the current study, male mummichog were injected with 0 (control), 1 pg/g, 1 ng/g or 1  $\mu$ g/g 11-ketotestosterone (11KT) to induce plasma hormone depression. Steroidogenic genes, including *cyp17a1* and *3 $\beta$ hsd* were quantified during steroid depression and recovery. Fish were sampled 6 h, 12 h, 18 h, 24 h, 36 h, 48 h, 60 h or 72 h post-injection and testis tissue was either snap frozen or incubated for 24 h at 18°C. *In vitro* gonadal incubations were conducted without (basal) or with steroid precursors progesterone (P), dehydroepiandrosterone (DHEA), P and DHEA or androstenedione (AD) to determine if addition of these precursors resulted in increased gonadal sex hormone synthesis. Plasma testosterone was depressed 12 h post-injection, and recovered by 36 h. Plasma 11KT was depressed at 24 h post-injection and recovered by 72 h post-injection. P and DHEA did not stimulate gonadal sex steroid production, indicating impairment in the pathway at P metabolism to 17 $\alpha$ -hydroxyprogesterone via CYP17A1 and DHEA metabolism to AD via 3 $\beta$ HSD. AD stimulated testosterone but not 11KT production, indicating that 17 $\beta$ -hydroxysteroid

dehydrogenase (17 $\beta$ HSD) is not inhibited but 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD) is. Gene transcripts of most steroidogenic genes correlated with periods of plasma hormone depression, with *cyp17a1* and *3 $\beta$ hsd* having the largest fold change and the longest duration of depression. Gonadal responses of 11KT-injected fish mirrored gonadal sex hormone production in excised testis incubated with the 3 $\beta$ HSD inhibitor trilostane, and the CYP17A1 inhibitor SU-10603. Overall, this study provides evidence that decreased gonadal sex steroid synthesis is linked with the inability to metabolize P or DHEA to downstream metabolites within the steroidogenic pathway.

## 1. Introduction

Androgenic chemicals capable of acting as endocrine disrupting compounds (EDCs) are introduced into the aquatic environment from a variety of anthropogenic sources including wastewater treatment plants (Cavallin et al., 2016), aquaculture practices (Liu et al., 2017), pulp mill effluents (Milestone et al., 2012) and runoff from agricultural lands (Huang et al., 2019). These androgens may be present due to natural excretion from humans and other mammals (Gomes et al., 2009) or because of their use in pharmaceuticals (Kaufman et al., 2019) or their use as growth promoters in beef and swine (Johnson and Hanrahan, 2010). Although androgen concentrations are typically in the parts per billion to parts per trillion range (Leet et al., 2015), these potent chemicals are still able to elicit responses in exposed fish by interacting with the hypothalamus-pituitary-gonadal (HPG) axis (Leet et al., 2011).

Responses from androgen exposed fish include impaired immune function (Massart et al., 2015), reduced fecundity (Liu et al., 2018), intersex (Niemuth and Klaper, 2018), male-skewed sex ratios (Ankley et al., 2018), and depressed plasma testosterone and 11-ketotestosterone (11KT) levels (Schultz et al., 2013; Wu et al., 2015; Passini et al., 2018; Rutherford et al., 2019). Plasma hormone depression is a well-established response in fish exposed to various androgenic EDCs and can be linked to impaired spermatogenesis (Schulz et al., 2010), altered mating behaviour (Stacey, 2015), or offset of reproductive cycling (Alavi et al., 2012).

Exogenous androgens elicit effects in teleosts primarily through androgen receptor (AR) agonism. Sex hormone depression is hypothesized to occur due to negative feedback within the HPG axis during times of exogenous androgen exposure and subsequent AR agonism (Shao et al., 2013; Weber et al., 2019). There is currently weak quantitative evidence for where along the HPG axis negative feedback exerts its effects (Ankley et al., 2020). Some androgens alter the



secretion rate of follicle stimulating hormone (FSH) or luteinizing hormone (LH) from the pituitary (Mateos et al., 2002; Wylie et al., 2018), resulting in depression of gonadal steroidogenesis. Other studies report no changes, or even slight increases, in FSH or LH levels in androgen exposed fish (Melo et al., 2015; Harding et al., 2016) indicating negative feedback may occur within the gonad itself. Gonadal production of testosterone and 11KT from androgen exposed fish, as determined through *in vitro* protocols, has found depressed production of these sex steroids (Feswick et al., 2014; Zheng et al., 2016; Rutherford et al., 2019).

Previous work with male mummichog (*Fundulus heteroclitus*), an estuarine species inhabiting the eastern coast of North America, has shown this species to be physiologically responsive to androgenic exposure. Androgens depress hormones in mummichog (Sharpe et al., 2004; Rutherford et al., 2015; 2019); a recent study injecting male mummichog with 1 µg/g body weight 5α-dihydrotestosterone (DHT) induced depression of plasma testosterone and 11KT 18 h post-injection, which was recovered by 36 h post-injection (Rutherford et al., 2019). Depressions in steroidogenic genes steroidogenic acute regulatory protein (*star*), 11β-hydroxysteroid dehydrogenase (*11βhsd*), 17β-hydroxysteroid dehydrogenase (*17βhsd*), 3β-hydroxysteroid dehydrogenase (*3βhsd*) and cytochrome P450 17a1 (*cyp17a1*) coincided with the period of plasma hormone depression. *3βhsd* and *cyp17a1* had higher fold-changes and longer durations of depression compared to other steroidogenic genes (Rutherford et al., 2019).

Several questions arose from Rutherford et al. (2019), including whether the DHT effects could be generalized to other androgens, whether *3βhsd* and *cyp17a1* are the most responsive genes during exposure to other androgens, and whether depressed sex steroids could be explained by depression of *3βhsd* and *cyp17a1*. To test if the activity of enzymes 3βHSD and CYP17A1 (coded for by the genes *3βhsd* and *cyp17a1*, respectively) are altered during exposure

to exogenous androgens, select steroidogenic substrates upstream of these two enzymes, and inhibitors of the enzymes, were used during testis *in vitro* incubations in the current study. Specifically, progesterone (P) and dehydroepiandrosterone (DHEA) can be converted to downstream metabolites via CYP17A1 or 3 $\beta$ HSD, respectively. Quantifying sex hormone production after addition of these substrates will identify if either CYP17A1 or 3 $\beta$ HSD is inhibited by exogenous androgens, as there will be no increase in sex hormone synthesis even with the addition of the substrate. Androstenedione (AD) is converted to testosterone via 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ HSD), utilizing neither 3 $\beta$ HSD or CYP17A1, and will identify if inhibition of sex hormone production is downstream of both CYP17A1 and 3 $\beta$ HSD. This study also compared the magnitude of change in testis tissue from 11KT-injected fish to testis tissue incubated with either the CYP17A1 inhibitor SU-10603 or the 3 $\beta$ HSD inhibitor trilostane to indirectly identify if androgen injected fish have sex hormone production rates observed when CYP17A1 or 3 $\beta$ HSD are inhibited.

## **2. Materials and methods**

### **2.1 Fish collection and housing**

Wild mummichog were caught by seine netting (1/4" mesh size) from an uncontaminated estuary near Shemogue Harbour, NB, Canada (N 40°10, W 64°08) in June, 2019. Fish were transported to Wilfrid Laurier University, Waterloo, ON, Canada in aerated totes and housed in 380 L recirculating stock tanks (Aquabiotech Inc, Coaticook, QC, Canada). Water parameters of stock tanks were 18  $\pm$  1 ppt seawater (dechlorinated City of Waterloo water with Crystal Sea Salts; Marine Enterprise International, Baltimore, MD, USA), photoperiod 16 h light: 8 h dark, dissolved oxygen (DO) > 80%, temperature 21  $\pm$  1° C. Fish were fed to satiation daily with

commercial trout pellets ( 2mm pellets, EWOS Pacific, Vancouver, BC, Canada). Water quality parameters were maintained within a range of 0-0.25 mg/L ammonia, 0-1.25 mg/L nitrite, 0-5 mg/L nitrate and  $7.5 \pm 0.1$  pH. Minimal mortalities occurred in stock tanks (< 5%). All protocols were conducted according to the Canadian Council of Animal Care guidelines, as approved by the Wilfrid Laurier Animal Care Committee.

## 2.2 Stock solutions

For the injection studies, 11-ketotestosterone (CAS # 564-35-2) was purchased from Steraloids (Newport, RI, USA) and dissolved in ethanol for an initial stock of 1 mg/ml. Subsequent stocks were diluted from this original stock using sterile filtered (0.1  $\mu$ m) phosphate buffered saline (GE Healthcare Life Sciences, UT, USA) for final concentrations of 0.25  $\mu$ g/ $\mu$ l, 0.25 ng/ $\mu$ l or 0.25 pg/ $\mu$ l. Stock solutions were made immediately before use and stored on ice prior to injection. Treatments were 0 pg/g body weight (control; saline only), 1 pg/g body weight, 1 ng/g body weight (first study only) and 1  $\mu$ g/g body weight (first study only). Stock concentrations were created to limit volume injected to  $\leq 75$   $\mu$ l. Injection volume was calculated for each fish by:

$$\text{Fish weight (g)} / 0.25 = \text{injection volume } (\mu\text{l})$$

For the *in vitro* assays, AD (CAS # 63-05-8), P (CAS # 53-87-0), DHEA (CAS # 53-43-0) and trilostane (CAS # 13647-35-3) were purchased from Sigma-Aldrich Canada (Oakville, ON) and SU-10603 (CAS # 786-97-0) was purchased from Novartis Pharmaceuticals (NJ, USA). Stock solutions of 1 mg/ml ethanol were created for each chemical and further diluted with ethanol so addition of a 5  $\mu$ l aliquot stock solution to 995  $\mu$ l *in vitro* medium (Medium 199) achieved final *in vitro* concentrations of 100 ng/ml (AD, P, DHEA), 10  $\mu$ M (trilostane) or 200

nM (SU-10603). Samples receiving both DHEA and P as substrates received 5  $\mu$ l aliquots of both stocks, added to 990  $\mu$ l *in vitro* medium. Stock concentrations were chosen as they have shown effects in fish in previous studies (Condeca and Canario, 1999; Leusch and MacLatchy, 2003; Arnold et al., 2007; DeQuattro et al., 2015; Hou et al., 2017). Solutions were made immediately prior to use and stored on ice during the *in vitro* protocol.

## 2.3 11KT injection studies

### 2.3.1 Establishing time to plasma hormone depression (first study)

Male mummichog were non-selectively placed into seventeen 40 L glass aquaria (n = 6 fish per tank). Each aquarium contained 36 L of 18 ppt saltwater, was  $19 \pm 1^\circ\text{C}$  and maintained at > 80% DO. Fish were fed to satiation daily and acclimated for five days prior to 11KT injection. Daily water renewal (75% water volume) was conducted to control ammonia levels (0-0.1 mg/L). The injection protocol in Rutherford et al. (2019) was followed for 11KT injections, with no mortalities occurring over the course of the exposure. Treatments for this study were control (saline only), 1 pg/g, 1 ng/g and 1  $\mu$ g/g body weight 11KT. Fish were sampled 6 h, 12 h, 18 h, 24 h and 36 h post-injection. The 1  $\mu$ g/g treatment was only sampled at 6 h and 36 h, to determine if this concentration resulted in supraphysiological elevation of plasma 11KT, masking inhibitory effects. For each treatment, an entire aquarium was sampled at each timepoint (n = 6 fish per timepoint, per treatment). For sampling, mummichog were anaesthetized with 0.05 % buffered tricaine methane sulfonate (TMS; Sigma-Aldrich), length was measured (nearest 0.1 cm), weighed (nearest 0.01 g) and blood collected from caudal vasculature using heparinized 26 G, 1 ml tuberculin syringes (Beckton-Dickinson, Franklin Lakes, NJ). Fish were then killed by spinal severance. Collected blood was centrifuged (12 min,

4°C, 3200 g) to isolate plasma. Plasma was stored at -20°C until ether extraction and EIA analysis of plasma steroid concentrations.

### 2.3.2 Correlating plasma hormone depression to gene responses and testis sex hormone production (second study)

Male mummichog were non-selectively removed from stock tanks and placed into twelve 40 L glass aquaria (n = 12 fish per tank). Aquarium parameters were identical to study one, including acclimation period, water quality parameters and feeding. Based on plasma hormone findings from study one, treatments for study two were control (saline only) and 1 pg/g body weight 11KT. Injection protocol was the same as study one and fish were sampled 6 h, 18 h, 36 h, 48 h, 60 h and 72 h post-injection. There were no mortalities during the exposure period. At each timepoint, an entire aquarium was sampled for each treatment (n = 12 fish per timepoint, per treatment). Fish were anaesthetized with TMS, weighed (nearest 0.01 g), length was measured (nearest 0.1 cm), bled as per study one, and then killed by spinal severance. Blood was centrifuged and plasma collected and stored as in study one. Liver and testis were excised and weighed (nearest 0.001 g) for calculation of somatic indices. Testis tissue was sectioned, with one portion (minimum 60 mg) snap frozen and stored at -80°C for gene expression analysis and tissue hormone extraction. The remaining testis tissue was sectioned (20-30 mg pieces) and placed in borosilicate tubes containing 1 ml Medium 199 (Sigma Aldrich) for *in vitro* treatments. Medium 199 was made immediately prior to fish sampling according to McMaster et al. (1995) without the addition of 3-isobutyl-1-methylxanthine.

Condition factor (CF) was calculated as  $CF = (\text{total weight (g)} / \text{length (cm)}^3) * 100$ , gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated as  $GSI \text{ or } HSI = (\text{tissue weight (g)} / [\text{total weight (g)} - \text{tissue weight (g)}]) * 100$ .

## 2.4 *In vitro* protocol

The protocol optimized in MacLatchy et al. (2003) was followed. Briefly, testis tissue was excised from the fish, blotted to remove excess moisture, weighed (nearest 0.001 g) and then sectioned into 20-30 mg portions. Each portion was then minced with a scalpel (while submerged in Medium 199) and placed in 1 ml Medium 199, on ice. Immediately prior to the start of incubation, Medium 199 was removed and replaced with 995  $\mu$ l fresh medium + 5  $\mu$ l aliquot of ethanol containing appropriate substrate (P, DHEA, or AD). The treatment with both P and DHEA was composed of 990  $\mu$ l Medium 199 with the addition of 5  $\mu$ l aliquot of P stock and 5  $\mu$ l aliquot of DHEA stock. The number of samples per *in vitro* substrate treatment was maximized, but was dependant on original testis size. Treatments included basal (ethanol only, no added substrates), + P, + DHEA + P + DHEA or + AD (n = 6 wells per treatment). After new medium addition, samples were covered and incubated at 18°C for 24h on an orbital shaker for gentle mixing. After incubation, tissue was removed from media and snap frozen for tissue hormone extraction and media were snap frozen for EIA analysis of sex hormone production. All samples were stored at -80°C until subsequent analysis.

The current study utilized P, DHEA and AD as substrates for *in vitro* incubations. P requires CYP17A1 to be metabolized to downstream hormones, while DHEA requires 3 $\beta$ HSD for conversion to downstream metabolites (Figure 4.1). AD can be metabolized to testosterone via 17 $\beta$ HSD without either CYP17A1 or 3 $\beta$ HSD enzymatic conversion and serves to identify if sex hormone inhibition is downstream of both CYP17A1 and 3 $\beta$ HSD in the current study (Figure 4.1). If inhibition of sex steroid synthesis is due to inhibition of CYP17A1, addition of P will not increase final concentrations of testosterone and 11KT in the *in vitro* media. Similarly, if sex hormone production is depressed due to inhibition of 3 $\beta$ HSD, addition of DHEA to the *in*

*vitro* media will not result in an increase in testosterone and 11KT. Comparison of sex hormone production rates from testis tissue incubated with P, DHEA or AD will identify which (if any) of CYP17A1, 3 $\beta$ HSD or 17 $\beta$ HSD are inhibited after 11KT injection.

#### 2.5 Inhibiting 3 $\beta$ HSD and CYP17A1 to quantify testis sex hormone production (third study)

Male mummichog were non-selectively removed from stock tanks, anaesthetized with TMS, lengthed (nearest 0.1 cm), weighed (nearest 0.01 g) and killed by spinal severance. Testis tissue was removed for *in vitro* hormone production analysis, following the same protocol as above. Immediately prior to starting incubations, old medium was removed and replaced with 995  $\mu$ l new medium + 5  $\mu$ l aliquot of ethanol containing ethanol only (basal), the 3 $\beta$ HSD inhibitor trilostane or the CYP17A1 inhibitor SU-10603. Treatments were basal (ethanol only), + trilostane (10  $\mu$ M), + SU-10603 (200 nM) or + trilostane and + SU-10603. Samples were covered and incubated at 18°C for 24 h with gentle mixing on an orbital shaker. After incubation, tissues were removed from media and snap frozen for tissue hormone extraction. Media were snap frozen for EIA hormone production analysis. Both tissues and media were stored at -80°C until subsequent analysis.

Trilostane and SU-10603 were selected as they inhibit 3 $\beta$ HSD and CYP17A1, respectively. Comparison of the magnitude of change of P and DHEA levels between testis incubated with inhibitors and testis tissue from 11KT-injected fish indirectly identifies if 11KT-injected fish have levels of P and DHEA expected when 3 $\beta$ HSD and CYP17A1 are inhibited.

#### 2.6 Enzyme immunoassay for hormone quantification

Plasma hormone extractions were processed in triplicate, according to McMaster et al., (1992). Extracts were reconstituted in 500  $\mu$ l EIA buffer (Cayman Chemical, Ann Arbor, MI,

USA) and stored at -80°C prior to EIA analysis. Steroid levels from *in vitro* media were measured directly. Tissue hormone extractions were conducted with 30-50 mg testis tissue added to homogenizing buffer (phosphate buffered saline + 1mM ethylenediaminetetraacetic acid (EDTA), pH 7.4) at a ratio of 2:1 (homogenizing buffer to tissue weight). Samples were sonicated, on ice, and then methanol (MeOH) was added at a ratio of 4:1 (MeOH volume to homogenizing buffer). Samples were placed at 4°C for 1 h with periodic mixing. After this incubation, samples were centrifuged (5 min, 4°C, 3000 g) and the supernatant transferred to a new vial. This process was done in triplicate. The supernatant was then dried under nitrogen, and samples were reconstituted in 1 ml acetate buffer (50 mM) and incubated at room temperature for 30 min. Samples were stored at -20°C until solid phase extraction (SPE). Supelclean LC-18 SPE columns with 500 mg packing (Sigma-Aldrich) were used for hormone retention. Columns were conditioned under vacuum with 5 ml methanol, then 5 ml deionized water, followed by room temperature sample. After passing the sample through the SPE cartridge, 5 ml methanol was passed through the columns and collected to be dried under nitrogen. Once dried, samples were reconstituted in 500 µl EIA buffer (Cayman Chemical) for EIA analysis.

EIA kits for testosterone and 11KT were purchased from Cayman Chemical and used to quantify plasma levels and *in vitro* production of both hormones. Progesterone EIA kits were also purchased from Cayman Chemical to analyze tissue levels of progesterone from both snap frozen and *in vitro* testis tissue. DHEA EIA kits were purchased from MP Biomedicals (Solon, OH, USA) to analyze tissue levels of DHEA from snap frozen and *in vitro* tissue. All kits were used according to manufacturers' instructions. Standards were analyzed in triplicate and samples analyzed in duplicate. Plates were read at 420 nm after 60-, 90- and 120-min incubations (testosterone), 90- or 105-min incubations (11KT) or 60-, 75- or 90-min incubations



(progesterone). DHEA plates were read at 450 nm after 60-min incubations. All inter- and intra-assay variations were within acceptable limits for all assays (< 12% for inter-assay and < 9% for intra-assay).

## 2.7 Gene expression analysis

Snap frozen testis tissue was processed according to Rutherford et al. (2019). Briefly, 800  $\mu$ l TRIzol (Invitrogen, Carlsbad, CA, USA) was added to 20-30 mg testis tissue to extract total RNA according to manufacturer's instructions. RNA pellets were then air dried and reconstituted in 20-30  $\mu$ l molecular grade water (Invitrogen) depending on pellet size. Quantification of RNA was completed using the NanoDrop-8000 spectrophotometer (Agilent Technologies, Mississauga, ON) at an absorbance of 260 nm and 280 nm. Using molecular grade water, samples were then diluted to 1  $\mu$ g/ $\mu$ l  $\pm$  0.1  $\mu$ g and requantified to confirm concentration. RNA quality was tested on a subset (~15%) of samples using the Experion Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, CA). All checked RNA was intact (RNA Quality Index  $\geq$  8.7).

Samples were then treated with 1  $\mu$ l DNase enzyme and 1  $\mu$ l 10X reaction buffer (AMP-D1 kit; Sigma-Aldrich) prior to reverse transcription. Reverse transcription was identical to Rutherford et al. (2019), and samples were stored at -80°C until quantitative polymerase chain (qPCR) analysis.

The relative standard curve/SsoFast Evagreen method was used to determine primer pair efficiencies, with a standard curve of serially diluted RT product. Primer specificity and single product amplification were demonstrated through melting curve analysis, applied to all samples.

Selected primer pairs generated a single peak during melt curve analysis, had R<sup>2</sup> values  $\geq 0.96$  and efficiencies between 91 and 105%.

Primer sequences for steroidogenic acute regulatory protein (*star*), cytochrome P450 11A1 (*cyp11a1*), cytochrome P450 17A1 (*cyp17a1*), 3 $\beta$ -hydroxysteroid dehydrogenase (*3 $\beta$ hsd*), 11 $\beta$ -hydroxysteroid dehydrogenase (*11 $\beta$ hsd*), 17 $\beta$ -hydroxysteroid dehydrogenase (*17 $\beta$ hsd*) and  $\beta$ -actin ( *$\beta$ actin*) were based on Rutherford et al. (2019). Accession numbers and primer sequences are reported in Rutherford et al. (2019). Genes of interest were normalized to  *$\beta$ actin*, as transcript levels of this gene were not altered by 11KT injection.

Samples were run in duplicate through qPCR using the CF96X Real-time System and C1000 touch thermocycler (Bio-Rad Laboratories). For reaction volumes and qPCR cycling conditions, see Rutherford et al. (2019). Pooled mummichog testis cDNA was run in duplicate on each plate to assess coefficients of variation among runs. Inter-assay variability was  $\leq 9.1\%$  for each gene. During analysis, any sample with an intrasample variation  $\geq 0.5$  cq between raw data duplicates was rerun to assess variation. All retested samples resulted in acceptable levels ( $< 0.5$  Cq) of intrasample variation.

## 2.8 Statistical analysis

Sigmaplot 11.0 (Systat Software INC, Chicago, IL, USA) and IBM SPSS Statistics v 22 (IBM, Armonk, NY, USA) were used to complete statistical analyses. Shapiro-Wilk and Levene's tests were used to check assumptions of normality and homogeneity of variances, respectively, prior to parametric analysis. LSI and GSI were analyzed using an analysis of covariance (ANCOVA), with total fish weight as the co-variate. An ANCOVA with total body weight and body length as the two variates was conducted for evaluation of CF. A one-way

analysis of variance (ANOVA;  $\alpha = 0.05$ ) was used to test for differences in plasma concentrations, tissue concentrations, or *in vitro* production of testosterone, 11KT, progesterone and DHEA. Gene expression data were also evaluated using ANOVA. If applicable, a Tukey's post-hoc test was used to determine the site of differences.

### 3. Results

#### 3.1 First study

Mummichog weight (9.11-15.23 g), length (89-112 mm), HSI (3.04-3.86%), GSI (1.44-1.71%) and CF (1.58-2.33%) were similar among all treatments ( $p > 0.05$ ). Plasma testosterone was depressed 12 h, 18 h and 24 h post-injection in both the 1 pg/g 11KT and 1 ng/g 11KT treatments ( $p = 0.031$ ; Figure 4.2A). Injection of 1 ng/g or 1  $\mu$ g/g 11KT artificially increased plasma 11KT concentrations at all times sampled (Figure 4.2B), so these treatments were removed from subsequent studies. Plasma 11KT was elevated at 6 h post-injection, and depressed at 24 h and 36 h post-injection in the 1 pg/g 11KT treatment ( $p = 0.001$ ; Figure 4.2B).

#### 3.2 Second study

##### 3.2.1 Plasma hormones and fish morphometrics

Based on results from study one, only control and 1 pg/g 11KT treatments were used. No differences in fish weight (8.85-14.93 g), length (89-126 mm), HSI (3.47-4.19%), GSI (1.29-2.14%) and CF (1.36-2.51%) occurred among treatments ( $p > 0.05$ ). Plasma testosterone was depressed only at 18 h post-injection ( $p = 0.036$ ; Figure 4.3A). Similar to study one, plasma 11KT was initially elevated 6 h post-injection, depressed at 24 h, 48 h and 60 h post-injection, and recovered to normal levels by 72 h post-injection ( $p = 0.024$ ; Figure 4.3B).

### 3.2.2 *In vitro* testosterone production

In basal samples (receiving no additional precursors), testis testosterone production was depressed by 1 pg/g 11KT at 18 h ( $p = 0.008$ ; Figure 4.4B) and 36 h ( $p = 0.012$ ; Figure 4.4C). Addition of precursors (P, DHEA or AD) increased testosterone production in all control treatments at all timepoints (Figure 4.4 A-D, all  $p < 0.05$ ). In 11KT-injected fish, the precursors P, DHEA, or P and DHEA together did not increase testosterone production to the same degree as it did in control fish at 6 h ( $p = 0.017$ ; Figure 4.4A), 18 h ( $p = 0.008$ ; Figure 4.4B), 36 h ( $p = 0.012$ ; Figure 4.4C) or 72 h ( $p = 0.015$ ; Figure 4.4D), except for P at 72 h. In 11KT-injected fish, AD stimulated testosterone production to the same degree as in control fish, at all timepoints ( $p < 0.05$ , Figure 4.4 A-D).

### 3.2.3 *In vitro* 11KT production

Testis 11KT production was depressed in basal 11KT treatments at 6 h ( $p = 0.004$ ; Figure 4.5A), 18 h ( $p = 0.009$ ; Figure 4.5B) and 36 h ( $p = 0.011$ ; Figure 4.5C) timepoints. Stimulation with precursors in the media increased 11KT production in all control testis samples compared to basal control (Figure 4.5 A-D, all  $p < 0.05$ ). No precursor increased testis production of 11KT in 11KT-injected fish to the degree that they recovered to basal levels (Figure 4.5 A-D; all  $p < 0.05$ ).

### 3.2.4 Tissue P and DHEA levels

P levels from snap frozen testis tissue were elevated at the 18 h timepoint in 11KT treated fish (Figure 4.1A; Supplementary Material). Testis tissue from fish injected with 1 pg/g 11KT sampled following *in vitro* incubation had elevated P levels in the 18 h and 36 h post-injection groups ( $p = 0.033$ ; Figure 4.6A). DHEA from snap frozen tissue was elevated at 6 h, 18 h and 36

h post-injection in 11KT-injected fish (Figure 4.1B Supplementary Material). DHEA concentrations from *in vitro* tissue were increased in the 18 h, 36 h and 72 h timepoints in 11KT-injected fish ( $p = 0.037$ ; Figure 4.6B).

### 3.2.5 Steroidogenic gene expression

Gene expression analysis was completed for 6 h, 18 h, 36 h and 72 h timepoints only, to determine if transcript levels of steroidogenic genes correlated with periods of plasma testosterone or 11KT depression. The selected timepoints generate a sampling time correlation for plasma testosterone depression (no depression (6 h) → depression (18 h) → recovery (36 h)) and plasma 11KT depression (no depression (18 h) → depression (36 h) → recovery (72 h)). Transcript levels of *cyp11a1* were not altered at any timepoint ( $p = 0.687$ ; data not shown). Depression of *star* occurred 18 h and 24 h post-injection ( $p = 0.037$ ; Figure 4.7A). Depression of *cyp17a1* was found at 6 h, 18 h and 36 h post-injection ( $p = 0.029$ ; Figure 4.7B). *17βhsd* expression was depressed at 18 h post-injection ( $p = 0.025$ ; Figure 4.7C). Transcript levels of *11βhsd* were depressed 6 h, 18 h and 36 h post-injection ( $p = 0.038$ ; Figure 4.7D), similar to *cyp17a1* expression. Depression of *3βhsd* occurred in all tested timepoints ( $p = 0.017$ ; Figure 4.7E).

## 3.3 Third study

### 3.3.1 Fish Morphometrics

Fish from this study were similar to fish from the first two studies (length (98-157 mm), weight (9.52- 15.16 mm), CF (1.44-2.29 %) and GSI (1.39-1.82 %)). A comparison of GSI showed no differences across the three studies, indicating the fish were in similar stages of their reproductive cycle at sampling ( $p = 0.119$ , data not shown; Biran and Levavi-Sivan, 2018).

### 3.3.2 *In vitro* testosterone production

Testis production of testosterone from untreated samples were similar to basal testosterone production from *in vitro* samples in study two (data not shown). Addition of trilostane or SU-10603 resulted in depressed T production (Figure 4.2A; Supplementary Material). Addition of P to Medium 199 increased T production in trilostane exposed samples but did not increase T production in SU-10603 exposed samples (Figure 4.2A; Supplementary Material). Addition of DHEA increased T production in SU-10603 exposed samples, but did not increase production in trilostane exposed samples (Figure 4.2A; Supplementary Material).

### 3.3.3 *In vitro* 11KT production

11KT production from inhibitor-free samples from this study were similar to basal 11KT production from *in vitro* samples in study two. Addition of trilostane or SU-10603 depressed production of 11KT in the testis (Figure 4.2B; Supplementary Material). Addition of P increased 11KT production in trilostane exposed samples, but not in SU-10603 exposed samples, while addition of DHEA increased 11KT production in SU-10603 exposed samples, but not in trilostane exposed samples (Figure 4.2B; Supplementary Material).

### 3.3.4 Tissue P and DHEA levels

P levels from untreated samples were similar to P levels from control samples of study two. Testis incubated with SU-10603 had increased levels of P, while trilostane had no effect on testis P levels ( $p = 0.033$ ; Figure 4.6A).

DHEA levels from untreated samples were similar to DHEA levels from control samples of study two ( $p = 0.037$ ; Figure 4.6B). Testis incubated with trilostane had increased levels of DHEA, while testis incubated with SU-10603 did not ( $p = 0.037$ ; Figure 4.6B).

#### **4. Discussion**

The results of the current study confirm that androgen injection leads to depression of plasma hormones testosterone and 11KT. Hormone depression is a commonly observed result of exposure to exogenous androgens in fish (Schultz et al., 2013; Passini et al., 2018; Yue et al., 2018; Rutherford et al., 2019), and may lead to reduced fertility (Tang et al., 2018), improper spermatogenesis (Schulz et al., 2010) or altered mating behaviour (Stacey, 2015). The current study observed plasma testosterone depression 12 hours after 11KT injection, and reduced testis production of 11KT 6 hours post-injection (Figures 4.2A and 4.5A, respectively). This is similar to the timeline in Rutherford et al. (2019) in which mummichog were injected with DHT. Slight variations in time to plasma hormone depression may be a result of different androgen receptor binding affinities between 11KT and DHT used in the studies (Larsson et al., 2002).

Endogenous levels of 11KT in male mummichog peak at approximately 10.1 ng/ml during the breeding season, and fall as low as 0.3 ng/ml during testis regression (Cochran, 1987). Injection with either 1 ng 11KT/g or 1  $\mu$ g 11KT/g resulted in supraphysiological levels of plasma 11KT (Figure 4.2), and these treatments were removed from analysis. However, injection with 1 pg 11KT/g body weight resulted in approximately 3 ng/ml 11KT 6 hours post-injection (Figure 4.2). This is within the normal range of 11KT for male mummichog, thus providing a physiologically-valid model for this experiment.

Exogenous androgens elicit endogenous responses through androgen receptor (AR) agonism (as reviewed in Golshan and Alavi, 2019). Depressed steroid levels due to AR agonism are well documented, and may be due to induction of negative feedback within the HPG axis (Shao et al., 2013; Weber et al., 2019). The exact points of perturbation within the HPG pathway during these times of decreased hormone synthesis are not currently known (Villeneuve, 2018; Ankley et al., 2020), but gonadal hormone production results, including in this study, indicate these mechanisms may occur within the testis itself.

Reduced synthesis of testosterone and 11KT in the testis occurred at 18 h and 6 h post-injection, respectively (Figure 4.4). As the testis is the primary area of sex steroid synthesis in male teleosts (Stocco, 2001), this reduction may be responsible for reduced circulating levels of these sex hormones (Ankley et al., 2011). Testosterone is less responsive to androgen exposure in teleosts compared to 11KT (Bosker et al., 2017; Marjan et al., 2018), and this is supported in the current study, as 11KT production was depressed more quickly than testosterone production. The use of 11KT as the exogenous androgen in the current study may result in a faster inhibition of 11KT production due to its affinity for the androgen receptor compared to the previous study where DHT was used as the exogenous androgen. In DHT-injected mummichog, no changes in testosterone production were observed, and 11KT production was depressed 24 h post-injection, but had recovered by 36 h post-injection (Rutherford et al., 2019). Throughout the time points sampled, plasma 11KT measured  $0.8 \pm 0.2$  ng/ml in the control fish, a level representative of non-reproducing male mummichog (Cochran, 1987). 11KT injection increased plasma 11KT levels to 3 ng/ml initially, subsequently initiating apparent negative feedback loops to compensate for the increased androgen levels. This induction was at a faster rate than mummichog injected with DHT, most likely because feedback loops present between the testis



and hypothalamus/pituitary sections of the HPG axis are more sensitive to circulating 11KT than DHT (Ozaki et al., 2019; Schuppe et al., 2020). As the endogenous role of DHT is not well-understood in fish, it is possible that 11KT induces a longer period of inhibition in male teleosts and does so at a faster rate than other androgens that are not naturally synthesized nor as integral to fish reproduction and development.

Previous work in male teleosts has identified 3 $\beta$ HSD and CYP17A1 as two potential enzymes responsible for reduced testicular sex steroid synthesis (Zhai et al., 2018; Chi et al., 2019). The current study utilized steroidogenic substrates immediately upstream of these two enzymes (P for CYP17A1 and DHEA for 3 $\beta$ HSD; Figure 4.1) to test if increasing the concentration of these substrates resulted in downstream increases in testosterone and 11KT production. While use of either substrate increased sex steroid production in all control samples, their addition did not result in a production rate recovery in 11KT-injected fish (Figures 4.4 and 4.5). Addition of AD to *in vitro* media resulted in increased testosterone production but did not increase 11KT production rates in any timepoint. This supports the evidence that 11 $\beta$ HSD is responsive to excess 11KT and acts as a control of 11KT synthesis rates (Gonzalez et al., 2015; Garcia-Garcia et al., 2017; Schiffer et al., 2017). AD is downstream of both DHEA and P in the steroidogenic pathway. As AD caused a stimulation in hormone synthesis rate, but neither DHEA nor P did, it is assumed that the enzymes between these substrates (3 $\beta$ HSD and CYP17A1) are inhibited during 11KT exposure.

Inhibition of 3 $\beta$ HSD alters normal regulation of the HPG axis (Sun et al., 2014). Depression of 3 $\beta$ HSD in the teleost steroidogenic pathway causes decreased androgen synthesis (Ohta et al., 2007; Sambroni et al., 2013) and reductions in levels of circulating sex steroids (Villeneuve et al., 2008). Trilostane, an inhibitor of the 3 $\beta$ HSD enzyme (Shears and Boyd,

1981), has been used in numerous studies to examine the effects of inhibition of 3 $\beta$ HSD on teleost steroidogenesis (Gilman et al., 2003; Watanabe et al., 2016; Shi et al., 2017). Effects of trilostane exposure include reduced spawning frequency (Villeneuve et al., 2008), inhibition of spermatogenesis (Miura et al., 1991) and reduction in sex steroid synthesis (Sambroni et al., 2013). In the current study, similarities in testis sex hormone production levels between testis from 11KT-injected fish and trilostane treated testis indicates that 3 $\beta$ HSD is a primary inhibitor in androgen exposed mummichog. Additionally, tissue levels of DHEA, the main substrate for 3 $\beta$ HSD, are elevated in 11KT-injected fish in the 18 h, 36 h and 72 h timepoints (Figure 4.6B). This increased level of DHEA may indicate that 3 $\beta$ HSD is not metabolizing its substrate at the same rate as those from the control fish, causing its buildup in the tissue. Decreased levels of the gene *3 $\beta$ hsd*, which codes for the enzyme 3 $\beta$ HSD, correlate to the timing of increased testis levels of DHEA except for in the 6 h timepoint, where gene expression is depressed but tissue concentrations of DHEA have not increased. It is possible that the depression of *3 $\beta$ hsd* in the 6 h timepoint may be a precursor to decreased DHEA metabolism, as gene transcription and translation is not an immediate process.

The steroidogenic enzyme CYP17A1 is integral to testosterone synthesis (Kumar et al., 2000) by facilitating the production of DHEA and AD (Ma et al., 2011; Zhai et al., 2018). Deficiency of CYP17A1 in teleosts causes depressed androgen synthesis (Meng et al., 2019), which has been linked with compromised development of secondary sexual characteristics and mating behaviour (Zhai et al., 2018). In the current study, the CYP17A1 inhibitor SU-10603 (SU) was utilized to inhibit conversion of progesterone to 17 $\alpha$ -hydroxyprogesterone (Bakker et al., 1978), which resulted in lower rates of both testosterone and 11KT production (Figure 4.2; Supplementary Material). SU treated testis had increased levels of tissue progesterone (Figure

4.6A), indicating that this inhibitor did impede progesterone metabolism to downstream metabolites. Testis tissue from 11KT-injected fish also had increased levels of progesterone, at the 18 h and 36 h timepoints, coinciding with depressed testis testosterone production and depression of *cyp17a1* expression. Combining these results, it is possible that the reduction in CYP17A1 facilitated metabolism of progesterone is responsible for depressed androgen synthesis within the testis. When progesterone levels return to control-level concentrations there is a simultaneous recovery of the rate of testosterone production.

Inhibition of *cyp17a1* expression is indicative of a reduction of the androgen synthesis capacity of the steroidogenic pathway (Rajakumar and Senthilkumaran, 2014; Zhai et al., 2018). Zebrafish (*Danio rerio*) deficient in *cyp17a1* have reduced plasma testosterone and 11KT (Zhai et al., 2018), evidence of the integral role this gene plays in androgen synthesis (Yao et al., 2009; Tokarz et al., 2015). In the present study, recovery of *cyp17a1* expression coincided with recovery in plasma 11KT levels. Recovery of *cyp17a1* has been linked with recovery of plasma testosterone levels in mummichog injected with DHT (Rutherford et al., 2019), but this is not evident in the current study. It is possible that the use of different model androgens during exposure could elicit different feedback mechanisms within the steroidogenic pathway, but this was not investigated in the current study.

Reduction of *3 $\beta$ hsd* transcript levels has been reported in fish exposed to exogenous androgens (Bluthgen et al., 2013; Liu et al., 2014; Rutherford et al., 2019). This gene is typically the first steroidogenic gene to respond to androgenic exposure and has been suggested as a biomarker for androgens (Kim et al., 2007). In the current study, *3 $\beta$ hsd* was depressed in all sampled timepoints and had one of the largest fold-changes of all steroidogenic genes. This response is similar to those elicited by DHT injection (Rutherford et al., 2019) and indicates that

androgenic disruption of the HPG axis could be via perturbation of *3βhsd* gene expression (Ankley et al., 2011).

The correlation between gene transcript levels and reductions in plasma sex hormones is not well-established in the literature. Gene responses to androgen exposure are influenced by fish sex, exposure length, reproductive status and many other variables (Chi et al., 2018; Guzman et al., 2018; Wang et al., 2019). Depression of most steroidogenic gene transcript levels coincide with periods of plasma hormone depression in the current study (Figure 4.7). With the exception of *cyp11a1*, all quantified genes were depressed in the 18h timepoint, corresponding with depressed testis production and plasma levels of testosterone. These gene responses agree with studies done with *Fundulus heteroclitus* (Rutherford et al., 2019), *Gobiocypris rarus* (Liu et al., 2014), *Sparus auratus* (Garcia-Garcia et al., 2017) and *Epinephelus akaara* (Li et al., 2006). Other studies report no effect (Andersen et al., 2006; Feswick et al., 2014; Gonzalez et al., 2015) or increased expression (Zheng et al., 2016; Hou et al., 2018) of steroidogenic genes in fish exposed to exogenous androgens. This variability in gene expression responses may be due to study design factors (Simon et al., 2014), compensatory responses (Cavallin et al., 2016) or exposure to different model androgens. While the literature is currently sparse, there is growing evidence that steroidogenic genes correlate to periods of plasma hormone depression in studies quantifying changes on an hourly scale. Chronic exposures (lasting 7+ days) may elicit compensatory responses in fish that are not seen during acute (24-48h) exposures; this may account for the variability in gene expression responses observed among studies.

The linkage between enzymatic activity and steroidogenic gene expression is currently lacking in studies of fish exposed to exogenous androgens (Wang, 2008; Fernandino et al., 2012; Tokarz et al., 2015; Muth-Kohne et al., 2016). Discrepancy between gene expression and

enzyme function may occur in the steps between gene activation and enzymatic activity, such as ribosomal protein translation (Cao et al., 2019). The current study supports the correlation between depressed transcripts of *3βhsd* and *cyp17a1* and inhibited enzymatic function by demonstrating the absence of increased sex hormone production from 11KT-exposed testis tissue supplemented with the substrates P or DHEA. Establishing a strong correlation between gene expression levels and enzymatic activity will permit gene expression to be used as a surrogate for enzymatic activity (Ohtsuki et al., 2012). Future studies may include protein quantification methods, such as Western blot analysis, to determine if changes in gene transcript levels correspond to differences in protein levels, as this data is lacking in the current study. Establishing a relationship between these two endpoints strengthens the biological plausibility of changes in gene expression underpinning alterations of sex hormone synthesis.

The current study utilized gene expression data, incubation of testis tissue with steroidogenic precursors to indirectly test enzyme efficiencies, and quantified testicular and plasma sex hormone levels. These endpoints considered together provide strong evidence that inhibition of steroidogenic genes, especially *3βhsd* and *cyp17a1*, are linked with depressed enzymatic efficiencies of the respective enzymes. These depressions in gene transcripts and enzyme functions are also linked with depressed testis sex hormone production and lowered levels of plasma sex hormones. Further evidence that 3βHSD and CYP17A1 are primarily involved in depression of testis sex hormone production comes from comparison between 11KT-injected fish and testis tissue incubated with trilostane and SU-10603 having similar levels of P and DHEA.

Overall, the current study provides evidence that exposure to exogenous 11KT alters the rate of testis sex steroid synthesis by decreasing conversion of P and DHEA to downstream

metabolites via enzymes controlled by *3 $\beta$ hsd* and *cyp17a1* expression. This study also demonstrates that steroidogenic gene expression coincides to timing of plasma hormone depression during acute exposure, with *3 $\beta$ hsd* and *cyp17a1* being the most androgen-sensitive genes quantified. This study helps to narrow the focus to specific areas of the HPG axis perturbed by exposure to androgens that can possibly account for observed decreases in plasma sex hormone levels.

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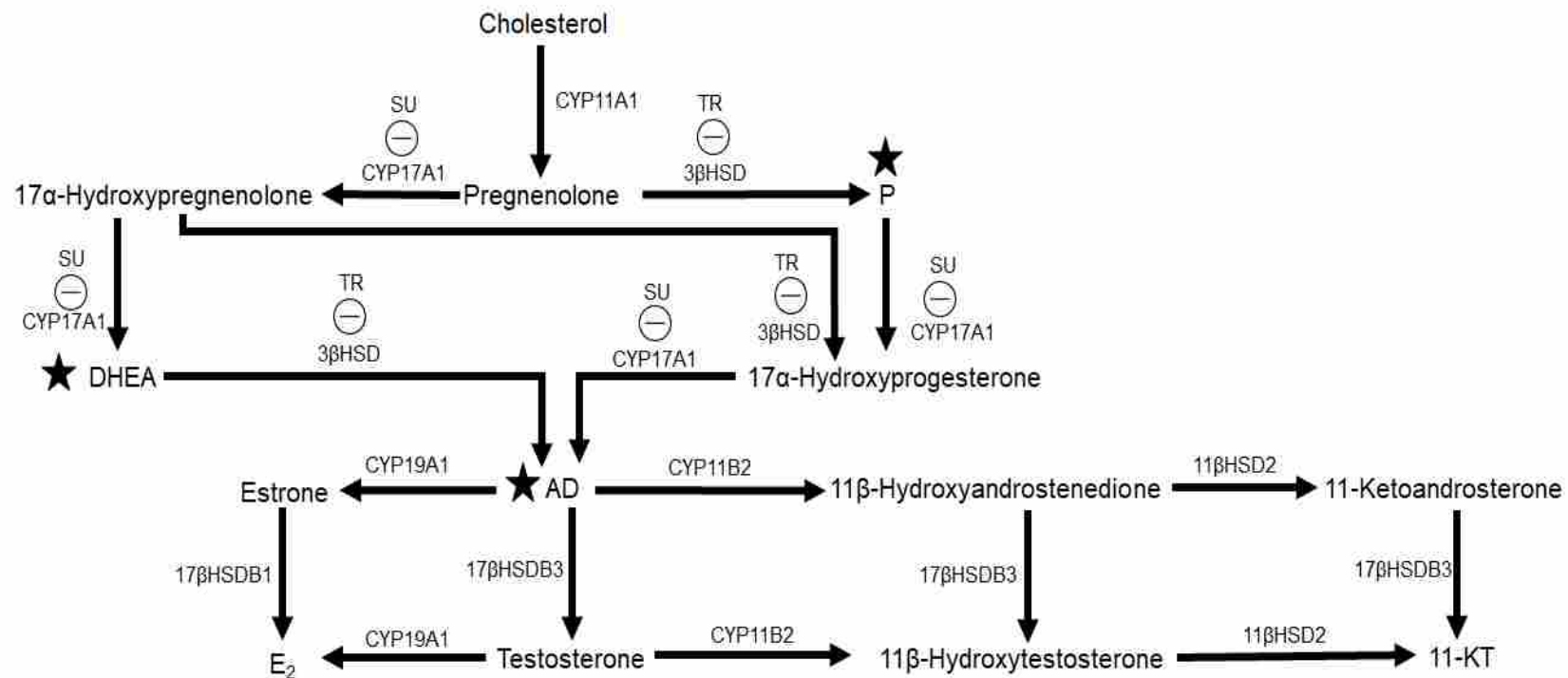


Figure 4.1. Main steroidogenic pathway in Leydig cells of testes in male teleosts. P = progesterone, DHEA = dehydroepiandrosterone, AD = androstenedione, E<sub>2</sub> = 17β-estradiol, 11KT = 11-ketotestosterone, CYP11A1 = cytochrome P450 11a1, CYP17A1 = cytochrome P450 17a1, 3βHSD = 3β hydroxysteroid dehydrogenase, 11βHSD2 = 11β hydroxysteroid dehydrogenase b2, 17βHSDB1 = 17β hydroxysteroid dehydrogenase b1, 17βHSDB3 = 17β hydroxysteroid dehydrogenase b3, CYP11B2 = cytochrome P450 11b2. Precursors labelled with a star (★) used for certain *in vitro* incubations at 100 ng/ml. Inhibition of 3βHSD achieved by addition of trilostane (TR), inhibition of CYP17A1 achieved by addition of SU-10603 (SU). Areas of pathway inhibition indicated by ⊖



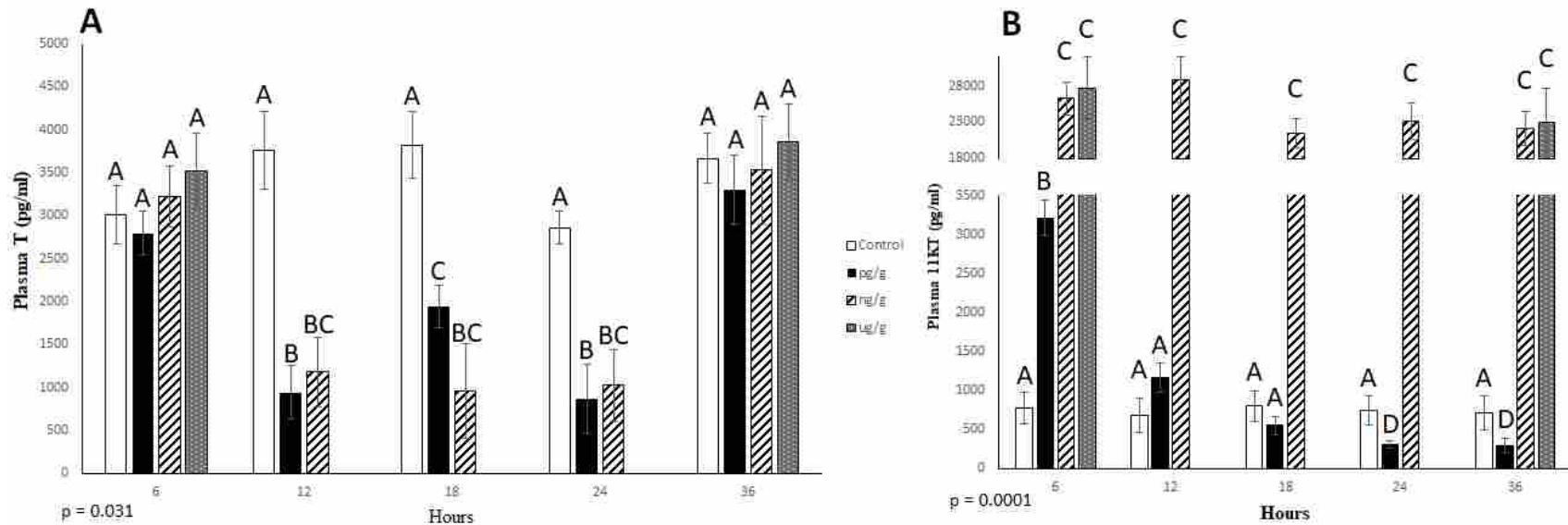


Figure 4.2. Mean ( $\pm$  1SEM) plasma testosterone (T; A) or 11-ketotestosterone (11KT; B) levels (pg/ml) in male mummichog (*Fundulus heteroclitus*) injected with saline only (control), 1pg/g body weight 11KT, 1 ng/g 11KT or 1 µg/g 11KT and sampled 6 h, 12 h, 18 h, 24 h or 36 h post-injection (n = 6 fish per timepoint, per treatment). 1 µg/g treatment sampled only at 6 h and 36 h timepoints to test for compatibility in enzyme immunoassay. Significant differences ( $p < 0.05$ ) as indicated by Tukey's post-hoc analysis represented by different letters.

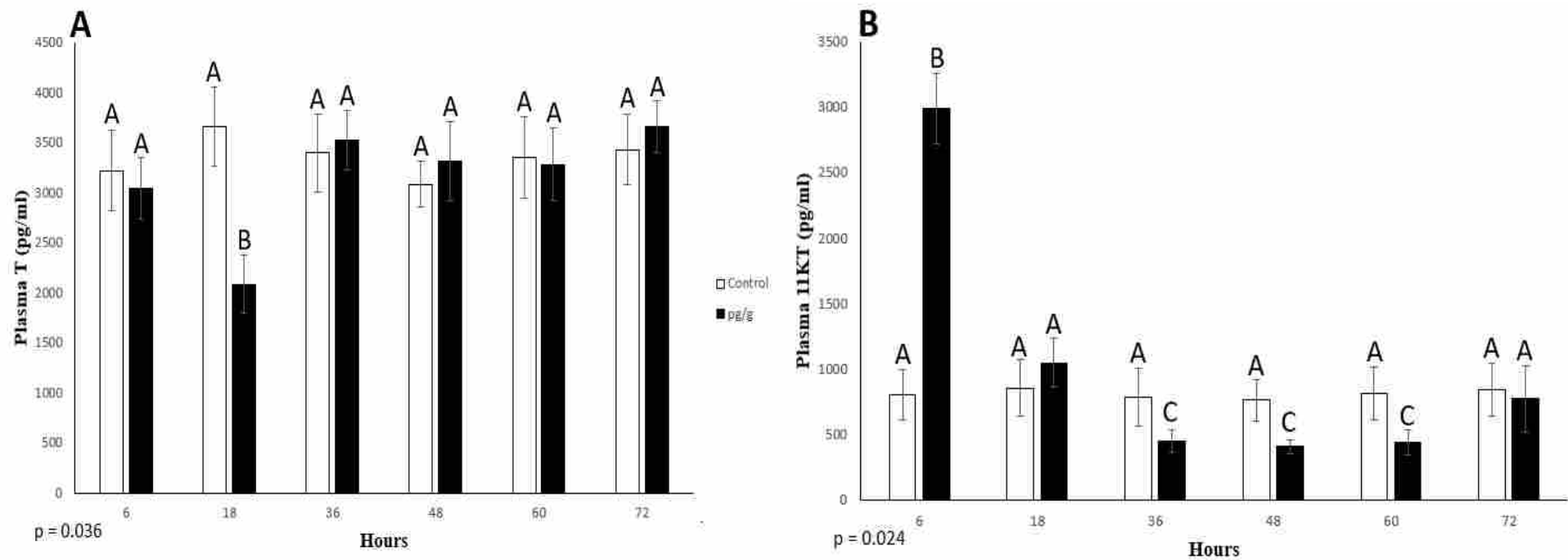


Figure 4.3. Mean ( $\pm$  1SEM) plasma testosterone (T; A) or 11-ketotestosterone (11KT; B) levels (pg/ml) in male mummichog (*Fundulus heteroclitus*) injected with saline only (control) or 1pg/g body weight 11KT and sampled 6 h, 18 h, 36 h, 48 h, 60 h or 72 h post-injection (n = 12 fish per timepoint, per treatment). Significant differences ( $p < 0.05$ ) as indicated by Tukey's post-hoc analysis represented by different letters.

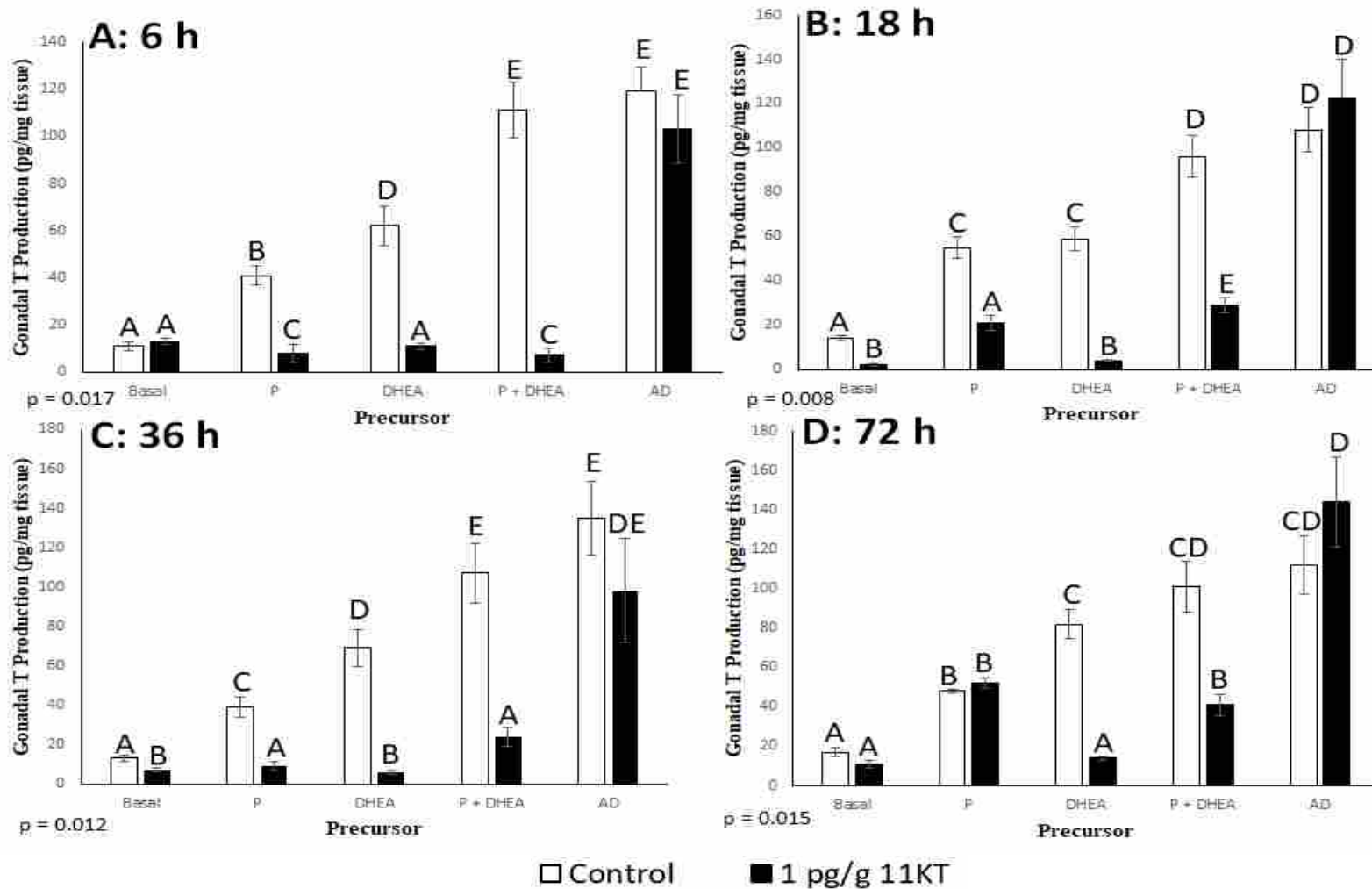


Figure 4.4. Mean ( $\pm$  1SEM) testis *in vitro* production (pg/mg tissue) of testosterone (T) in male mummichog (*Fundulus heteroclitus*) injected with control (saline only) or 1 pg/g 11KT and incubated at 18°C for 24 h without steroidogenic precursors (basal) or with 100 ng/ml progesterone (P), 100 ng/ml dehydroepiandrosterone (DHEA), both P and DHEA, or 100 ng/ml androstenedione (AD). Fish sampled 6 h (A), 18 h (B), 36 h (C) or 72 h (D) post-injection. Significant differences ( $p < 0.05$ ), as indicated by Tukey's post-hoc analysis, represented by different letters.

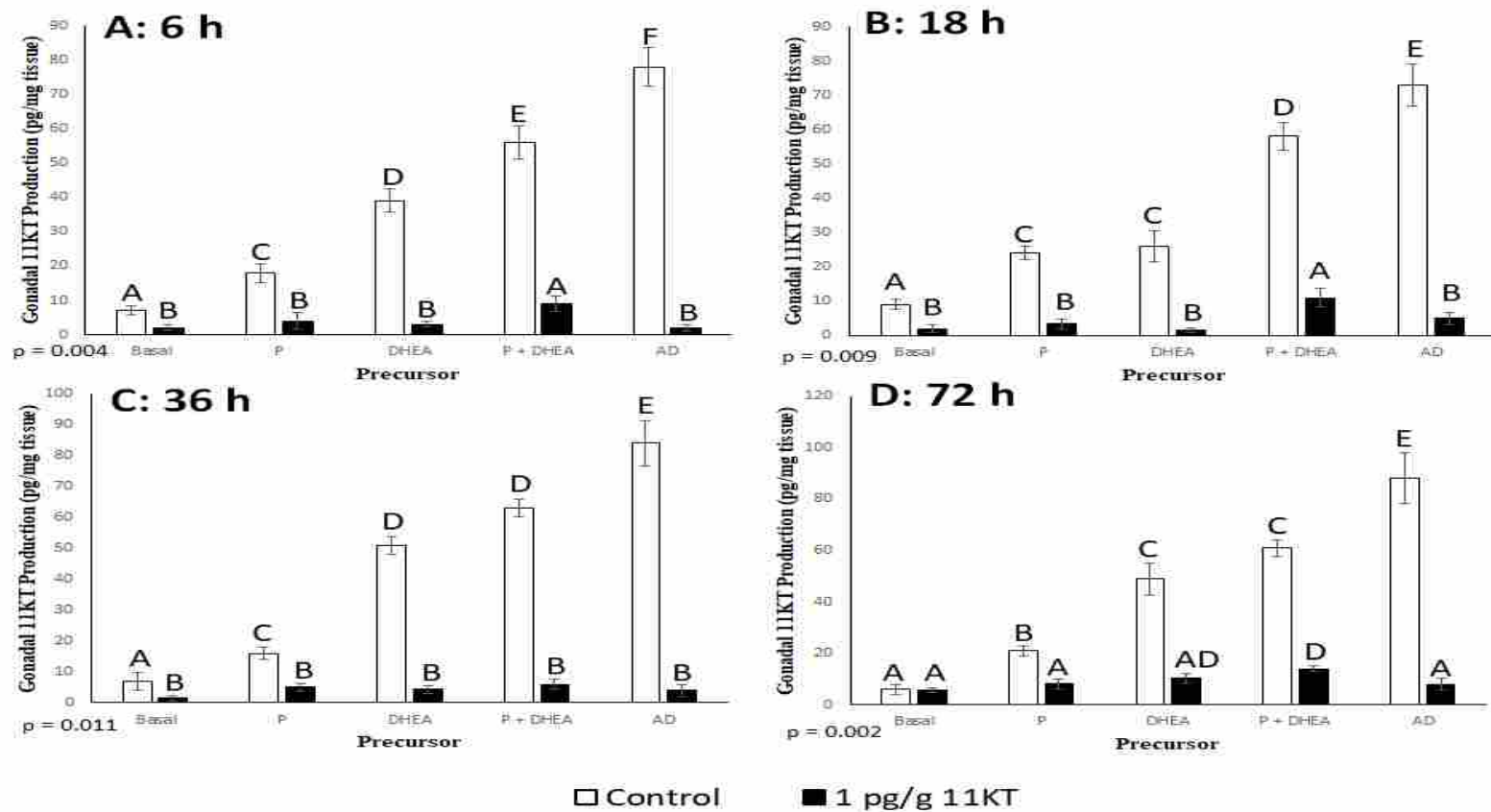


Figure 4.5. Mean ( $\pm$  1SEM) testis *in vitro* production (pg/mg tissue) of 11-ketotestosterone (11KT) in male mummichog (*Fundulus heteroclitus*) injected with control (saline only) or 1 pg/g 11KT and incubated at 18°C for 24 h without steroidogenic precursors (basal) or with 100 ng/ml progesterone (P), 100 ng/ml dehydroepiandrosterone (DHEA), both P and DHEA, or 100 ng/ml androstenedione (AD). Fish sampled 6 h (A), 18 h (B), 36 h (C) or 72 h (D) post-injection. Significant differences ( $p < 0.05$ ), as indicated by Tukey's post-hoc analysis, represented by different letters.

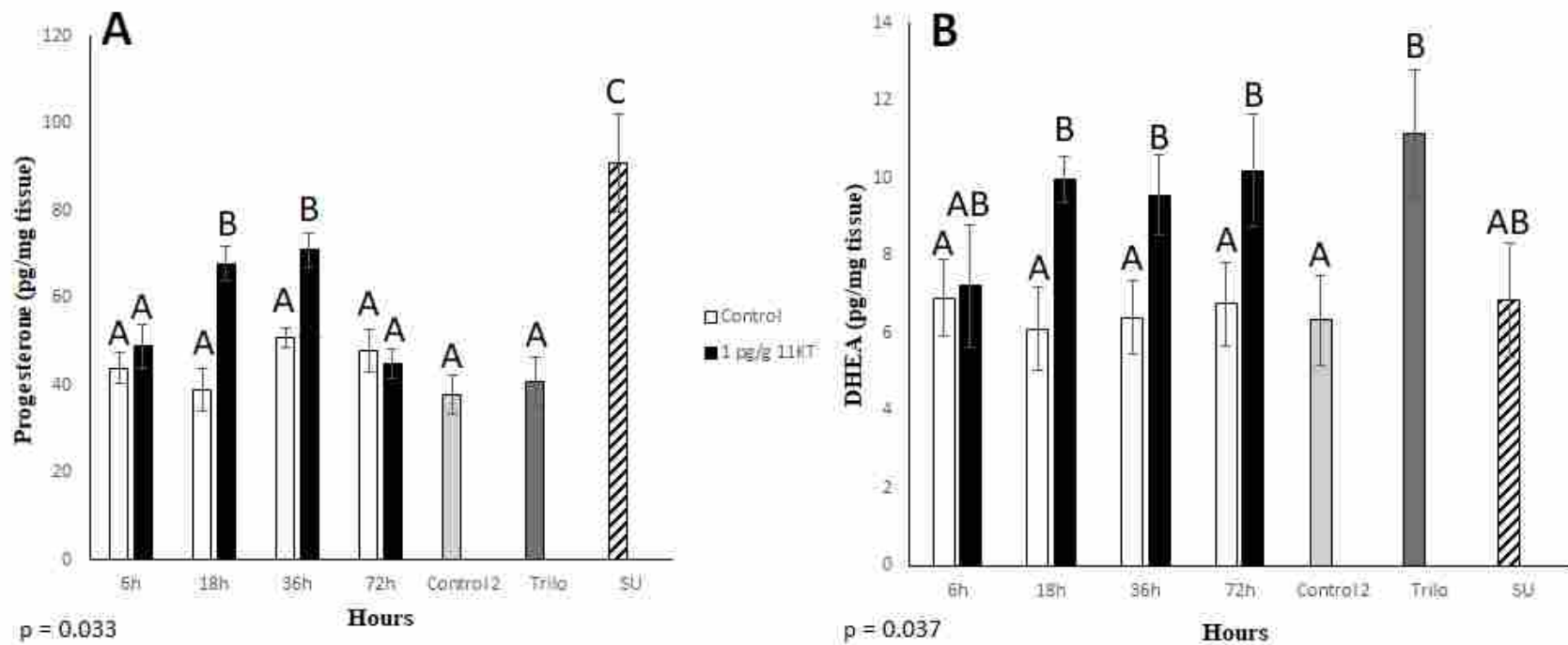
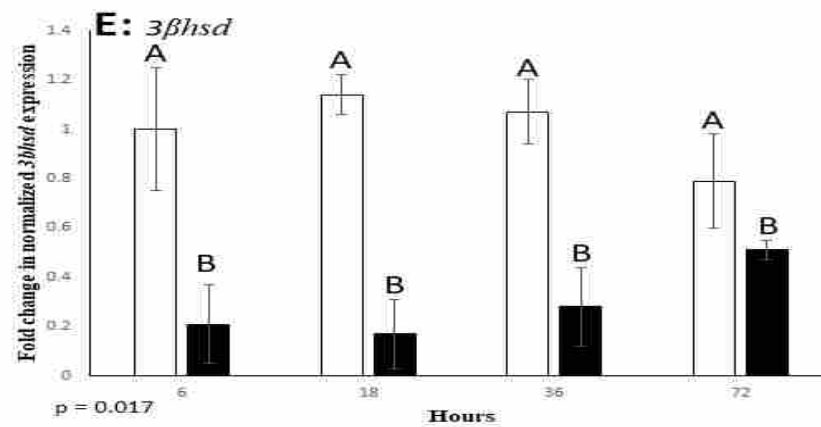
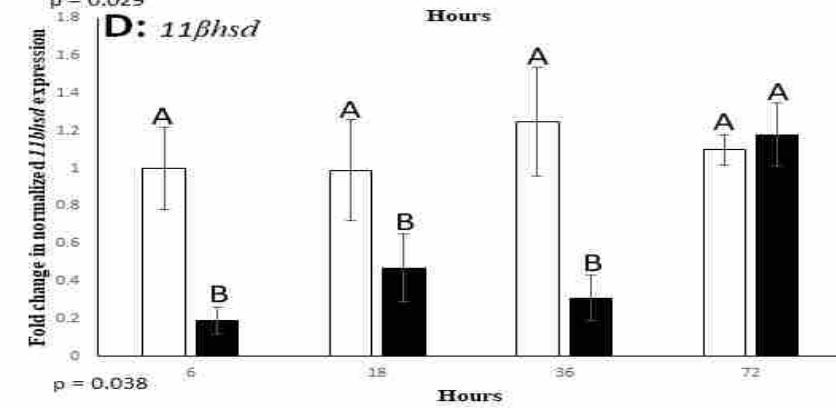
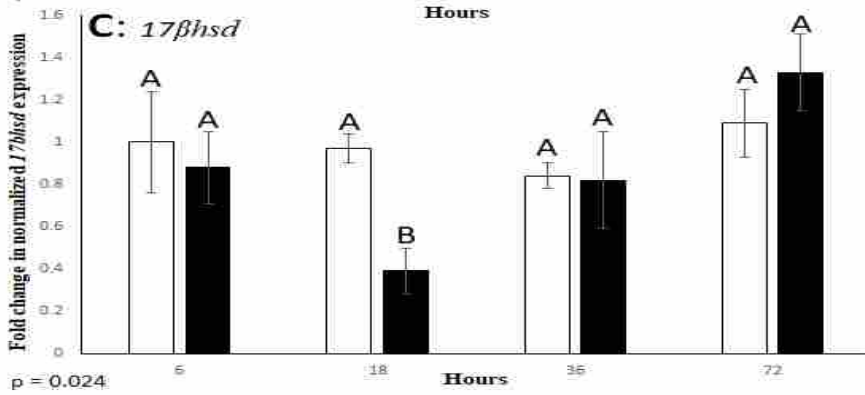
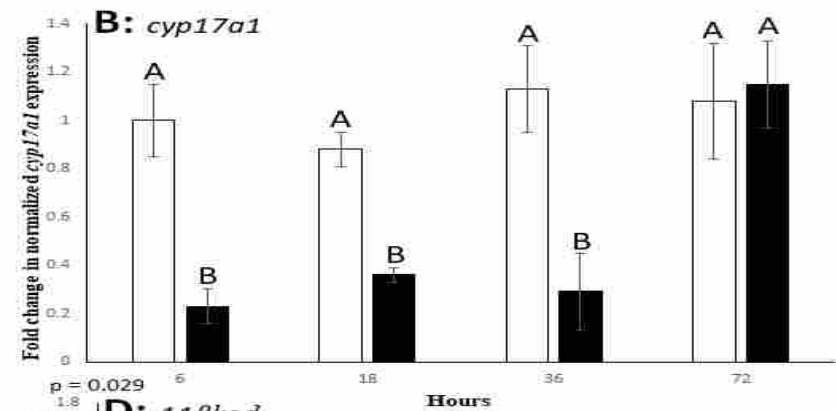
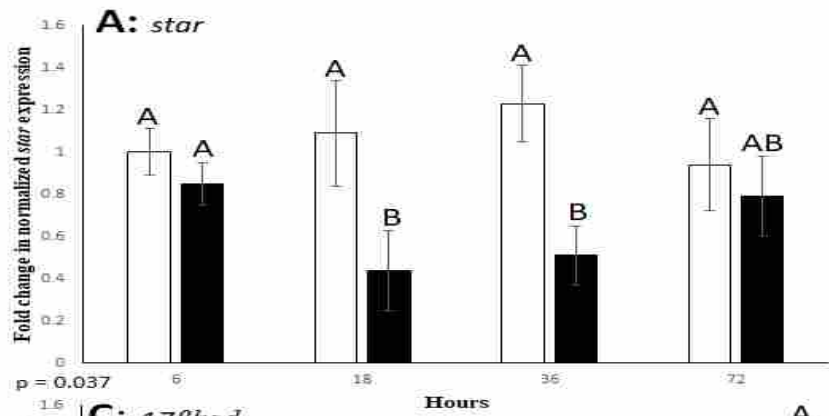


Figure 4.6. Mean ( $\pm$  1SEM) testis concentration of progesterone (A) or dehydroepiandrosterone (DHEA; B) in male mummichog (*Fundulus heteroclitus*) injected with control (saline only) or 1 pg/g 11-ketotestosterone (11KT) and sampled 6 h, 18 h, 36 h or 72 h post-injection. Testis were incubated in Medium 199 at 18°C for 24 h before frozen for hormone analysis. Control 2, trilostane (trilo) and SU-10603 (SU) treatments from study three, in which testis tissue was incubated with ethanol only (control), 10  $\mu$ M trilo or 200 nM SU in Medium 199 for 24 h at 18°C. Tissue was then frozen for hormone analysis. Significant differences ( $p < 0.05$ ), as indicated by Tukey's post-hoc analysis, represented by different letters.

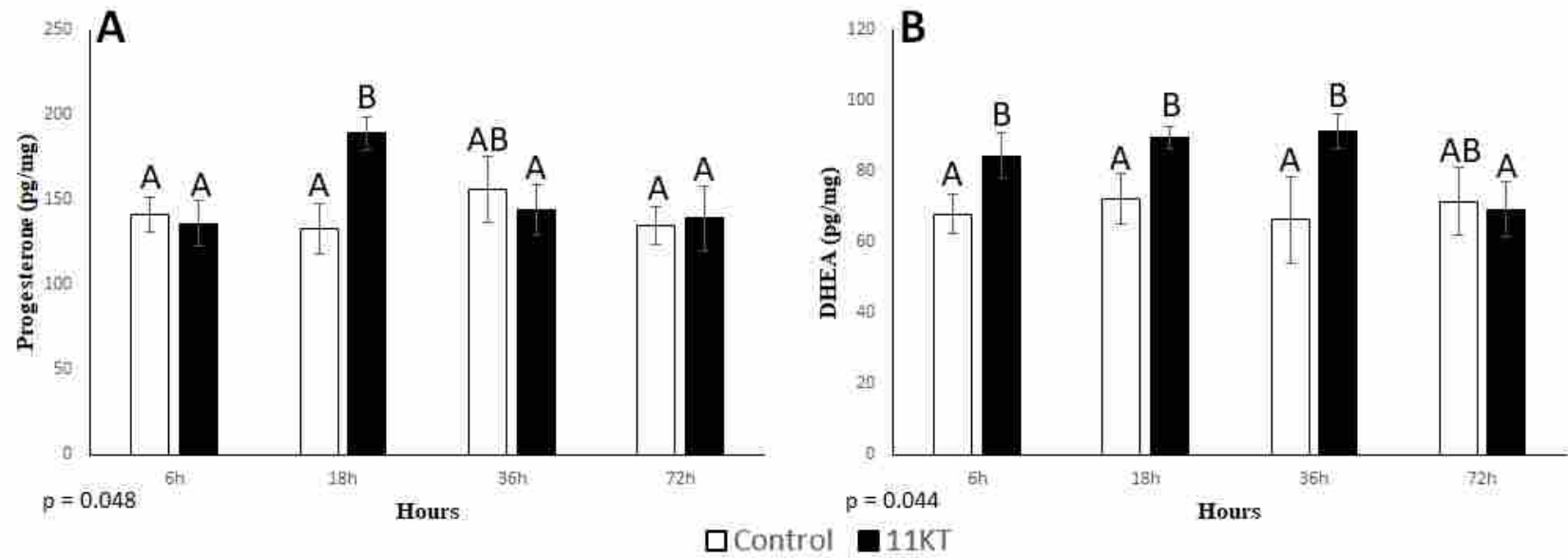


□ Control    ■ 1 pg/g 11KT

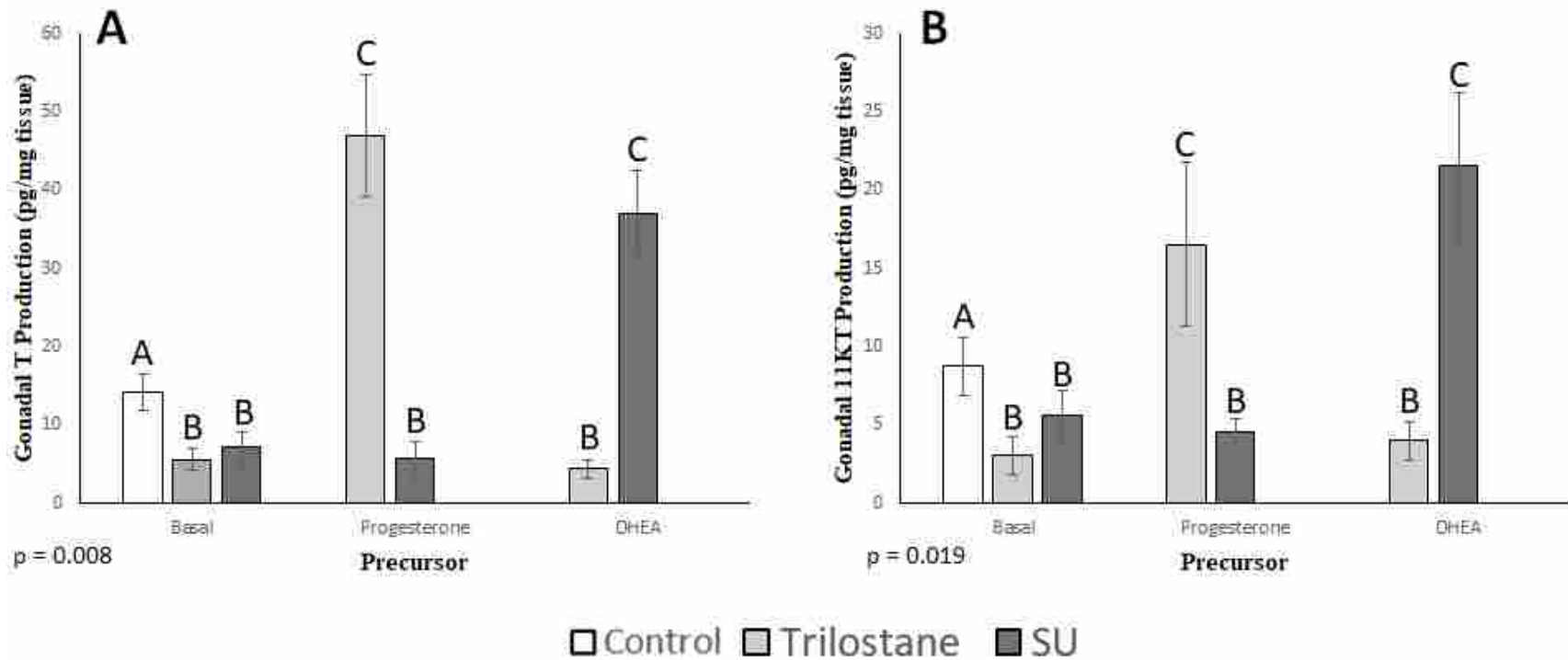
Figure 4.7. Testis expression ( $\pm$  1SEM) of steroidogenic acute regulatory protein (*star*; A), cytochrome P450 17A1 (*cyp17a1*; B), 17 $\beta$ -hydroxysteroid dehydrogenase (*17 $\beta$ hsd*; C), 11 $\beta$ -hydroxysteroid dehydrogenase (*11 $\beta$ hsd*; D) or 3 $\beta$ -hydroxysteroid dehydrogenase (*3 $\beta$ hsd*; E) in male mummichog (*Fundulus heteroclitus*) injected with control (saline only) or 1 pg/g 11-ketotestosterone (11KT) and sampled 6 h, 18 h, 36 h or 72 h post-injection (n = 12 per treatment, per timepoint). Expression levels normalized to  $\beta$ actin. Significant differences (p < 0.05), as indicated by Tukey's post-hoc analysis, represented by different letters.

## **Supplementary Material**





Supplementary Figure 4.1. Mean ( $\pm$  1SEM) testis concentration of progesterone (A) or dehydroepiandrosterone (DHEA; B) in male mummichog (*Fundulus heteroclitus*) injected with control (saline only) or 1 pg/g 11-ketotestosterone (11KT) and sampled 6 h, 18 h, 36 h or 72 h post-injection. Testis was then frozen for hormone analysis. Significant differences ( $p < 0.05$ ), as indicated by Tukey's post-hoc analysis, represented by different letters.



Supplementary Figure 4.2. Mean ( $\pm$  1SEM) testis *in vitro* production (pg/mg tissue) of testosterone (T; A) or 11-ketotestosterone (11KT; B) in male mummichog (*Fundulus heteroclitus*) testis tissue incubated with ethanol only (control), 10  $\mu$ M trilostane or 200 nM SU-10603 (SU). Significant differences ( $p < 0.05$ ), as indicated by Tukey's post-hoc analysis, represented by different letters.

## **Chapter Five:**

### **General discussion and conclusion**

Research on the impacts of androgenic endocrine disrupting compounds in fish is understudied compared to the estrogenic counterparts. Androgens are regularly introduced into the aquatic environment through numerous anthropogenic sources and their potency makes them capable of eliciting negative effects on the endocrine system in fish even at extremely low levels. While apical responses such as skewed sex ratio (Gonzalez et al., 2015) and reduced fertility (Tang et al., 2018) are established in androgen exposed fish, underlying molecular changes leading to these apical effects are not well understood. Plasma hormone depression, a typical teleost response to exogenous androgens, is thought to occur due to negative feedback along the hypothalamus-pituitary-gonadal (HPG) axis after androgen receptor agonism by exogenous androgens (Ankley et al., 2018; Weber et al., 2019). The specific points within the HPG axis that are consistently inhibited during exogenous androgen exposure, and could potentially account for depressed plasma hormone levels, are currently unknown.

## 1. Significance

This thesis adapted existing *in vitro* gonadal incubation protocols (McMaster et al., 1995; MacLatchy et al., 2003) to allow sampling of RNA following the 24-hour incubation period. It was the first to directly compare, from the same fish, steroidogenic gene expression in testes snap frozen at the time of sampling and following *in vitro* testis incubation. This comparison identified response discrepancies between the two methodologies, and indicates that gene expression analysis from *in vitro* tissue incubation is not representative of *in vivo* responses. Future work should not include gene expression analysis from *in vitro* tissue as a substitute for *in vivo* tissue gene responses.

This thesis is among the first to compare steroidogenic gene transcript levels to plasma hormone depression in male teleosts on an hourly scale. Most studies focus on chronic exposures which may not exemplify the acute changes caused during initial exogenous androgen exposure. The correlation between most steroidogenic genes and plasma hormone depression is substantiated in this thesis with two different model androgens, and can serve as a guide for future studies targeting specific genes and enzymes within the steroidogenic pathway.

The data in this thesis provide evidence that negative inhibition resulting in depressed plasma sex hormones is occurring within the gonadal steroidogenic pathway of male mummichog. Identification of *3 $\beta$ hsd* and *cyp17a1* as the two genes most responsive to exogenous androgen perturbation helps to strengthen understanding of molecular changes underpinning alterations at higher levels of biological organization. Combined with the evidence that neither dehydroepiandrosterone nor progesterone cause a recovery in gonadal sex hormone production, while androstenedione does, the results of this thesis are integral to the identification of specific points of the HPG axis inhibited during exogenous androgen exposure that may account for observed decreases in plasma sex hormones.

This thesis is the first to demonstrate changes in steroidogenic gene transcript levels coinciding with decreases in enzymatic activity in male teleosts during injection with 11KT. The linkage between depression of both the genes and the enzymes that they are coding for is a major step towards understanding key relationships and biological plausibility of changes in genes predicting changes in other endpoints, such as sex hormone synthesis. While some genes, such as *cyp19a1*, have been well studied in teleosts and the relationship between transcript levels, enzymatic activity and estradiol synthesis robustly outlined (Thomas, 2012; Bohne et al., 2013),

the current thesis is the first to begin to characterize other steroidogenic gene/enzyme relationships by focusing on *3 $\beta$ hsd*, *cyp17a1* and *11 $\beta$ hsd*.

The enzyme 11 $\beta$ -hydroxysteroid dehydrogenase, coded by the gene *11 $\beta$ hsd*, converts testosterone to 11-ketotestosterone in the Leydig cells of teleost testis (Lokman et al., 2003). Data from this thesis have identified this gene and enzyme as being sensitive to increased 11KT, which has not been reported in teleosts before. Transcript levels of *11 $\beta$ hsd* are depressed by high testosterone levels (Gonzalez et al., 2015; Garcia-Garcia et al., 2017) in teleosts, and this gene has been suggested to be sensitive to androgen perturbation. This thesis supports this hypothesis and future work can determine if changes in *11 $\beta$ hsd* are biomarkers of androgen exposure.

Investigations on teleost reproductive biology is currently aimed at identifying endocrine active compounds via high-throughput testing regimes (Ankley et al., 2016), understanding the molecular events that occur during exposure which lead to adverse effects on apical endpoints such as fecundity (Villeneuve et al., 2014) or mating behaviour (Davis et al., 2017), and developing potential biomarkers of exposure that can serve as predictive tools for reproductive toxicology (Zhou et al., 2015). The data in this thesis help identify molecular changes in the steroidogenic pathway during exposure to exogenous androgens in male teleosts, and can aid future research by providing evidence of more targeted changes to specific genes instead of a general depression of the entire steroidogenic pathway. The quantified endpoints in this thesis incorporated multiple levels of biological organization, quantifying changes in gene expression (molecular), steroid production (biochemical) and plasma steroids (organismal), to produce strong evidence that changes to *cyp17a1*, *3 $\beta$ hsd* and *11 $\beta$ hsd* are indicative of changes to enzymatic activity and testis sex hormone synthesis capacity. Additionally, the use of *3 $\beta$ hsd* as a potential biomarker of androgen exposure is supported by the findings of this thesis. Through

understanding molecular changes and linking them with changes in enzyme activity and sex hormone synthesis, this thesis aids in the establishing of biological plausibility of using gene transcripts as predictive tools for future reproductive toxicology studies.

## 2. Future Work

The next step in establishing causal linkages between changes to steroidogenic genes and impacts to plasma sex hormone levels is quantifying enzyme reaction rates during exogenous androgen exposure. There is currently limited information available on normal enzymatic conversions within teleost steroidogenesis (Tokarz et al., 2015), so preliminary work to establish baseline functions and rates will be needed before research focuses on periods of exogenous androgen exposure. Through a similar testing regime as chapters 3 and 4 of the current thesis, enzyme reaction rates could be tested prior to, during, and after plasma hormone depression and correlated to gene expression levels. This would strengthen the evidence that changes in gene transcript levels are associated with similar changes to the efficiencies of the enzymes they code for.

The estrogenic effects that aromatizable androgens can elicit must be tested with respect to steroidogenic gene expression. Expanding model androgens to include testing of aromatizable androgens is necessary to establish if these androgens elicit similar responses as non-aromatizable androgens. As aromatizable androgens can be metabolized to estrogenic compounds, they may not elicit the same degree of androgen receptor agonism, or can serve as estrogen receptor ligands, and therefore might generate a different gene response pattern that was not quantified in the current thesis. A comparative experiment using both aromatizable and non-aromatizable androgens and quantifying the suite of steroidogenic genes may show whether

estrogenic metabolites of the aromatizable androgens elicit different responses in genes compared to strictly androgenic compounds. This information is needed for comprehensive understanding of androgenic mechanisms of action on the steroidogenic pathway in teleosts.

The current thesis used model androgens 11KT and DHT in injection experiments to quantify steroidogenic gene changes and link them to changes in sex hormone synthesis within the testes of male mummichog. These two model androgens are found in some anthropogenic effluents, although typically in low concentrations (as reviewed by Liu et al., 2009). Future work should test additional androgens that can be found in higher concentrations in effluent receiving waters, such as 17 $\beta$ -trenbolone (Ankley et al., 2018), androstenedione (Hernandez et al., 2013), epiandrosterone (Zhang et al., 2018) or androsterone (Williams et al., 2019). While androgen receptor agonism is the hypothesized mechanism of action for each of these androgens and the two tested in the current thesis, establishing the impacts of a wider range of model androgens will increase the knowledge base of androgenic effects on steroidogenic genes and strengthen extrapolation to field-based exposures.

The linkage between androgen receptor agonism and depressed testis synthesis of sex hormones is not well understood. To further investigate this relationship, studies can utilize androgen receptor inhibitors such as cyproterone acetate, in tangent with exposure to model androgens, to determine if gene responses are altered when androgen receptors are incapable of acting as transcription factors. Cyproterone acetate alters paternal care behaviour, such as nest defense, in male smallmouth bass (*Micropterus dolomieu*) even when exposed to high levels of 11KT (Dey et al., 2010), possibly due to lack of androgen receptor agonism. Despite the evidence that inhibition of androgen receptor leads to changes in reproductive behaviour, the effect of androgen receptor inhibition on steroidogenic gene responses is not well reported in the



literature. An earlier study in the MacLatchy lab has shown steroidogenesis to be depressed by cyproterone acetate exposure in mummichog (Sharpe et al., 2004); using the current thesis as a foundation, additional studies can be undertaken to identify whether effects on the same or different genes coincide with these decreases in steroid hormone production.

In the current thesis, gene responses were elicited at much lower concentrations by 11KT (1 pg/g body weight; Chapter 4) than DHT (1 ng/g body weight; Chapter 3). Investigation into why these two androgens require different concentrations to elicit similar gene effects is necessary to fully understand if androgen receptor activation is the main factor in eliciting gene responses. In the mummichog, androgen receptor affinities for 11KT and DHT have not been quantified. Androgenic affinities are conserved across many teleost species, with 11KT having the highest androgen affinity, followed by DHT, then testosterone (Olsson et al., 2005; Bain et al., 2015). Whether this difference in affinity is the reason for the observed disparity between bioactive concentrations of 11KT and DHT in the current thesis should be investigated.

The methods developed in this thesis can be expanded to test other classes of endocrine disrupting compounds, such as estrogens or antiandrogens. Utilizing the same *in vitro* protocol, the steroidogenic genes tested in this thesis would be intact and quantifiable after exposure to other compounds. This would allow comparison of gene response patterns, and potentially identify differential responses elicited on steroidogenic genes from different chemical classes. Once response patterns are established, the same testing regime could be used for complex effluents, with the ability to quantify changes in steroidogenic gene transcript levels after exposure and identify if a complex effluent contains androgenic, estrogenic or other bioactive compounds based on gene responses.

For the past three decades, gonadal sex hormone synthesis has been quantified *ex vivo* through validated *in vitro* protocols (McMaster et al., 1995). Through the addition of select precursors, the standardization of tissue weights and hormone production calculations, these assays are valuable in identifying if hormone synthesis is impacted during exposure to endocrine active compounds. While the hormone production information is a valuable diagnostic tool for researchers, there are some drawbacks to the *in vitro* testing system which may make it inappropriate as a testing regime for changes in steroidogenic gene transcripts. Traditional *in vitro* testing uses the gonad only for determination of hormone production, removing the ability for cross-talk and feedback between the gonad and various other endocrine systems such as the hypothalamus, pituitary, liver or head kidney (Wong et al., 2006; Johnstone et al., 2014). The removal of these systems inhibits the delivery of gonadotropins to the gonad, which is normally the cue for steroidogenesis to proceed (Mukjerhee et al., 2017), and removes any extra-gonadal feedback loops which would be present during *in vivo* exposure. Extrapolating *in vitro* responses to *in vivo* activity returns inconsistent accuracy (Johnstone et al., 2016), as evidenced by the gene expression responses seen in chapters two and three of this thesis. Co-culturing of multiple tissue types, including brain, liver and gonad, during *in vitro* incubation has elicited responses more exemplary of *in vivo* changes (Ekman et al., 2011; Johnstone et al., 2016), indicating if the goal of *in vitro* exposure is for anything other than hormone production, a co-culture system should be utilized.

The disconnect between gene responses from incubated tissue and from snap frozen tissue may be an artifact of testis incubation. Data from this thesis indicate that while gene transcripts are increased in incubated testis, this does not correspond to increases in enzymatic activity (chapter 4) or sex hormone synthesis (chapters 3 and 4). While no studies are currently

known to investigate post-transcriptional gene modifications from gonadal *in vitro* samples in teleosts, there is evidence that stress induced by tissue excision can interfere with RNA capping (Kiledjian, 2019), mRNA binding to ribosomes, and subsequent protein synthesis (Popis et al., 2016) and tRNA binding to mRNA codons (Orioli et al., 2016) in mammalian brain and hepatocytes. Further investigation into factors uncoupling mRNA expression from protein synthesis are needed in teleosts, but could include any of the previously mentioned responses of tissues to excision.

Comparative studies between mummichog and other model teleosts are needed to confirm that steroidogenic responses seen in this thesis are not species-specific. Mummichog are a model estuarine species, and though they are sensitive to androgenic endocrine disrupting chemicals, they may not be representative of freshwater species. Mummichog appear to be less sensitive to estrogenic endocrine disrupting compounds (Rutherford et al., 2020), but this comparison between mummichog and other species has not been explored for androgenic compounds.

The exposure period from the current thesis should be increased to determine if changes observed within the first few hours of injection are indicative of long-term changes in exposed organisms. Typical reproductive toxicology bioassays are conducted on a scale of 7 + days, during which time the initial responses to exposure may be ameliorated as the organism undergoes homeostatic compensatory mechanisms to offset extended exposure (Leet et al., 2015; Ali et al., 2017). Compensatory mechanisms elicited during longer exposures may alter, or mask, the initial depression of steroidogenic genes correlating to plasma hormone depression observed in this thesis. These potential responses need to be characterized to fully understand the shifts that occur in steroidogenic genes during long-term exposure. Subsampling of treatments over the

course of 7-14 days, beginning with frequent samplings at exposure start and then a gradual spreading of sampling timepoints until cessation, can help classify whether the changes found in steroidogenic genes in this study are representative of changes after longer exposure or if these responses are just the initial reaction of a fish to exogenous androgens which is then altered over the course of the exposure. It is important to note that time frames for exposure and sampling need to be aligned with the particular endocrine system being investigated; stress responses in fish, e.g., can be stimulated in minutes with effects to days, so even the time frame of sampling within hours may be too long (Winberg et al., 2016). As well, when cross-talk between various endocrine systems occurs it can have a significant bearing on when sampling is most appropriate (Gorissen et al., 2016; Sanchez-Vazquez et al., 2019).

### 3. Conclusion

Overall, data from this thesis provides insight into gene expression changes as underlying mechanisms of inhibition of sex hormone synthesis in testis tissue. The results show that depression of most steroidogenic gene transcripts coincide with depression of sex hormone synthesis in the testis and plasma sex hormone levels. The data also begin to establish linkages between depressed transcripts of *3 $\beta$ hsd* and *cyp17a1* and changes in the enzymatic activities of their respective proteins, and suggests that these gene expression alterations can be predictive of altered androgen synthesis in testis tissue. Future work should strengthen the understanding between androgen receptor agonism and depression of plasma sex hormones through targeted investigations utilizing androgen receptor agonists and antagonists in addition to exogenous androgens.

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