Thyroid hormones and androgens differentially regulate gene expression in testes and ovaries of sexually mature *Silurana tropicalis*

Campbell, D.E.K.[1], and Langlois, V.S.[1,2,3*]

[1] Biology Department, Queen's University, Kingston, ON, CA
[2] Institut national de la recherche scientifique - Centre Eau Terre Environnement (INRS-ETE), Quebec City, QC, CA
[3] Department of Chemistry and Chemical Engineering, Royal Military College of Canada, Kingston, ON, CA

***Author for correspondence and reprint requests:**

Dr. Valerie S. Langlois
Associate Professor
Institut national de la recherche scientifique - Centre Eau Terre Environnement (INRS-ETE)
490, de la Couronne, Quebec City (Quebec) G1K 9A9, CANADA
valerie.langlois@inrs.ca
Tel: 418-654-2547
Fax: 418 654-2600

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Abstract

A series of *ex vivo* exposures using testicular and ovarian tissues of sexually mature Western clawed frogs (*Silurana tropicalis*) were designed to examine molecular mechanisms of thyroid hormone (TH) and androgen crosstalk sans hypophyseal feedback as well as investigate potential sex-specific differences. Tissues were exposed *ex vivo* to either triiodothyronine (T3), iopanoic acid (IOP), one co-treatment of IOP + 5α-dihydrotestosterone (5α-DHT), 5α-DHT, 5β-dihydrotestosterone (5β-DHT), or testosterone (T). Direct exposure to different androgens led to androgen specific increases in thyroid receptor and deiodinase transcripts in testes (*trβ* and *dio1*) but a decrease in expression in ovaries (*trβ* and *dio3*), suggesting that male and female frogs can be differently affected by androgenic compounds. Moreover, exposure to select androgens differentially increased estrogen-related transcription (estrogen receptor alpha (*erα*) and aromatase (*cyp19*)) and production (estradiol) in ovaries and testes indicating the activation of alternate metabolic pathways yielding estrogenic metabolites. Sex-steroid-related transcription (steroid 5α-reductase type 2 (*srd5α2*) and *erα*) and production (5α-DHT) were also differentially regulated by THs. The presence and frequency of transcription factor binding sites in the putative promoter regions of TH- and sex steroid-related genes were also examined in *S. tropicalis*, rodent, and fish models using *in silico* analysis. In summary, this study provides an improved mechanistic understanding of TH- and androgen-mediated actions and reveals differential transcriptional effects as a function of sex in frogs.

1. Introduction

The actions of thyroid hormones (THs) are highly diverse and impact nearly every biological endocrine system (Cortés et al., 2014; Duarte-Guterman et al., 2014; Mullur et al., 2014; Cooke et al., 2004). The challenge remains to characterize and predict the interactions among THs and the major endocrine axes. THs have been shown to cross-regulate with the hypothalamus–pituitary–gonadal axis (HPG) targeting gonadotropin synthesis, steroidogenesis, and gonadal cellular differentiation in vertebrates (reviewed in: Cortés et al., 2014; Duarte-Guterman et al., 2014; Flood et al., 2013; Habibi et al., 2012; Wajner et al., 2009; Wagner et al., 2008; Cooke et al., 2004; Maran, 2003). A large body of literature exists on the molecular mechanisms underlying TH-mediated reproductive effects in gonadal tissue (Duarte-Guterman et al., 2014; Flood et al., 2013; Habibi et al., 2012; Wagner et al., 2008; Cooke et al., 2004; Maran, 2003), however relatively little is known with regard to sex specific effects. Transcripts of thyroid hormone-related machinery have been detected in testicular and ovarian tissues of numerous species (Mammals: Carosa et al., 2017; *Physalaemus pustulosus*: Duarte-Guterman et al., 2012; *Silurana tropicalis*: Duarte-Guterman and Trudeau, 2011; *Scarus iseri*: Johnson and Lema, 2011; *Oncorhynchus mykiss*: Sambroni et al., 2001; *Podarcis sicula*: Cardone et al., 2000). TH-related transcripts have moreover been shown to develop sexually-dimorphic patterns with higher mRNA levels of TH receptors (*trs*: *trα* and *trβ*) and deiodinases (*dios*: *dio1, dio2,* and *dio3*) reported in testes than in ovaries of frog and fish species (*S. tropicalis*: Duarte-Guterman and Trudeau, 2011; *S. iseri*: Johnson and Lema, 2011). Sex specific transcriptional mechanisms in reproductive tissues may not be evident until after the completion of sexual development in anamniotes once the animal has reached sexual maturity. The main goal of this study was to examine the differential effects of THs as a function of gender in amphibians and to elucidate possible sex specific mechanisms of crosstalk in juvenile *S. tropicalis*.

Understanding mechanisms of sex steroid-regulation of the TH axis is highly relevant to amphibians due to the dependence of metamorphosis on THs. Estrogenic compounds (e.g., estradiol (E_2) and 17α-ethinylestradiol (EE_2)) have been shown to repress TH function and impede growth in aquatic species (*X. laevis*: Sharma and Patiño, 2010; *Rana pipiens:* Hogan et al., 2008; *R. pipiens* and *Rana*

sylvatica: Hogan et al., 2006; Teleost fish: reviewed in Orozco and Valverde-R, 2005; Brown et al., 2004). In contrast, androgens appear to stimulate the TH system in vertebrates. Exposure to testosterone (T) and 17α-methyltestosterone has shown to elevate circulating TH levels and peripheral TH metabolism in fish (reviewed in: Orozco and Valverde-R, 2005; Brown et al., 2004; Cyr and Eales, 1996; *Salvelinus alpinus*: MacLatchy and Eales, 1988; *Salmo gairdneri, Richardson*: Hunt and Eales, 1979). More recently, exposure to anti-androgenic compounds have been shown to alter TH-related transcription and activity in developing *S. tropicalis* tadpoles (Langlois et al., 2011; Langlois et al., 2010b; Duarte-Guterman et al., 2009), which substantiates the potential for this crosstalk in amphibians. Androgen response elements (AREs) have also been identified in the promoter regions of *trs* and *dios* in model fish and tetrapod species (*Mus musculus*, *S. tropicalis,* and *Oryzias latipes*: Flood et al., 2013). Therefore, we can hypothesize for direct androgenic regulation of TH-related transcription.

Isolating direct TH- or androgen-mediated crosstalk *in vivo* is difficult as this assay encompasses all pathways of regulation, including compensatory feedback mechanisms by higher regulatory centres, such as the hypothalamus–pituitary axis. An *ex vivo* assay ascertains direct and independent molecular responses by eliminating factors, such as hormonal feedback loops and biotransformation of the chemical by other organs (e.g., liver; Scholz et al., 2013). To characterize molecular mechanisms of TH- and androgen-action as a function of sex, testicular and ovarian tissues of juvenile *S. tropicalis* were exposed *ex vivo* to either triiodothyronine (T3), iopanoic acid (IOP), one co-treatment of IOP + 5α-dihydrotestosterone (5α-DHT), 5α-DHT, 5β-dihydrotestosterones (5β-DHT), or T for 6 h. Gonadal TH- and sex steroid-related transcript levels and sex-steroid media levels were examined in testis and ovary tissue to elucidate molecular mechanisms of crosstalk with regard to the function of sex. We also conducted a novel *in silico* promoter analysis to examine the presence and frequency of putative thyroid-, androgen- and estrogen-response elements (TREs, AREs, and EREs, respectively) in *S. tropicalis* TH- and sex steroid-related genes and made species comparisons with rodent and fish models.

2. Material and methods

2.1 Animals and exposure

Juvenile male and female *S. tropicalis* frogs were raised and housed in the Queen's University Animal Care Facility (Kingston, ON, Canada). Animals were kept in dechlorinated and aerated water (25 ± 1°C) on a 12:12 h light:dark regime (light commencing at 0700 h). All aspects of animal care were performed in accordance with the guidelines of the Queen's University's Animal Care Committee and the Canadian Council on Animal Care.

Two *ex vivo* assays were performed following methods of Bissegger et al. (2014). In the first *ex vivo* assay, we examined whether TH status affects sex steroid-related transcription and hormone production in testes and ovaries of sexually mature juvenile *S. tropicalis*. Juvenile frogs were anaesthetized by immersion in 2% of 3-aminobenzoic acid ethyl ester (MS-222; Sigma Canada Ltd., Oakville, ON, Canada) and euthanized by decapitation. Four males were used per treatment. Each testis was evaluated independently resulting in a total of eight whole testes per treatment. Four females were used per treatment and two pieces of ovary tissue – each piece weighing between 5 to 25 mg – were removed per frog. Each piece was evaluated independently resulting in a total of eight ovary pieces per treatment. Once dissected – tissues were weighed and placed in separate 1.5 mL centrifuge tubes filled with 500 µL of ice-cold Lebovitz (L-15 media, Sigma, Oakville, ON, Canada) containing 10 mM HEPES, 50 µg/mL gentamicin (Fisher Scientific, Ottawa, ON, Canada) and 2% synthetic serum replacement (Sigma, Oakville, ON, Canada) at pH 7.4. Tissues were kept on ice until the exposure commenced. Previous time dependent experiments (2–10 h) performed by Bissegger et al., (2014) showed that RNA degradation was not evident with the time elapsed between dissection of tissues and treatment incubations, and an incubation times of 6 h or less. The individual eight whole-testes or ovary-pieces were then placed in eight separate designated wells in 24-well plates containing either 500 µL L-15 media (control samples) or L-15 media containing T3 (50 nM; Sigma, Oakville, Ontario, CA), IOP (10 µM; TCI America), or one co-treatment of IOP (10 µM, TCI America) + 5α-DHT (1 µM; Steraloids, Newport, RI, USA). The individual treatments occupied a total of eight wells or two columns with the whole testes

assay spanning two 24-well plates (32 wells total) and ovary pieces assay spanning two 24-well plates (32 wells total). T3 is a potent TH and the concentration was chosen based on *in vivo* studies conducted with *S. tropicalis* (Campbell and Langlois, 2017; Duarte-Guterman and Trudeau, 2011; Duarte-Guterman et al., 2010). IOP is a TH antagonist that inhibits local deiodinase (*dio*) function. *Dios* are enzymes responsible for the activation and deactivation of THs within individual tissues. IOP is non-specific impeding all *dio* function, as a result the chemical locally induces both hypo- and hyperthyroid conditions: (i) leading to the accumulation of THs and (ii) preventing further local synthesis of active hormones. The IOP concentration was chosen based on *in vivo* studies conducted with *S. tropicalis* and *X. laevis* (Campbell and Langlois, 2017; Fini et al., 2007). The 24-well plates were incubated for 6 h at 26°C using an orbital shaker. After 6 h, the tissues and media were collected and flash frozen on dry ice. Samples were stored at −80°C for subsequent gene expression and sex steroid hormone analyses.

In the second *ex vivo* assay, we investigated androgen-mediated regulation of sex steroid- and TH-related transcription in isolated testis and ovary tissue of juvenile *S. tropicalis*. Animals were euthanized and tissues were collected the same way as described above. The individual eight whole-testes or ovary-pieces were transferred from the 1.5 mL centrifuge tubes filled with 500 µL of cold Lebovitz into eight separate designated wells in 24-well plates containing either 500 µL of L-15 media (control samples) or L-15 media containing 1 µM of T, 5α-DHT, or 5β-DHT (Steraloids, Newport, RI, USA). The individual treatments occupied a total of eight wells or two columns with the whole testes assay spanning two 24-well plates (32 wells total) and ovary pieces assay spanning two 24-well plates (32 wells total). These concentrations were chosen based on an *ex vivo* study conducted with *S. tropicalis* (Bissegger and Langlois, 2016). The 24-well plates were incubated for 6 h at 26°C using an orbital shaker. After 6 h, the organs were collected and flash frozen on dry ice. Samples were stored at −80°C for subsequent gene expression analysis.

2.2 Sex steroid analysis

Media concentrations of E_2, T, and 5α-DHT were measured using commercially available enzyme-linked immunosorbent assays (ELISAs; E_2 and T: Cayman Chemical, Cedarlane, Burlington, ON, Canada; 5α-DHT: IBL America, Cedarlane, Burlington, ON, Canada). Media samples were thawed on ice and diluted two-fold in the immunoassay buffer. All media samples were run in duplicate. The immunoassay protocols were then followed as described by the manufacturer. The absorbance of samples were measured using an Infinite® M1000 PRO plate reader (Tecan, Montreal, QC, Canada) at 405 nm for E_2 and T, and 450 nm for 5α-DHT. The limit of detection according to the manufacturer was 15 pg/mL for E_2, and 6 pg/mL for both T and 5α-DHT. Note that the T and 5α-DHT levels could not be accurately quantified in the co-treatment IOP + 5α-DHT because the antiserums to both T and 5α-DHT were reported to cross-react with 5α-DHT by 27.4% and 100%, respectively.

2.3 *In silico* promoter analysis

To further characterize potential mechanisms of molecular crosstalk, we examined the presence and frequency of TREs, AREs, and EREs in the putative promotor regions of *S. tropicalis* sex steroid-related genes (*erα* and *cyp19*). The presence and frequency of EREs were examined in TH-related genes (*trβ*) and androgen-related genes (*ar* and *srd5α2*). For information on *tr* and *ar* half-site motifs in these genes please refer to Flood et al. (2013). All sequences used for analysis were collected from the Ensembl Project (http://www.ensembl.org). Weighted matrices of *tr*-, *ar*-, and *er*-binding sites were obtained using the PROMO matrices search engine (v.3.0.2; Farré et al., 2003) in conjunction with the TRANSFAC matrices database (v.7.0). We then used the FIMO software (v.4.11.1; Grant et al., 2011) to scan for the *tr*-, *ar*-, and *er*-motifs within the putative promoter region (−2000 to +1) of our target genes applying a *p*-value output threshold of 0.001. The matched *tr*, *ar*, and *er* motif sequences were searched against the core recognition motif sequence with the criterion of allowing no mismatches as a final validation step. The frequency on single half-sites (TRE: 5'-TGACCT-3', 5'-TGTCCT-3'; ARE: 5'-TGTTCT-3'; ERE: 5'-TGACC-3'), direct repeats, and palindrome sequences were evaluated.

2.4 Gene expression analysis

Total RNA from ovary pieces and whole testes was isolated using TRIzol (Life Technologies, Burlington, ON, CA) following in accordance with the manufacturer's protocol and was purified using the TURBO DNA-free™ Kit (Ambion; ThermoFisher Scientific, Ottawa, ON). The quantity of RNA was determined using a NanoDrop-2000 spectrophotometer (Thermofisher, Ottawa, ON, Canada). First strand cDNA was synthesized following the GoScript Reverse Transcription kit protocol with random primers (Promega, Madison, WI, USA) in a Mastercycler Pro S Thermocycler (Thermo Fisher, Ottawa, ON, Canada). The cDNA products were diluted 80-fold prior to qPCR amplification.

Primer sequences for androgen receptor (*ar*), aromatase (*cyp19*), estrogen receptor (*erα*), deiodinases (*dio1*, *dio2*, and *dio3*), 5α-reductases (*srd5α1*, *srd5α2*, *srd5α3*), TH receptors (*trα* and *trβ*), and the reference genes ornithine decarboxylase (*odc*) and ribosomal protein L8 (*rpl8*) were previously designed and validated by Langlois et al. (2010b). Real-time PCR primers for *dax-1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) were previously designed and validated by Campbell and Langlois (2017). All qPCR assays were performed using a CFX 96 Real-Time System (Bio-Rad Laboratories Inc, Mississauga, ON) and GoTaq qPCR MasterMix with bryt green (Promega, Madison, WI, USA). The thermocycler program included an enzyme activation step at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, and 1 min at a gene-specific annealing temperature of 58°C, 60°C, or 62°C. After this amplification phase, there was a denaturation step of 1 min at 95°C. A dissociation curve was subsequently generated to confirm the presence of a single amplicon. The threshold for each gene was assessed automatically by the Bio-Rad CFX Manager Software 3.0. Pooled cDNA from each treatment were serially diluted (1:4) to produce a standard curve with a starting concentration of 50 ng. Each assay met the required a reaction efficiency of 100 ± 15% and an $R^2 \geq 0.989$. For quality control purposes negative control reactions were also included (i.e., no reverse-transcriptase (noRT) and no-template-controls (NTC)). The standard curve, control reactions, and samples were run in duplicate. Gene expression data are presented as the fold change relative to the mean control treatment. Fold change data were then normalized to the mean fold change of a reference gene. The expression of

198 reference genes can differ between some tissue types and treatments. A series of reference genes were
199 therefore profiled for ovary and testis samples (data not shown) and were only considered once the
200 absence of treatment effects were confirmed. Fold change data of testis and ovary tissue exposed to
201 androgenic compounds were normalized to the mean fold change of the reference gene *odc* (Fig. 2 and
202 Fig. 4). Fold change data of testis and ovary tissue exposed to TH-related compounds were then
203 normalized to the mean fold change of the reference genes *odc* or *rpl8,* respectively (Fig. 3, 5, and 6).
204
205 **2.5 Statistical analysis**
206 Statistical analyses were performed using Prism 6 (GraphPad Software Inc., San Diego, CA, USA) and
207 JMP (Version 12; SAS, Cary, NC, USA). Data and residuals were tested for normality and
208 homoscedasticity using the Shapiro–Wilk and Levene tests, respectively. Data were log transformed when
209 necessary to improve the fit to normality. Outlier analysis was performed using the Grubbs Test. Media
210 sex steroid data are presented as means ± 95% CL. Testis and ovary samples were analyzed as
211 independent variables. Treatments were compared to controls through one-way ANOVAs and Dunnett's
212 post hoc analyses. Differences were accepted as significant at an alpha level of $p < 0.05$.

3. Results

3.1 Testis and ovary tissue modulate sex steroid levels in response to T3 and 5α-DHT

Exposure to T3, IOP, or a co-treatment of IOP + 5α-DHT differentially altered levels of sex steroids (i.e., E_2, T, and 5α-DHT) in the media surrounding treated ovary and testis tissues (Table 1). Treatment with T3 significantly increased production of 5α-DHT from testis tissue by 50%. We found that sex steroid hormone levels were unaffected by IOP treatment alone; however, co-treatment with 5α-DHT significantly increased E_2 levels in the media surrounding testes by 50% and ovaries by 100%. Gonadal tissues did not respond to TH-related compounds by modulating T levels ($p > 0.05$; Table 1).

3.2 Promoter analysis reveals potential for crosstalk

The identified putative response elements (TREs, AREs, and EREs), their relative positions to the start codon, along with the core recognition motifs are shown in Fig. 1. The putative promoters of *trβ*, *ar*, and *srd5α2* are characterized by one to three *er* half-site motifs (5'-TGACC-3') in all species. For all three species, a single *tr* half-site motif (5'-TGACCT-3') was detected in the putative promoter of *erα*. The *M. musculus erα* promoter contained four AREs; however, EREs were not detected. In contrast, five EREs were identified in the promoter region of *erα* in *S. tropicalis*, and a single *er* palindrome motif plus four *er* half site motifs were identified in *O. latipes*. One to two AREs were found in the frog and fish *erα* promoter. In the putative *cyp19* promoter, the number of TREs decreased in a stepwise fashion in mice, frogs, and fish. Five *tr* half site motifs and a single direct half site repeat were observed in the putative promoter of *cyp19* in *M. musculus*. A total of three *tr* half site motifs were identified in S. *tropicalis*, and a single tr half site motif was detected in *O. latipes*. We identified two to three *ar* half site motifs in the putative *cyp19* promoter region of each species. Two *er* half site motifs were detected in *M. musculus*, a single half site motif and a direct half site repeat was identified in *S. tropicalis* and a single palindromic sequence was observed in *O. latipes*.

3.3 Gene expression

3.3.1 Androgens and T3 share analogous regulatory mechanisms of TH-related gene expression in gonadal tissues

The relative abundances of *tr* and *dio* mRNAs were differentially modulated by testes and ovaries in response to all three androgens (i.e., T, 5α-DHT, or 5β-DHT; Fig. 2). Expression of *trβ* increased on average by 1.5-fold in 5α-DHT or 5β-DHT treated testes ($F_{3, 26} = 3.8$, $p = 0.02$); whereas, *trβ* transcripts decreased by approximately 50% in 5α-DHT treated ovaries ($F_{3, 26} = 5.5$, $p = 0.005$). We found that *trα* expression in gonadal tissues of male and female juvenile *S. tropicalis* was not affected by T, 5α-DHT, or 5β-DHT *ex vivo* ($p > 0.05$; data not shown). Exposure to 5α-DHT significantly increased *dio1* expression by 1.7-fold in testis tissue ($F_{3, 22} = 3.6$, $p = 0.02$), while *dio2* and *dio3* mRNA levels remained unchanged. Ovary tissue did not respond to androgenic compounds by modulating *dio1* or *dio2* expression, but exposure to T or 5α-DHT significantly decreased *dio3* expression by approximately 40% ($F_{3, 27} = 8.2$, $p = 0.001$).

Testes and ovaries responded to T3 and IOP by differentially modulating the expression of *trs* and *dios* in sex-specific manner analogous to that observed for androgens (Fig. 3). Exposure to T3 significantly increased *trβ* transcripts 2-fold in testes ($F_{3, 26} = 20.6$, $p < 0.0001$), in contrast to ovary tissue, which did not modulate *trβ* expression in response to the TH exposure. A 40% decrease in *trβ* mRNA levels was observed however in IOP-treated ovary tissue ($F_{3, 22} = 3.3$, $p = 0.04$). Transcriptional regulation of *dio3* was similar between sexes with T3 increasing *dio3* expression by 3.5-fold in testes ($F_{3, 25} = 13.2$, $p < 0.0001$) and 5-fold in ovaries ($F_{3, 21} = 16.2$, $p < 0.0001$). We found that TH-related gene expression in ovary tissue was unaffected by the co-treatment of 5α-DHT; however, co-treatment with IOP + 5α-DHT significantly increased *dio1* transcripts by 1.5-fold in testes ($F_{3, 21} = 3.3$, $p = 0.04$). This finding compliments the previously observed increase in *dio1* expression in 5α-DHT treated testes. Transcripts of *trα* and *dio2* were not affected by T3 or IOP treatments ($p > 0.05$; data not shown).

3.3.2 Androgenic compounds positively regulate estrogen-related gene expression in testis and ovary tissue

Androgens differentially regulated the transcription of estrogen-related genes between sexes (Fig. 4). Both 5α-DHT and 5β-DHT significantly increased *erα* expression in ovary tissue by 2-fold ($F_{3, 26} = 6.3$, $p = 0.002$). A significant 1.6-fold increase in *cyp19* expression was also noted in 5α-DHT-treated testes ($F_{3, 25} = 3.6$, $p = 0.03$). We observed that *cyp19* transcription was however unaffected by androgenic compounds in ovaries ($p > 0.05$). Gonadal tissues did not modulate *ar*, *srd5α1*, srd*5α2,* and *srd5α3* transcription in response to different androgens ($p > 0.05$; data not shown). Exposure to T significantly decreased *dax-1* expression by 50% in testis tissue ($F_{3, 25} = 5.20$, $p = 0.006$); while, different androgens did not modulate *dax-1* transcription in ovary tissue ($p > 0.05$). Furthermore, we observed a sexually dimorphic pattern in the gene expression of *dax-1* where the mRNA levels of *dax-1* in testis tissue were 23-fold higher than in ovary tissue (Two-tailed t-test, $t_{11} = 11.0$, $p < 0.0001$).

3.3.3 TH-related compounds differentially regulate sex steroid-related gene expression in testis and ovary tissue

Gonadal tissues responded to compounds with TH-, anti-thyroid-, and androgen-related modes of action by differentially modulating the relative abundance of sex steroid-related transcripts (Fig. 5 and 6). Exposure to T3 significantly increased *erα* expression by 2.3-fold in treated ovaries ($F_{3, 22} = 6.4$, $p = 0.003$). Testis tissue did not respond to T3 or IOP by modulating *erα* transcription ($p > 0.05$); however, co-treatment with IOP + 5α-DHT significantly increased *erα* expression by 1.6-fold in treated-testis tissue relative to the control ($F_{3, 24} = 4.3$, $p = 0.01$), indicating an androgen mediated effect. Exposure to T3 significantly decreased *srd5α2* expression by 47% in treated ovary tissue relative to the control ($F_{3, 22} = 3.4$, $p = 0.04$). A thyroid-mediated effect was indicated as the expression of *srd5α2* did not increase in ovary tissue co-treated with IOP + 5α-DHT ($p > 0.05$). Testis tissue did not respond to treatments by modulating *srd5α2* transcription ($p > 0.05$). Gonadal tissues did not modulate *cyp19, dax-1, ar*, *srd5α1*, or *srd5α3* transcription in response to TH related compounds ($p > 0.05$; Fig. 5 and 6).

4. Discussion

Androgens and THs have been shown to be extensively involved in sexual development; however, relatively little is known with regard to the molecular mechanisms underlying androgen and TH crosstalk as a function of sex. This study therefore investigated (i) androgenic regulation of TH-related gene expression and (ii) TH-related regulation of sex-steroid gene expression in frogs using an *ex vivo* approach to elucidate potential sex-specific mechanisms of androgenic- and TH-mediated actions in reproductive tissues.

The transcription of several *tr*- and *dio*-isoforms within the reproductive tissues was significantly altered following exposure to different androgens (T, 5α-DHT, or 5β-DHT) indicating direct crosstalk. Few studies to date have reported on the molecular mechanisms underlying androgenic regulation of the TH-axis between sexes, however we previously identified AREs in the putative promoter regions of *trs* and *dios* in *S. tropicalis* using *in silico* analysis (Flood et al., 2013). Moreover, transcription of TH-related genes has been observed within reproductive tissues and over the course of both testicular and ovarian development in a wide range of vertebrate species (Duarte-Guterman et al., 2014; Flood et al., 2013; Habibi et al., 2012; Wagner et al., 2008; Cooke et al., 2004; Maran, 2003). Exposure to 5α-DHT or 5β-DHT significantly increased *trβ* expression in testicular tissue *ex vivo*; whereas, exposure to 5α-DHT significantly decreased *trβ* expression in ovary tissue suggesting that different androgens regulate gonadal *trβ* expression via positive or negative mechanisms in male and female frogs, respectively. The androgen-mediated increases in *trβ* expression in testicular tissue may be indicative of a more masculinized profile. The basal endogenous TH-related gene expression exhibits a natural sexual dimorphism in *S. tropicalis,* with testes characterized by higher *trβ* mRNA levels than ovaries (Duarte-Guterman and Trudeau, 2011). Moreover, elevated TH-related gene expression and enzyme activity is associated with a more masculinized profile (reviewed in Flood et al., 2013). Conversely, exposure to the potent androgen 5α-DHT further reduced *trβ* mRNA levels in ovary tissue, which could potentially indicate a more feminized profile. We previously examined the effects of 5α-DHT on TH-related gene expression during embryogenesis and observed that exposure to the androgen negatively regulated *trβ* expression in NF

316 stage 46 *S. tropicalis* larvae while also decreasing androgen-related transcription (Campbell and Langlois,
317 2017). Expression of *dio1* and *dio3* were also modulated in an androgen-, isoform- and sex-specific
318 manner. Exposure to 5α-DHT and IOP + 5α-DHT significantly increased *dio1* expression in testes
319 compared to the control and IOP treatments, respectively. Langlois et al. (2010b) demonstrated that
320 exposure to finasteride (a srd5 inhibitor) significantly decreased *dio1* expression in *S. tropicalis* larvae.
321 Exposure to androgenic compounds has also been shown to increase *dio1* mRNA levels in hepatic tissue
322 of mice (Šošić-Jurjević et al., 2015; Miyashita et al., 1995) and fish (MacLatchy and Eales, 1988). The
323 expression of *dio3* decreased following exposure to T or 5α-DHT in ovary tissue. The transcription of
324 *dio3* has been proposed as biomarker for tr activation and TH levels (reviewed by Nelson and Habibi,
325 2009; Shi et al., 1996). The T3-induced response of hepatocyte *dio3* gene was reduced to approximately
326 50% or 25% of the control, with inhibition of *trβ* expression in goldfish (Nelson and Habibi, 2008). These
327 findings suggest that TH-related gene expression can be directly regulated by different androgens and
328 highlight possible sex-specific effects of androgen-mediated actions on transcription of *tr*-and *dio*-
329 isoforms in amphibians. However, the degree and direction of regulation were androgen, isoform- or sex-
330 specific.

331 The androgen-mediated decreases in *trβ* and *dio3* expression in ovarian tissue may be indicative
332 of a more feminized profile. For example, exposure to pesticides with known feminizing and TH-related
333 properties have been shown to decrease *dio3* transcripts while increasing *erα* transcripts in various tissues
334 in fish and frogs (butachlor: Zhu et al., 2014; atrazine: Langlois et al., 2010a). In the present study, the
335 expression of *erα* significantly increased in 5α-DHT and 5β-DHT treated ovary tissues. We moreover
336 identified two AREs in the putative promoter regions of *erα* in *S. tropicalis*, indicating potential direct
337 androgenic regulation. Studies have also shown however that a metabolite of 5α-DHT, 5α-androstane-3β,
338 17β-diol, is weakly estrogenic in fish and rodent models (Mouriec et al., 2009; Oliveira et al., 2007). 5α-
339 androstane-3β, 17β-diol can bind to either *erα* or *erβ* significantly increasing transcription of both
340 isoforms (Miller et al., 2013; Sikora et al., 2009). The increase in *erα* in ovary tissue was presumably
341 associated with significant increases in E_2 levels of the media surrounding ovary tissue treated with IOP +

342 5α-DHT. Plasma levels of E_2 were found to be unaffected by *in vivo* exposure to 5α-DHT in adult frogs
343 (*X. laevis*: Urbatzka et al., 2007; Coady et al., 2005), possibly as a result of hypophyseal-feedback
344 mechanisms causing the peripheral degradation and elimination of produced steroids *in vivo*.
345 Nevertheless, exposure to 5α-DHT has been shown to result in a rapid and consistent increase in E_2
346 production *in vitro* from ovarian explants of *P. promelas* (Ornostay et al., 2016; Ornostay et al., 2013).
347 These 5α-DHT-mediated increases in E2 production are also consistent with findings in female fish
348 species exposed to the androgenic compounds 17α-methyltestosterone or 17β-trenbolone (*Carassius*
349 *auratus* of Pengze: Zheng et al., 2016; *G. rarus*: Gao et al., 2015; *Gadus morhua*: Kortner and Arukwe,
350 2009). Exposure to 5α-DHT did not modulate *srd5α2* transcript levels in testes. Bissegger and Langlois
351 (2016) previously confirmed that *srd5a2* expression was unaffected by exposure to T, 5α-DHT, or 5β-
352 DHT *ex vivo* in testis tissue of frogs. However, *cyp19* transcripts in- and E_2 production from- whole testis
353 significantly increased following exposure to 5α-DHT. Production of E_2 from testes of male *S. tropicalis*
354 exposed to 5α-DHT were similar to findings in male fish species exposed to 17β-trenbolone (Ankley et
355 al., 2003). Exposure to T also significantly decreased *dax-1* expression by 50% in testis tissue. The
356 expression and activity of *dax-1* has been shown to be essential for normal testicular development in
357 vertebrates (reviewed in: Iyer and McCabe, 2004; Lalli and Sassone-Corsi, 2003; Parker and Schimmer,
358 2002) and was identified only recently in the reproductive tissues of *S. tropicalis* post-metamorphosis
359 (Haselman et al., 2014). This suggests that the regulatory role of *dax-1* may extend past the period of
360 sexual differentiation and later into sexual development in amphibians. Overall, research on the
361 mechanism of *dax-1* in adult amphibians is limited and future studies should examine the role of *dax-1*
362 with regard to TH- and androgen-crosstalk in frogs. The function of these gene expression modifications
363 in the testes remains to be determined. Overall further investigation on the complex interplay between the
364 androgen and estrogen axes and the possible secondary effects on the TH-axis is warranted.
365 The present study demonstrated that exposure to T3 also increased *erα* expression in ovary tissue.
366 We identified one TRE half site (5' – AGGTCA – 3') in the putative *erα* promoter in *S. tropicalis*. Studies
367 have shown however that the *tr* can also bind to EREs - altering targeted gene expression as well as

interfering with the ability of the *er* to transactivate from *tr*-bound EREs (Vasudevan and Pfaff, 2005; Vasudevan et al., 2001). Exposure to T3 also significantly decreased *srd5α2* mRNA levels in ovary tissue. In support of negative regulation of *srd5α2* expression by T3, chronic exposure to the TH disruptor potassium perchlorate was shown to significantly increase *srd5α2* transcripts *in vivo* in hepatic (Flood and Langlois, 2014) and ovary tissue (Campbell et al., 2018). Although testis tissue did not respond to T3 by modulating *srd5α2* expression, a significant increase in 5α-DHT levels was observed in the media surrounding the T3-treated testis tissue. Exposure to methimazole, a known TH-disruptor, has been shown to decrease 5α-DHT production from testis tissue (*R. norvegicus*: Anbalagan et al., 2010; Kala et al., 2002). Morais et al. (2013) proposed that T3-modulation of steroidogenesis in Leydig cells is mediated by *trβ* in the fish *Danio rerio*. We previously identified TREs in *srd5α2* of *M. musculus*, *S. tropicalis*, and *O. latipes* (Flood et al., 2013). Taken together these findings indicate that THs can regulate sex-steroid related gene expression in testis and ovary tissue in amphibians, and sex-specific gene expression patterns are maintained *ex vivo*.

One explanation for the differential regulation of TH-related gene expression between sexes could be via DNA methylation and/or histone modification. DNA methylation has been associated with transcriptional repression leading to low mRNA levels of highly methylated genes (Chen and Riggs, 2005). Exposure to T3 has been shown to affect histone and polymerase II modification, but does not affect hyper-methylation in the promoter region of *trβ* in *X. laevis* tadpoles (Kasai et al., 2015). Moreover, sexually dimorphic DNA methylation patterning has been observed in sex steroid-related genes in frogs and fish (Bissegger and Langlois, 2016; Navarro-Martín et al., 2011; Contractor et al., 2004), but sex differences in methylation patterns have not yet been investigated with regard to TH-related genes. Tissue and age specific DNA methylation and histone modification patterns have however been thoroughly studied in amphibians. During metamorphosis, different tissues in developing tadpoles (e.g., hind limbs, tail tissues, etc.) have been shown to respond to exogenous T3 by differentially modulating histone modifications in trs and dios (Grimaldi et al., 2013; Shi et al., 2009), indicating the potential for differential epigenetic regulation of TH-related genes in reproductive tissues. In summary, epigenetic

394 regulation may play a role in differential modulation of TH- and sex steroid-related gene expression
395 between sexes.
396 This study is the first to characterize sex-specific differences in TH- and sex steroid-related gene
397 expression between testes and ovaries of sexually mature juvenile *S. tropicalis* following *ex vivo* exposure
398 to androgens (5α-DHT, 5β-DHT, or T), THs (T3), or TH-antagonists (IOP). Indeed, direct exposure to
399 different androgens led to androgen specific increases in *trβ* and *dio1* transcripts in testes but decreases in
400 *trβ* and *dio3* expression in ovaries, suggesting that male and female frogs can be differently affected by
401 androgenic compounds. Moreover, exposure to select androgens differentially increased estrogen-related
402 transcription (*erα* and *cyp19*) and production (E2) in ovaries and testes suggesting the activation of
403 alternate metabolic pathways yielding estrogenic metabolites. Sex steroid-related transcription (*erα and*
404 *srd5α2*) and production (5α-DHT) were differentially-regulated between sexes by T3, however sex-
405 specific gene expression patterns were maintained *ex vivo.* In summary, this study provides insight into
406 the molecular mechanisms underlying androgenic and TH-related actions and reveals potential
407 differential transcriptional effects as a function of sex in frogs. However, additional studies incorporating
408 *in vivo* and epigenetic approaches should be performed under longer-exposure conditions to firmly
409 establish these mechanisms of crosstalk.
410

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608 **Table 1.** Total estradiol (E_2), testosterone (T), and 5α-dihydrotestosterone (5α-DHT) produced by testes [pg] and ovaries [pg/g] during a 6 h *ex*
609 *vivo* incubation. Media sex steroid data are presented as means (least squares means [95% CL]. Testicular sex steroid hormone levels are
610 normalized by organ mass. Ovarian sex steroid hormone levels are reported per g tissue. Significant differences between treatments and the control
611 (*) were identified by one-way ANOVAs followed by post hoc Dunnett's tests ($p < 0.05$).
612

Sex	Sex steroid-hormones	Control	T3 (50 nM)	IOP (10 μM)	IOP + 5α-DHT (10 μM) (1 μM)
Male [pg]	T ($F_{2, 13} = 0.31$, $p = 0.74$)[b]	3,605 [2588, 4622]	4,094 [2980, 5208]	3,595 [2481, 4709]	NM
	5α-DHT ($F_{2, 21} = 5.79$, $p = 0.01$)	14,186 [10991, 17381]	21,436 [18241, 24631]*	16,558 [13363, 19753]	NM
	E_2 ($F_{3, 28} = 40.1$, $p < 0.0001$)	227.3 [204.0, 250.6]	203.7 [180.3, 227.0]	207.8 [184.5, 231.1]	355.6 [332.3, 378.9]*
Female[a] [pg/g]	T ($F_{2, 21} = 1.84$, $p = 0.18$)	14,533[5386, 23680]	22,870 [13723, 32016]	25,054 [15907, 34200]	NM
	5α-DHT ($F_{2, 21} = 2.39$, $p = 0.12$)	9,957 [7168, 12746]	13,976 [11187, 16765]	13,667 [10880, 16459]	NM
	E_2 ($F_{3, 28} = 3.94$, $p = 0.02$)	12,040 [6226, 17853]	16,677 [10864, 22491]	14,035 [8222, 19849]	25,814 [20000, 31627]*

613

614 [a] Female sex steroid-hormones were log-transformed to normalize residuals.
615 [b] For each sex per treatment n = 8, except n = 5 for levels of T in males in all treatments.

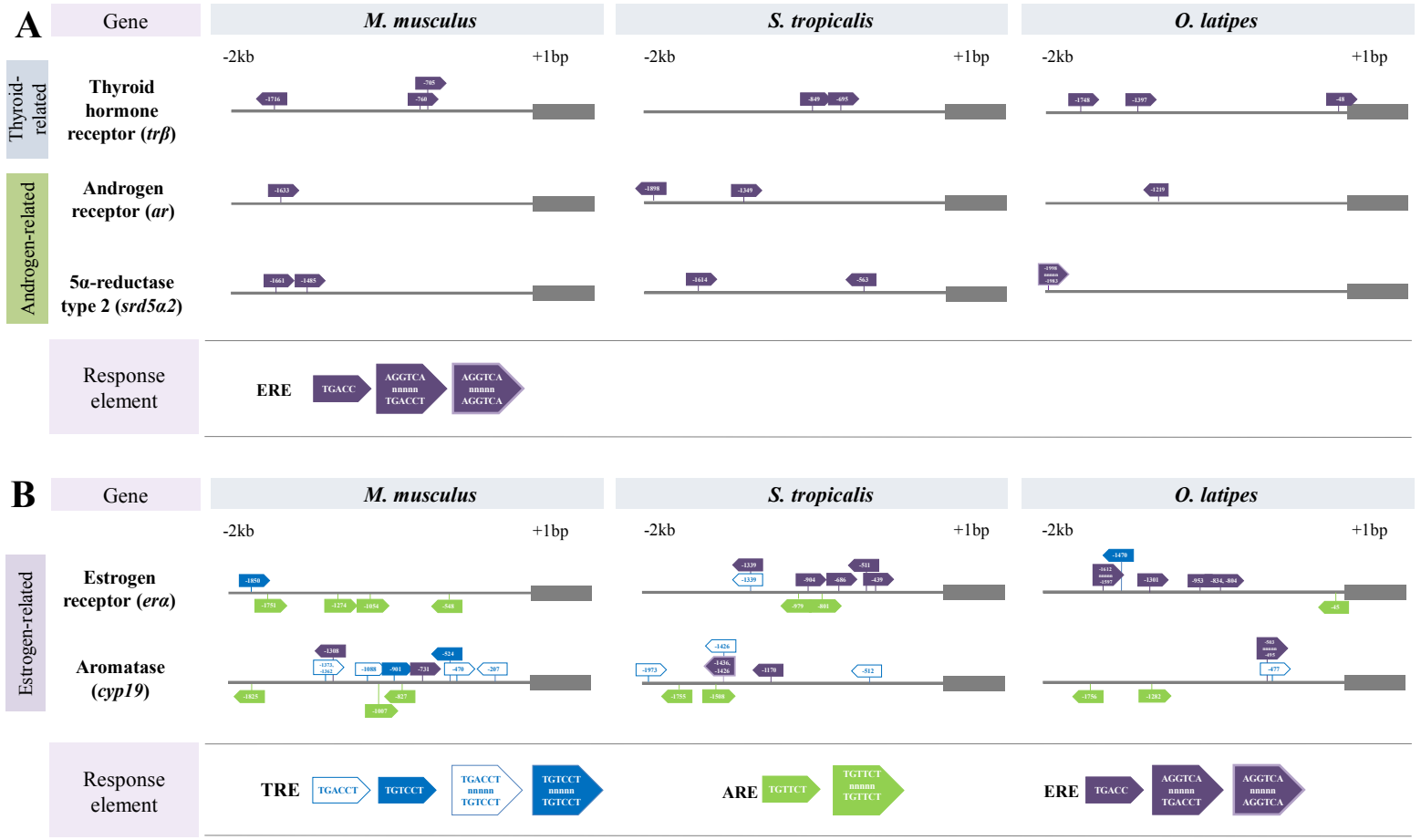

Figure 1. Promoter analysis of *M. musculus*, *S. tropicalis*, and *O. latipes* TH-related genes (*trβ*) and androgen-related genes (*ar*, *srd5α2*) (A), and estrogen related genes (*erα*, and *cyp19*) (B). For information on *tr* and *ar* half-site motifs in thyroid- and androgen-re;ated genes please refer to Flood et al. (2013). All sequences used for analysis were collected from the Ensembl Project (http://www.ensembl.org). Putative transcription factor binding sites within the putative promoter (-2000 to +1) were identified using PROMO (v.3.0.2; Farré et al., 2003) and FIMO (v.4.11.1; Grant et al., 2011) software. TREs are shown in blue, AREs are shown in green, and EREs are represented by purple arrows. This figure was adapted from Figure 1 in Flood et al., 2013 (Ch. 1).

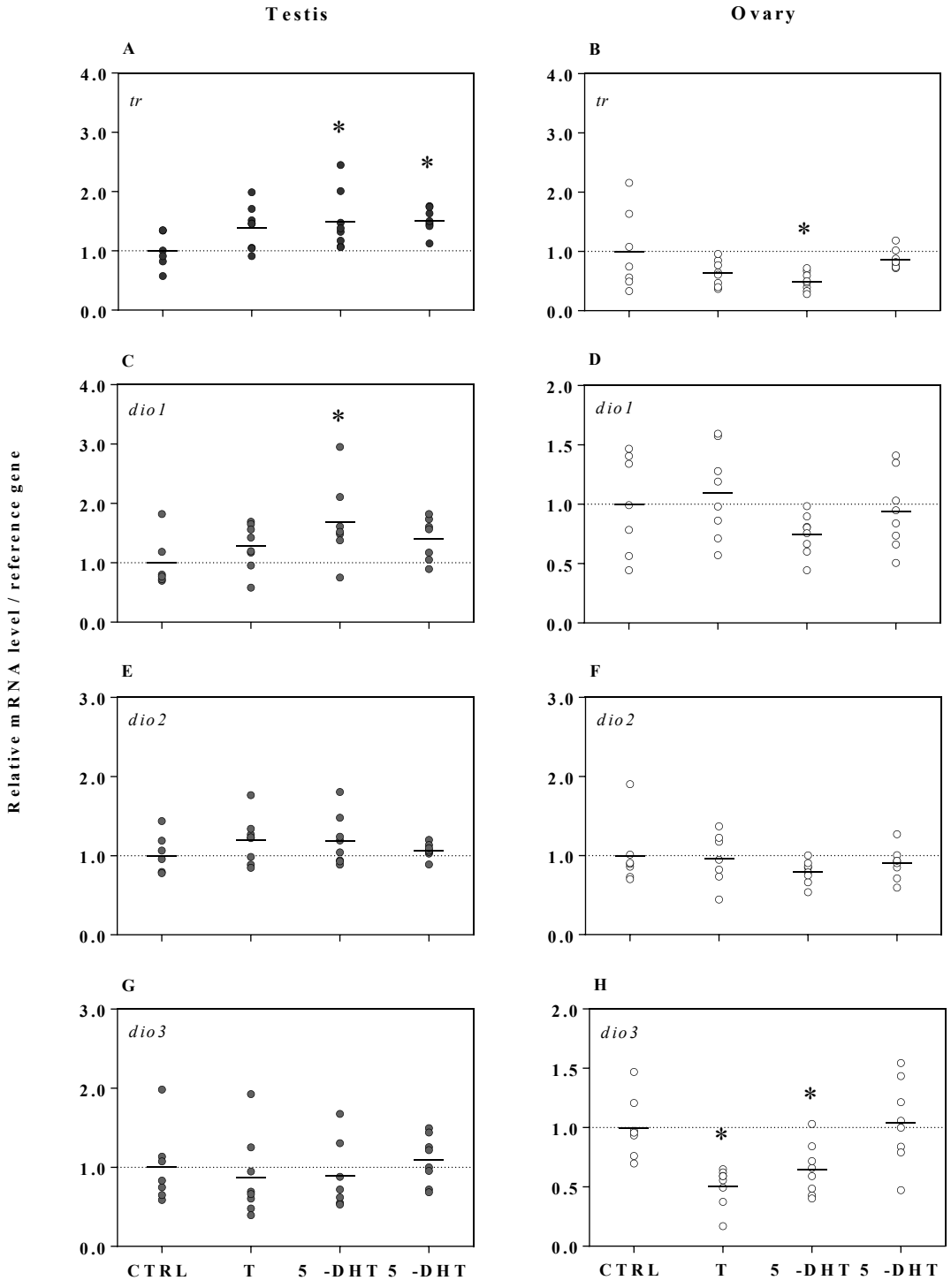

Figure 2. Expression of *trs* (*trβ*) and *dios* (*dio1*, *dio2*, and *dio3*) in testes (A, C, E, and G, respectively) and ovaries (B, D, F, and H, respectively) exposed *ex vivo* to testosterone (T; 1 μM), 5α-dihydrotestosterone (5α-DHT; 1 μM), and to 5β-dihydrotestosterone (5β-DHT; 1 μM) for 6 h. Symbols represent individual samples (n = 6–8 per treatment). Gene expression data are normalized to *odc* and presented as fold changes relative to the control treatment. Significant differences between treatments and the control (*) were identified by one-way ANOVAs followed by post hoc Dunnett's tests ($p < 0.05$). Note that the scales of the y-axis vary.

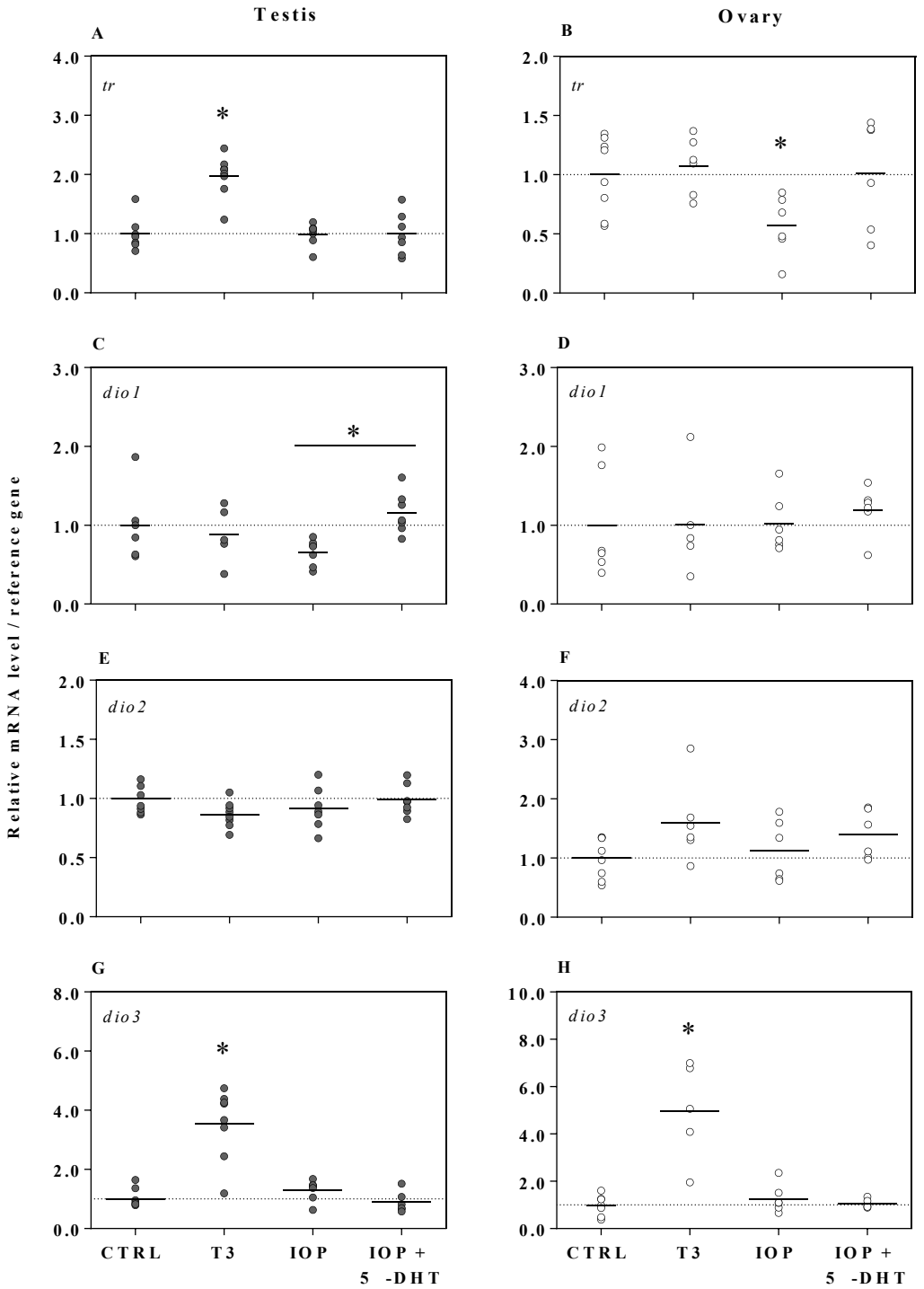

Figure 3. Expression of *trs* (*trβ*) and *dios* (*dio1*, *dio2*, and *dio3*) in testes (A, C, E, and G, respectively) and ovaries (B, D, F, and H, respectively) exposed *ex vivo* to triiodothyronine (T3; 50 nM), iopanoic acid (IOP; 10 µM), and to a co-treatment of IOP (10 µM) + 5α-dihydrotestosterone (5α-DHT; 1 µM) for 6 h. Symbols represent individual samples (n = 5–8 per treatment). Testis and ovary gene expression data are normalized to *odc* and *rpl8,* respectively and presented as fold changes relative to the control treatment. Significant differences between treatments and the control (*) were identified by one-way ANOVAs followed by post hoc Dunnett's tests ($p < 0.05$). Significant differences between treatments were identified by two-tailed t tests ($p < 0.05$). Note that the scales of the y-axis vary.

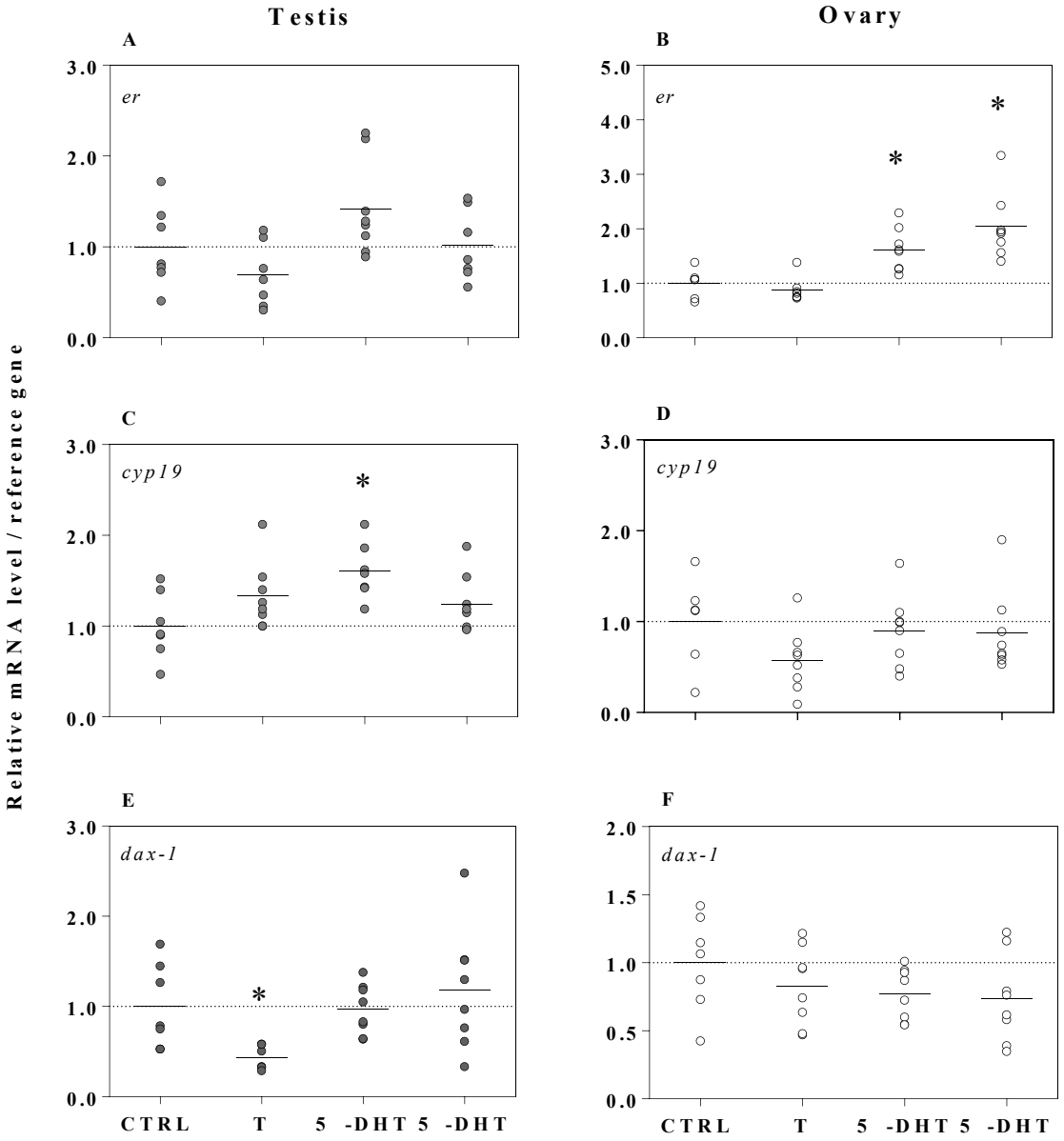

Figure 4. Expression of *erα*, *cyp19*, and *dax-1* in testes (A, C, and E respectively) and ovaries (B, D, and F respectively) exposed *ex vivo* to testosterone (T; 1 μM), 5α-dihydrotestosterone (5α-DHT; 1 μM), and to 5β-dihydrotestosterone (5β-DHT; 1 μM) for 6 h. Symbols represent individual samples (n = 6–8 per treatment). Gene expression data are normalized to *odc* and presented as fold changes relative to the control treatment. Significant differences between treatments and the control (*) were identified by one-way ANOVAs followed by post hoc Dunnett's tests ($p < 0.05$). Note that the scales of the y-axis vary.

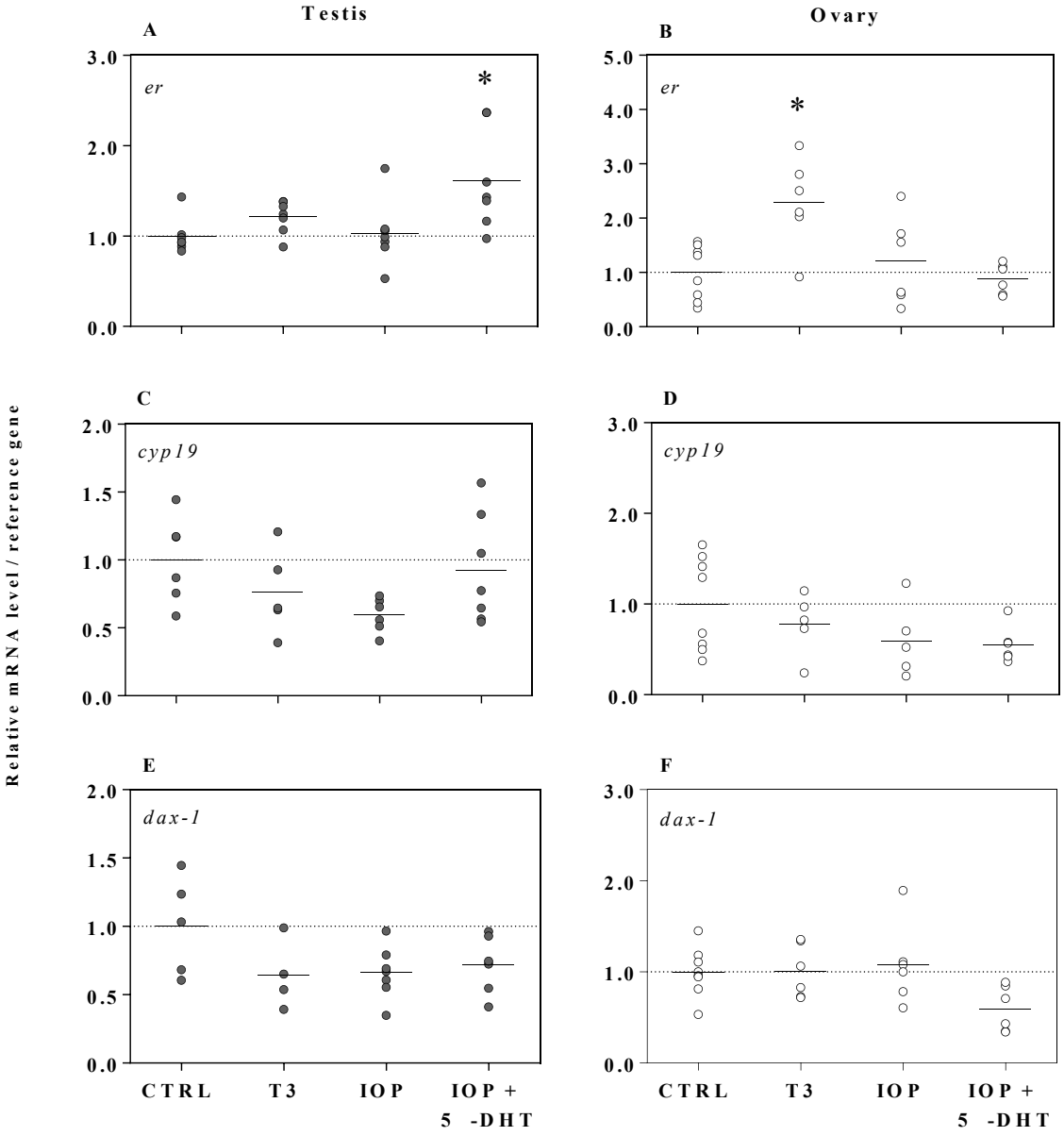

Figure 5. Expression of *erα*, *cyp19,* and *dax-1* in testes (A, C, and E respectively) and ovaries (B, D, and F respectively) exposed *ex vivo* to triiodothyronine (T3; 50 nM), iopanoic acid (IOP; 10 μM), and to a co-treatment of IOP (10 μM) + 5α-dihydrotestosterone (5α-DHT; 1 μM) for 6 h. Symbols represent individual samples (n = 5–8 per treatment). Testis and ovary gene expression data are normalized to *odc* and *rpl8,* respectively and presented as fold changes relative to the control treatment. Significant differences between treatments and the control (*) were identified by one-way ANOVAs followed by post hoc Dunnett's tests ($p < 0.05$). Note that the scales of the y-axis vary.

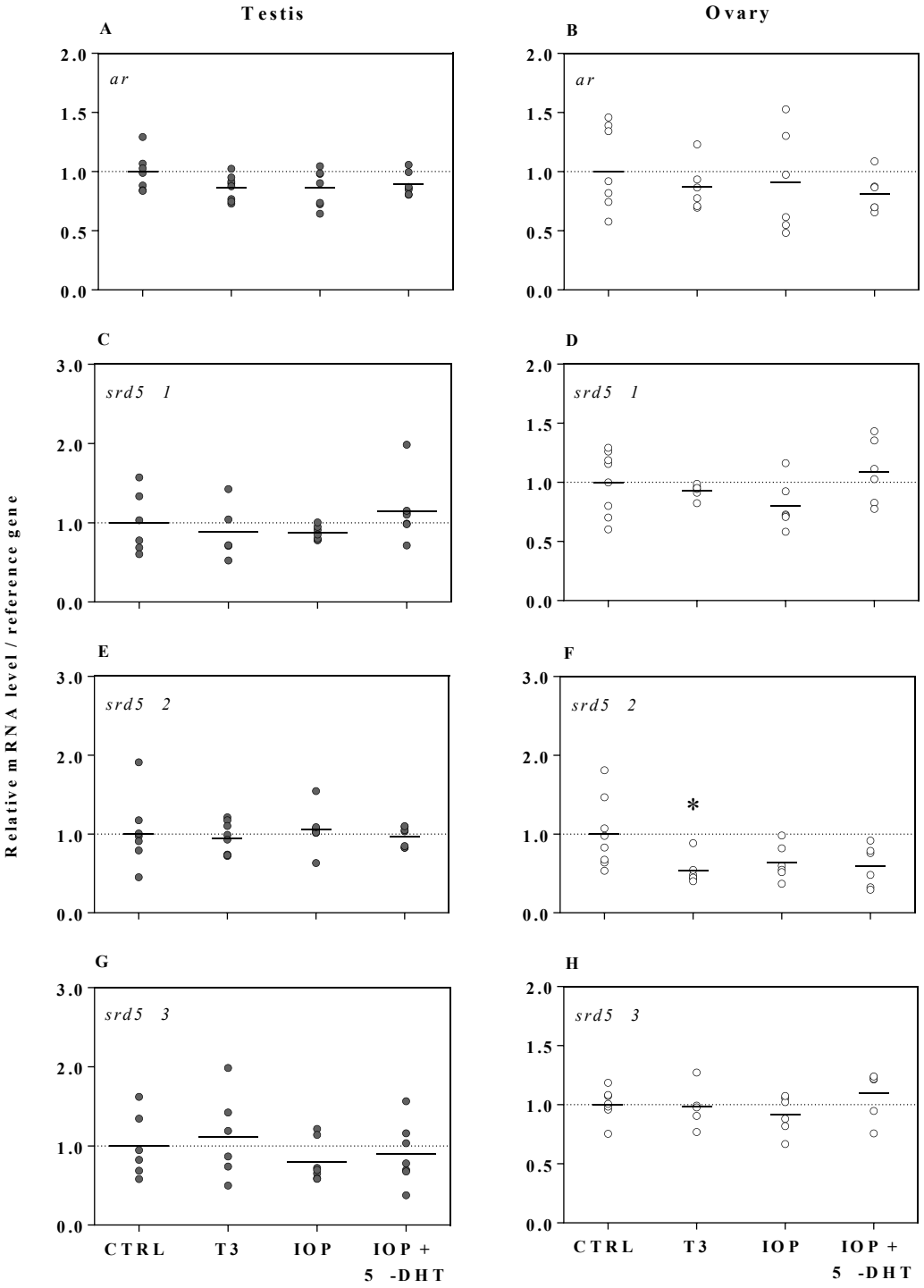

Figure 6. Expression of *ar*, *srd5α1*, *srd5α2*, and *srd5α3* in testes (A, C, E, and G, respectively) and ovaries (B, D, F, and H, respectively) exposed *ex vivo* to triiodothyronine (T3; 50 nM), iopanoic acid (IOP; 10 µM), or a co-treatment of IOP (10 µM) + 5α-dihydrotestosterone (5α-DHT; 1 µM) for 6 h. Symbols represent individual samples (n = 5–8 per treatment). Testis and ovary gene expression data are normalized to *odc* and *rpl8,* respectively and presented as fold changes relative to the control treatment. Significant differences between treatments and the control (*) were identified by one-way ANOVAs followed by post hoc Dunnett's tests ($p < 0.05$). Note: scales of the y-axis vary.