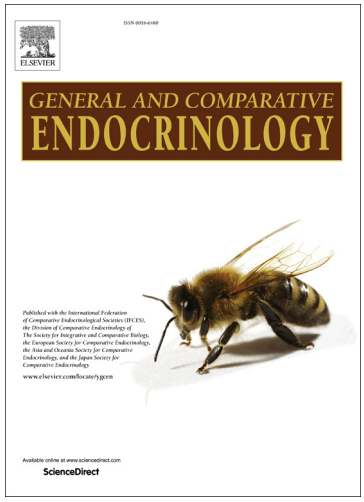

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PII: S0016-6480(17)30665-2
DOI: https://doi.org/10.1016/j.ygcen.2017.10.017
Reference: YGCEN 12789

To appear in: *General and Comparative Endocrinology*

Received Date: 26 September 2017
Revised Date: 25 October 2017
Accepted Date: 27 October 2017

Please cite this article as: Campbell, D.E.K., Langlois, V.S., Expression of *sf1* and *dax-1* are regulated by thyroid hormones and androgens during *Silurana tropicalis* early development, *General and Comparative Endocrinology* (2017), doi: https://doi.org/10.1016/j.ygcen.2017.10.017

Expression of *sf1* and *dax-1* are regulated by thyroid hormones and androgens during *Silurana tropicalis* early development

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Keywords: Sex determination; Thyroid hormones; Androgens; Crosstalk; Iopanoic Acid; Embryogenesis; Real-time RT-PCR; *Silurana tropicalis*

Abstract

Thyroid hormones (THs) and androgens have been shown to be extensively involved in sexual development; however, relatively little is known with regard to TH-related and androgenic actions in sex determination. We first established expression profiles of three sex-determining genes (*sf1*, *dax-1*, and *sox9*) during the embryonic development of Western clawed frogs (*Silurana tropicalis*). Transcripts of *sf1* and *sox9* were detected in embryos before the period in which embryonic transcription commences indicating maternal transfer, whereas *dax-1* transcripts were not detected until later in development. To examine whether TH status affects sex-determining gene expression in embryonic *S. tropicalis,* embryos were exposed to co-treatments of iopanoic acid (IOP), thyroxine (T4), or triiodothyronine (T3) for 96 h. Expression profiles of TH receptors and deiodinases reflect inhibition of peripheral deiodinase activity by IOP and recovery by T3. Relevantly, elevated TH levels significantly increased the expression of *sf1* and *dax-1* in embryonic *S. tropicalis*. Further supporting TH-mediated regulation, examination of the presence and frequency of transcription factor binding sites in the putative promoter regions of sex-determining genes in *S. tropicalis* and rodent and fish models using *in silico* analysis also identified TH motifs in the putative promoter regions of *sf1* and *dax-1*. Together these findings advocate that TH actions as early as the period of embryogenesis may affect gonadal fate in frogs. Mechanisms of TH and androgenic mechanisms of crosstalk in relation to the regulation of steroid-related gene expression were also investigated.

1. Introduction

Sex determination is highly diverse in vertebrates. Sex determining mechanisms have broadly been divided into either genotypic sex determination (GSD) or environmental sex determination (ESD). In GSD, inherited sex chromosomes at fertilization determine gonadal fate and the ensuing sex differentiation (Barske and Capel, 2008; reviewed in Vilain and McCabe, 1998); whereas in ESD, sexual fate is controlled by environmental factors experienced after fertilization (Sarre et al., 2004; Valenzuela and Lance, 2004; Bull, 1983). Endocrine processes provide the foundation for sex determination and subsequent gonadal formation in all vertebrate species. A growing body of literature advocates that endocrine disruption can overcome the sex-determining program irrespective of GSD or ESD (Mizoguchi and Valenzuela, 2016; Golan and Levavi-Sivan, 2014; Nakamura, 2010). Thyroid hormones (THs) have pleotropic effects in developing vertebrates, including effects on gonadal development. Several studies have demonstrated that TH status alters sex ratios in fish (Sharma and Patino, 2013; Mukhi et al., 2007; Bernhardt et al., 2006) and amphibians (Goleman et al., 2002). THs have been shown to crosstalk with both the estrogen and androgen axes regulating sex steroid-related transcription and production (reviewed in: 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). Although our knowledge of the molecular mechanisms underlying TH mediated reproductive effects is increasing, relatively little is known regarding sex determination.

We previously identified potential crosstalk via several candidate sex-determining genes imperative to bipotential gonad formation and differentiation (Flood et al., 2013). The steroidogenic factor 1 (*sf1*) is encoded by the NR5A1 gene and is important for sexual differentiation as it is expressed in primordial organ cell clusters fated to differentiate into mammalian adrenal glands, testes, and ovaries (reviewed in: Hoivik et al., 2010; Parker and Schimmer 2002; Vilain and McCabe, 1998). Moreover, *sf1* is considered a master regulator of steroidogenic-related genes. Numerous studies have demonstrated the ability of TH status to not only influence steroid hormone production, but also the underlying transcriptional activity (Duarte-Guterman et al., 2014; Flood et al., 2013). The widespread effects of THs on steroidogenesis could thus be mediated via *sf1*. Another sex-determining gene of interest is *dax-1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1). Encoded by NR0B1, *dax-1* expression is restricted to tissues directly involved in steroid hormone production and

reproductive function. This gene served primarily as a negative regulator by binding to the promoter of different genes, including but not limited to, *sf1*, androgen receptor (*ar*), and aromatase (*cyp19*) (reviewed in: Orekhova and Rubtsov, 2015; Lalli and Sassone-Corsi, 2003). The expression and activity of *Dax-1* has been shown to be essential for normal testicular development in vertebrates (reviewed in: Iyer and McCabe, 2004; Lalli and Sassone-Corsi, 2003; Parker and Schimmer, 2002). Studies have shown that Dax-1 can negatively regulate thyroid hormone receptor transcription (Valadares et al., 2008; Moore et al., 2004); however, reciprocal TH regulation has not yet been investigated. The sex-determining region Y box 9 (*sox9*) is a male-specific transcription factor that mediates testis differentiation (Kobayashi et al., 2005; Kent et al., 1996). Outside the reproductive axes, studies showed that THs can influence *sox9* transcript levels. Okubo and Reddi (2003) observed that *sox9* expression in *Mus musculus* chondrocytes significantly decreases with thyroxine (T4) exposure. Thus, THs may regulate *sox9* expression during the period of sexual determination and gonadal formation. Sex-determining genes initiate a cascade of genetic events that play a crucial role in vertebrate gonadal differentiation as well as sexual development. Consequently, TH mediated regulation of sex-determining genes may have long-lasting effects on subsequent development.

All three genes (*sf1*, *dax-1*, and *sox9*) are co-expressed in precursor testis and ovary cells within the gonadal ridge of embryonic vertebrates (Kobayashi et al., 2005; Hoyle et al., 2002; Kent et al., 1996; Ikeda et al., 2001, 1994). The genital ridge is formed during the period of embryogenesis (i.e., 72 hours post-fertilization (hpf) in amphibians; El Jamil et al., 2008) and 10 days postcotium (dpc) in mice (Tanaka and Nishinakamura, 2014; Kent et al., 1996). Thyroid gland organogenesis begins to form at approximately Nieuwkoop and Faber (NF) stage 40 (~72 hpf) with consequential I^- uptake at approximately NF stage 46 (~96 hpf) in the frog *Xenopus laevis* (Brown, 2005). Thyroid gland activity is not detected until post-partum in mice; however, the fetus' TH requirements are met via the placenta (reviewed in Darras et al., 2015). However, several studies have detected deiodinase (*dio*) transcription and activity during the period of embryogenesis in amphibians (*Silurana tropicalis*: Tindall et al., 2007; *X. laevis*: Morvan Dubois et al., 2006) and unlike their mammalian counterparts, *dio*s serve as the only source for *de novo* production of THs prior to thyroid gland activity. Therefore, dio-related transcription and activity may play a putative role in gonadal fate in amphibians.

To understand the putative role of THs on sex-determining gene expression prior to gonadal differentiation, we first established expression profiles of the *sf1, sox9, and dax-1* from the commencement of amphibian embryogenesis (NF stage 2) to the beginning of larvae development (NF stage 46). Embryos (NF stage 10–12) were exposed to co-treatments of iopanoic acid (10 μM; IOP), thyroxine (5 nM; T4), or triiodothyronine (50 nM; T3) for 96 h to examine whether TH status affects sex-determining gene expression in embryonic *S. tropicalis*. Embryos were also exposed to either T3 (0.5, 5 or 50 nM) or 5α-dihydrotestosterone (5α-DHT; 4, 40, or 400 nM) to further study TH and androgenic mechanisms of crosstalk in relation to the regulation of sex-determining gene expression. We also conducted novel *in silico* promoter analysis to assess the presence and frequency of putative transcription factor binding sites in *S. tropicalis* and compare it with rodent and fish models.

2. Materials and Methods

2.1 Animals

Sexually mature male and female *S. tropicalis* frogs were housed in the Queen's University Animal Care Facility (Kingston, ON, Canada). Adults were reared in tanks containing dechlorinated and aerated water (25 ± 1°C) on a 12:12 h light:dark regime (light commencing at 0700 h). Fertilized eggs were obtained from three pairs of adult frogs and were mixed together in order to remove any clutch effect. Spawning was artificially induced by injecting human chorionic gonadotropin hormone (hCG; 2500 IU/mL; Sigma Canada Ltd., Oakville, ON, Canada) into the dorsal lymph sac. Both males and females received a priming injection of 50 μL hCG (12.5 IU) followed by a boosting injection of 200 μL hCG (100 IU) after 24 h, as previously outlined by Flood and Langlois (2014). Eggs were present within 2 to 3 h post-injection. Developmental stages were determined following the Nieuwkoop and Faber (NF) developmental staging system (Nieuwkoop and Faber, 1994). Animal care was performed in accordance with the guidelines of the Animal Care Committee of Queen's University and the Canadian Council on Animal Care.

2.2 Developmental profile

Samples of whole embryos were taken at different NF stages of development: 2, 7, 16, 21, 27, 34, 41, and 46. At each stage, embryos were pooled (20 embryos for NF 2 to NF 34 and 10 embryos for NF 41 and NF 46) to ensure

sufficient material for RNA isolation. Pools (n = 6 to 8 per NF stage) were flash frozen on dry ice and stored at -80 °C for further analysis.

2.3 Exposures and material

Eggs were allowed to develop to NF stage 8, at which point they were collected and de-jellied with 2% (w/v) l-cysteine (pH 8.0; Sigma Canada Ltd., Oakville, ON, Canada) for 2 min. The eggs were washed three times with modified Ringer's solution (0.1 M NaCl, 1.8 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 300 mg/L $NaHCO_3$). Two experiments were run in parallel using methodology previously outlined by Flood and Langlois (2014). In the first experiment, we examined whether TH status affects sex-determining gene expression in embryonic *S. tropicalis*. Embryos (NF stage 10–12) were placed in either modified Ringer's solution (1:9 v/v) or one of six test solutions in 125-mL glass jars at a density of 50 embryos per jar. Embryos were exposed to DMSO (0.001%), 10 μM of IOP (TCI America), a co-treatment of 10 μM IOP + 50 nM T3 (Sigma, Oakville, Ontario, Canada), a co-treatment of 10 μM IOP + 5 nM T4 (Sigma, Oakville, Ontario, Canada), 5 nM T4 or 50 nM of T3. Of note, IOP is a known inhibitor of the enzymatic activity of both dio2 and dio3 in frogs (Becker et al., 1997). In all exposures, a concentration of 0.04 ppm of the antibiotic gentamycin (Sandoz Canada, Inc Boucherville, QC, Canada) was administered every 24 h. Water changes occurred every 24 h until NF stage 46. At NF stage 46 embryos were pooled (10 embryos per pool) to ensure sufficient material for RNA isolation. Pools (n = 6 to 8 per treatment) were flash frozen on dry ice and stored at -80 °C for further analysis. In the second experiment, we examined TH and androgenic mechanisms of crosstalk in embryonic *S. tropicalis*. Embryos (NF stage 10–12) were placed in either modified Ringer's solution (1:9 v/v) or one of the seven test solutions in 125-mL glass jars at a density of 50 embryos per jar. Embryos were exposed to DMSO (0.001%), T3 (0.5, 5 or 50 nM), or 5α-DHT (4, 40, or 400 nM; Steraloids, Newport, RI, USA). In all exposures, a concentration of 0.04 ppm of the antibiotic gentamycin (Sandoz Canada, Inc Boucherville, QC, Canada) was administered for every 24 h. Water changes occurred every 24 h until NF stage 46. At NF stage 46 embryos were pooled (10 embryos per pool) to ensure sufficient material for RNA isolation. Pools (n = 5-8 per treatment) were flash frozen on dry ice and stored at -80 °C for further analysis. Each experiment was conducted once to reduce animal use.

2.4 Gene expression analysis

Total RNA from NF stage 46 larvae was isolated using an e.Z.N.A. Total RNA Kit I (OMEGA Biotek; VWR International, Mississauga, ON, Canada) in accordance with the manufacturer's protocol. We purified total RNA using the TURBO DNA-free™ Kit (Ambion; ThermoFisher Scientific, Ottawa, ON, Canada). The quantity of RNA was determined on a NanoDrop-2000 spectrophotometer (Thermofisher, Ottawa, ON, Canada). First strand cDNA was synthesized following the GoScript Reverse Transcription kit protocol (Promega, Madison, WI, USA) using random primers in a Mastercycler Pro S Thermocycler (Thermo Fisher, Ottawa, ON, Canada). The cDNA products were diluted 20-, 40-, or 80-fold prior to PCR amplification. For quality control purposes, negative control reactions were also included (i.e., no reverse-transcriptase (noRT) and no-template-controls (NTC)). Primer sequences for androgen receptor (*ar*), deiodinases (*dio1*, *dio2*, and *dio3*), 5α-reductases (*srd5α1*, *srd5α2*, *srd5α3*) and TH receptors (*trα* and *trβ*) were previously designed and validated by Langlois et al. (2010). Real-time PCR primers for *sf1*, *dax-1*, and *sox9* were designed based on GenBank sequences (*sf1*: accession no. NM_001145741.1, forward 5' – 3': ACCCTGTGACTAAAAACCTCCC, reverse 5' – 3': GCATAGTCATTCCAAGCGGTG, amplicon size: 99 bp; *dax-1*: accession no. XM_002933615; forward 5' – 3': AATCCCACAACAGCAACCCA; reverse 5' – 3': GTGGGCTTTTTAGGCTGACTTT; amplicon size: 99 bp; *sox9*: accession no. NW_004668240.1; forward 5'–3': ATGCTGGAGGCAGAATGTGGGGAA; reverse 5'–3': CGGCGCAGAAAGTCCGTAAAGAATG; amplicon size: 125 bp) using the Primer-BLAST tool from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Elongation factor-1 alpha (*ef1α*) and 18s ribosomal RNA (*18s*) were used as normalizing genes to normalize the expression of target genes. All qPCR assays were performed using a CFX 96 Real-Time System (Bio-Rad Laboratories Inc, Mississauga, ON, Canada) and GoTaq qPCR MasterMix (Promega, Madison, WI, USA). The thermocycler program used 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 serial diluted (1:4) to produce a standard curve with a starting concentration

of 50 ng. Each assay required a reaction efficiency of 100 ± 15% and an R2 ≥ 0.989. To further quality control measures, the standard curve, control reactions, and samples were run in duplicate. For the developmental profile, gene expression data is presented as fold change relative to NF stage 2 for *sox9* and *sf1*, and NF stage 21 for *dax-1* and data were normalized to RNA content (as per Langlois et al., 2010). For exposures, gene expression data is presented as fold change relative to the mean control treatment and were normalized to the normalizing gene *ef1α* (Fig. 3) and to *18s* (Fig. 4) as their expression did not statistically change among treatments.

2.5 Promoter analysis

To further examine potential mechanisms of molecular crosstalk we examined the frequency of thyroid response elements (TREs), androgen response elements (AREs), and estrogen response elements (EREs) in the putative promotor regions of *S. tropicalis dax-1*, *sf1*, and *sox9*. 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 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 regions (−2000 to +1) of our target genes. A *p*-value output threshold of 0.001 was selected. 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 of 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.

2.6 Statistical analysis

Statistical analyses were performed using Prism 6 (GraphPad Software Inc., San Diego, CA, USA) and JMP (Version 12; SAS, Cary, NC, USA). Data and residuals were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene tests, respectively. Statistical outliers were removed and the data was log transformed when necessary to improve the fit to normality. Developmental profile data are presented as individual samples relative to the first stage mRNA was detected. Developmental stages were compared through one-way ANOVAs and Tukey

post hoc analyses. Treatments were compared to controls through one-way ANOVAs and Tukey's or Dunnett's post hoc analyses. Differences were accepted as significant at an alpha level of $p < 0.05$.

3. Results

3.1 Developmental profile

The expression profiles of the sex-determining genes differ in pattern and magnitude of change; however, for all of the genes, transcript levels increased during embryogenesis (Fig. 1). Levels of *sf1* and *sox9* mRNA were detected at all stages of development and low levels of maternally-derived mRNA were observed at NF stage 2 and NF 7. However, transcripts of *dax-1* were first detected at NF stage 21. Expression of *sf1* significantly increased 3.1- to 5.5-fold between NF stage 16 and NF 21, mRNA levels then increased 27- to 45-fold from NF stage 27 ($F_{7, 45} = 89.1$, $p < 0.0001$). Levels of *dax-1* mRNA dramatically increased 75- to 195-fold between NF stage 27 and NF 46 ($F_{4, 26} = 234.0$, $p < 0.0001$). In contrast, *sox9* expression remained relatively constant throughout embryonic development and only showed a 2-fold significant increase at NF stage 34 compared to NF stage 2 ($F_{7, 44} = 18.3$, $p < 0.0001$).

3.2 Gene expression

3.2.1 TH disrupting chemicals alter sex-determining gene expression

The expression profiles of *dio*s and *tr*s eloquently reflected the inhibitory properties of IOP (Fig. 2). The TH-disrupting chemical competitively inhibits *dio1*, *dio2*, and *dio3* activity (reviewed in Schweizer and Steegborn, 2015). Expression of *dio2* increased 2.7-fold in larvae co-treated with IOP + T3 and 4.9-fold in T3 treated NF stage 46 larvae. A similar expression pattern was observed for *dio3*; transcripts increased 6.4-fold in larvae co-treated with IOP + T3 and 8.6-fold in T3 treated NF stage 46 larvae. These findings clearly reflect IOP mediated inhibition of *dio* activity and subsequent altered TH status. In contrast, expression of *dio1* decreased by 50% in T3-treated larvae. Expression of *trα* appeared to be unaffected by IOP as transcripts increased 2.2-fold in larvae co-treated with IOP + T3 and 3.9-fold in T3 treated larvae. Expression of *trβ* however, increased 1.5-fold in IOP treated larvae, 2-fold in larvae co-treated with IOP + T4, 8.6-fold in larvae co-treated with IOP + T3, 2-fold in larvae treated with T4 alone

and 4.9-fold in T3 treated larvae. The higher expression of *trβ* in the co-treatment of IOP + T3 compared to the T3 treatment is presumably concomitant with the accumulation of THs following *dio3* inhibition.

The expression of *sf1* and *dax-1* were significantly altered by peripheral TH metabolism (Fig. 3). Expression of *sf1* increased 1.6-fold in larvae co-treated with IOP + T3 and 2.2-fold in T3 treated NF stage 46 larvae. Expression of *dax-1* significantly increased 1.7-fold in larvae co-treated with IOP + T3 however *dax-1* mRNA levels were unaffected in T3 treated larvae. In support of the *trβ* expression profile, the higher transcript levels of *dax-1* observed in the co-treatment of IOP + T3 is also presumably associated with the accumulation of THs. Expression of *ar* increased 1.3-fold in in larvae co-treated with IOP + T3 and 1.5-fold in T3 treated larvae. We found *sox9* expression to be unaffected by TH status in embryogenic *S. tropicalis* ($p > 0.05$; Fig. 3).

3.2.2 Androgens negatively regulate TH-, sex-steroid-, and sex determining- gene expression

In the second experiment, *sf1* and *dax-1* were not affected by T3; however, significant increases in *trα*, *trβ*, and *ar* expression were observed (Fig. 4). T3 increased the expression of *trβ* mRNA in a dose-related manner (0.5, 5, and 50 nM). Transcript levels of *trα* and *ar* increased on average by 1.5-fold in NF stage 46 larvae treated with 50 nM of T3. Also, a significant 60% decrease in *srd5α1* expression of NF stage 46 larvae exposed to 50 nM of T3 was observed.

Transcripts of TH-, sex steroid-, and sex-determining genes decreased in NF stage 46 larvae exposed to different concentrations of 5α-DHT (4, 40, and 400 nM; Fig. 4). Expression of *trβ*, *ar*, *sf1*, *dax-1*, and *sox9* significantly decreased by approximately 50% in 40 nM or 400 nM 5α-DHT treated larvae. Transcripts of *srd5α2* demonstrated the largest decrease of approximately 75% in the same treatments. The expression of *trα*, *srd5α1*, and *srd5α3* expression were unaffected by 5α-DHT treatments ($p > 0.05$). Of note, there is a discrepancy in the expression data between Fig. 3 and Fig. 4 at 50 nM T3 as the same treatment led to an increase in mRNA levels for *sf1*, *dax-1*, and *sox9* in Fig 3., but not in Fig. 4. A difference between nominal and experimental concentrations would explain this discrepancy, thus the actual concentration of T3 in the 50 nM treatment in Fig 3 would be higher than in the 50 nM T3 treatment in Fig 4 as the latter concentration was not high enough to reproduce the same change for the expression of *sf1*, *dax-1*, and *sox9*. Unfortunately, water analysis was not performed in this study, so this hypothesis cannot be tested further.

3.3 Promoter analysis of sex-determining genes

The relative positions of response elements (TREs, AREs, and EREs) to the start codon, along with the core recognition motifs are shown in Fig. 5. The putative *M. musculus*, *S. tropicalis*, and *O. latipes* promoters of *sf1* were characterized by the presence of TREs and EREs. Two *tr* half-site motifs (5'-TGACCT-3') and two *er* half-site motifs (5'-TGACC-3') were identified in the promoters of mice and frogs, respectively. While a combination of four *tr* and *er* half-site motifs were identified in the promoter region of fish. We distinguished five *tr* half-site motifs (5'-TGACCT-3'or 5'-TGTCCT-3'), one *ar* half-site motif (5'-TGTCCT-3'), one direct *ar* half site motif repeat (5'-TGTCCTnnnnnTGTCCT-3'), and three *er* half-site motifs in the putative promoter of *dax-1* in *M. musculus.* A similar diversity of response elements was observed in the putative promoter of *dax-1* in *O. latipes.* We identified a total of five *tr* half-site motifs, three *ar* half-site motifs, and one *er* half-site motif between -2000 bp and +1 bp upstream of *dax-1.* In contrast, we only identified a single *tr* half-site motif in the putative promoter of *dax-1* in *S. tropicalis.* The putative promoters of *sox9* contained a single *ar* half-site motif in mice, two *ar* half-site motifs in frogs and AREs were not detected in fish. The promoter of *sox9* in *S. tropicalis* was also characterized by a single *tr* half-site motif and *er* half site motif. The promoter of *sox9* in *O. latipes* contained a single *tr* half-site.

4. Discussion

Sex-determining genes initiate a cascade of genetic events that plays a crucial role in vertebrate gonadal differentiation as well as sexual development. However, current knowledge is limited on sex-determining gene expression and regulation, particularly in lower vertebrates such as amphibians. This study investigated the role of TH- and androgen-signaling on sex-determining gene expression in frogs to elucidate potential mechanisms of crosstalk during amphibian embryogenesis prior to gonadal differentiation.

Our data confirmed that transcription of *sf1*, *dax-1*, and *sox9* occurs in the frog embryo; however, the distinct developmental profiles of these genes suggest that they may be differently affected by exogenous THs and androgens during amphibian embryogenesis. Transcripts of *sf1* were detected in NF stage 2 and NF stage 7 embryos before the period in which embryonic transcription commences indicating maternal transfer. Homologs to *sf1* in fish are

similarly present in unfertilized eggs and early stages of embryogenesis (*Danio rerio*; von Hofsten et al., 2005). In mice, *sf1* is detected at 9.5 dpc and is one of the earliest transcription factors expressed in the precursor cells of the future gonad (Ikeda et al., 2001, 1994). Expression of *sf1* is associated with the onset of steroidogenesis in vertebrates and is thought to be a master regulator of steroidogenic genes (reviewed in Hoivik et al., 2010; Parker and Schimmer, 2002). Maternally transferred sex steroids in addition to *de novo* sex steroid synthesis via related biosynthetic enzyme mRNA and activities have been detected in the amphibian embryo (*S. tropicalis*: Langlois et al. 2010; *X. laevis*: Bögi et al., 2002). These observations indicate that a *sf-1* mediated pathway (enzyme – hormone – receptor) is established prior to gonadal formation (NF stage 48 – 49; El Jamil et al., 2008). Several reports have also implicated the transcription factor in the regulation of *dax-1* expression. Studies have shown that binding of Sf1 to response elements in the putative *dax-1* promoter is necessary to achieve high levels of *dax-1* expression in mammalian embryonic development (Hoyle et al., 2002; Kawabe et al., 1999). Transcripts of *dax-1* were only first detected at NF stage 21 in *S. tropicalis* embryos (this study). Similarly, the expression of *dax-1* has been shown to be similarly delayed in embryonic fish (3 dpf; *Pimephales promelas*; Wood et al., 2015) and in the genital ridge of mice (11.5 dpc; Swain et al., 1996). Here, we further demonstrated that levels of *sf1* and *dax-1* transcripts increased dramatically at NF stage 27 and continued throughout organogenesis. These gene expression level increases coincide with a rise in processes related to steroid metabolism and male gonadal development in *S. tropicalis* (Langlois and Martyniuk, 2013). Previous studies have demonstrated considerable crosstalk between TH and sex steroid axes, and THs are proposed to be involved in testicular development (Flood et al., 2013; Duarte-Guterman et al., 2010). Whether *sf1* and *dax-1* are potential targets of TH mediated action is a question we addressed in the second part of this study. In contrast to *sf1* and *dax-1* mRNA profiles, *sox9* transcript levels remained relatively constant throughout embryogenesis. Several studies have also demonstrated that *sox-9* is expressed maternally and comparable gene expression profiles were observed in fish and amphibian species (*P. promelas*, Wood et al., 2015; *S. tropicalis*, Yanai et al. 2011; *X. laevis*, Spokony et al., 2002). To the best of our knowledge, this is the first report of the developmental profiles of *sf1*, *dax-1*, and *sox9* mRNA levels in amphibian embryos.

We observed that two sex-determining genes, *sf1* and *dax-1*, changed significantly following direct exposure to T3. Several studies examining the role of THs on sexual development have also observed T3 mediated modulation

of sex steroid-related release and gene expression (reviewed in Flood et al., 2013). It has been proposed however that the T3-mediated actions on sex steroid-related mRNA levels are dependent on *sf1* expression. For example, inhibition of *sf1* expression considerably diminished T3-mediated regulation of *star* (steroidogenic acute regulatory protein) mRNA levels in mice (Manna et al., 2001). By altering *sf1* expression, THs could have considerable influence over steroidogenesis as a whole. The mRNA levels of *sf1* significantly increased by 2.2-fold in T3-treated larvae and 1.5-fold in larvae co-treated with IOP + T3. Transcripts of *sf1* were shown to increase with increasing T3 concentrations in mouse Leydig tumor cells (Manna et al. 1999). Sarkar et al. (2016) observed that exposure to BDE-209 (a brominated flame retardant with TH disrupting properties) decreased serum levels of THs (T4 and T3) and subsequently decreased mRNA and protein levels of *sf1* in testes of adult mice further demonstrating that TH status influences *sf1* expression. We did not observe TREs in the putative promoter region of *sf1* in *S. tropicalis*; however, (i) TREs may be located at other locations (e.g., enhancers) within the target locus, moreover (ii) studies have shown that the *tr*s can alternatively bind to EREs altering targeted gene expression (Vasudevan and Pfaff, 2005; Vasudevan et al., 2001). In the present study, EREs were identified in the putative promoter region of *sf1* in *S. tropicalis*. Comparisons between species furthermore revealed a combination of EREs as well as TREs in the putative promoters of *sf1* in *M. musculus* and *O. latipes*. The expression of *dax-1* was unaffected in T3-treated larvae; however, *dax-1* mRNA levels increased by 1.7-fold in larvae when co-treated with IOP + T3. This discrepancy may be due to the inhibition of dio3 enzymatic activity by IOP (i.e., T3 is accrued on top of the initial 50 nM of T3). Transcript levels of *trβ* were also significantly higher in IOP + T3 treated larvae compared to that of larvae in the T3 treatment further indicating higher TH levels in the IOP + T3 treatment. We identified several TREs in the putative promoters of *dax-1* in *M. musculus, S. tropicalis,* and *O. latipes*, which provides support for a vertebrate-wide control of *dax-1* expression by THs. Further experimental confirmation of putative transcription factor binding sites in the promoter regions of these species is required. Interestingly, exposures to neither TH-disruptors nor THs affect *sox9* mRNA, suggesting that this gene is not regulated directly by THs during embryogenesis. To the best of our knowledge, this is the first study to observe TH-mediated actions on sex-determining genes during embryogenesis in amphibians. TH mediated regulation of *sf1* and *dax-1* observed at NF stage 46 may have important consequences on subsequent gonadal

development, as NF stage 46 falls within the period of genital ridge formation just prior to the start of gonadal differentiation.

The TH-mediated increases in *sf1* and *dax-1* expression may be indicative of a masculinized phenotype. T3 treatment has previously been shown to elicit genetic, hormonal, and phenotypic changes in line with a more masculinized profile in a wide range of vertebrate species (reviewed in Flood et al., 2013). Increase in the expression of *sf1* leads to sexually dimorphic patterns with higher mRNA levels in developing testis than ovaries in mice (Ikeda et al., 2001). We demonstrated that *dax-1* transcript levels are also higher in testes than ovaries of adult *S. tropicalis* (Campbell and Langlois, *in preparation*). In the present study, significant 1.3-fold and 1.5-fold increases were observed in *ar* expression of larvae co-treated with IOP + T3 and larvae exposed to T3, respectively. Duarte-Guterman et al. (2011) similarly observed a significant increase in *ar* expression. A T3-mediated decrease in *srd5α1* expression was also observed in NF stage 46 *S. tropicalis* larvae. Duarte-Guterman et al. (2010) conversely demonstrated a T3-mediated increase in *srd5a1* in stage NF 46 *S. tropicalis*; however, exposure duration may make a difference as embryos were only exposed between NF stage 27 to NF 46. Transcripts of *srd5α1* were confirmed to decrease in the gonad-mesonephros complex of T3-treated *Physalaemus pustulosus* tadpoles (Duarte-Guterman et al., 2012). Transcript levels of *srd5α1* are moreover sexually dimorphic with higher mRNA levels observed in ovaries than testes in adult frogs (*P. pustulosus*, Duarte-Guterman et al., 2012; *S. tropicalis*, Duarte-Guterman and Trudeau, 2011). These results in combination with the decrease in sex-determining gene expression could indicate that T3 may also promote some aspects of masculinization in *S. tropicalis*. The masculinizing effects of these modulations on subsequent sexual development remain to be investigated.

To confirm a masculinizing effect of excess THs, we examined whether the potent androgen 5α-DHT would modulate gene expression in a similar fashion. Exogenous 5α-DHT concentrations above 4 nM appears to influence a number of genetic pathways as we observed concurrent decreases in *sf1*, *dax-1*, *ar*, *srd5α2* and *trβ* in NF stage 46 *S. tropicalis*. The lowest treatment tested (i.e., 4 nM 5α-DHT) is higher than endogenous androgen levels in larvae frogs (i.e., androgens have been detected at levels ranging from 0.01 to 0.02 nM in NF stage 20 to 40 *X. laevis* embryos (Bögi et al., 2002). However, significant changes in gene expression were not observed at this concentration suggesting that the animals can cope with this level of 5α-DHT. Transcriptional changes were observed at the two

highest concentrations of 5α-DHT investigated. Transcript levels of *sf1*, *dax-1,* and *sox9* significantly decreased by 50% in the 40 and 400 nM 5α-DHT treatments. Previous studies also reported androgenic modulation of *sf1* expression. For example, transcripts of *sf1* decreased along with several other steroidogenic genes in developing female trout exposed to androgen 11β-hydroxyandrostenedione (10 mg per kg of food; *Oncorhynchus mykiss*; Baron et al., 2008). Furthermore, Zheng et al. (2015) observed that 5α-DHT significantly decreased *sf1* expression in rat primary pituitary cell cultures. The 5α-DHT-mediated decrease in *sf1* expression may have influenced subsequent *dax-1* and *sox9* transcription in NF stage 46 *S. tropicalis* as studies have reported that Sf1 initiates transcription of *dax-1* (Hoyle et al., 2002) and *sox9* (Sekido et al., 2008, 2004). These observations suggest that a 5α-DHT mediated decrease in *sf1* expression may promote some aspects of feminization in *S. tropicalis* by decreasing *dax-1* and *sox-9* expression. Previous studies observed that transcript levels of *sf1*, *sox9*, *ar*, *srd5α2,* and *trβ* moreover exhibit sexually dimorphic patterns as gonadal differentiation progresses with higher expression in differentiating testes compared to ovaries (Kobayashi et al., 2005; Hoyle et al., 2002; Kent et al., 1996; Ikeda et al., 2001, 1994). Conditions experienced during embryogenesis may have long-lasting effects on subsequent development; consequently, the concomitant decrease in sex-determining gene expression could alter the sex-determining program in larval frogs. Further experimental confirmation of this hypothesis is required.

It was proposed that embryonic metabolism modulates steroid levels to buffer the process of gonadal differentiation from the effects of maternal steroids (Feist et al., 1990). Biosynthetic enzyme mRNAs and activities have been detected as early as NF stage 7 in *S. tropicalis* (Langlois et al., 2010). Langlois et al. (2010) demonstrated that exposure to the endocrine disruptors' finasteride and fadrozole regulate the activity and the expression of *cyp19* and *srd5α2* in NF stage 46 *S. tropicalis*. These observations suggest that activation of alternative metabolic pathways may protect the developing embryo from excessive androgenic action by eliminating 5α-DHT to estrogenic metabolites. Studies in fish and rodents have demonstrated that a metabolite of 5α-DHT, 5α-androstane-3β, 17β-diol, is weakly estrogenic (Mouriec et al., 2009; Oliveira et al., 2007). Other androgenic compounds have been reported to increase in estrogen-related gene expression and estradiol (E_2) production. Miller et al. (2013) demonstrated that 5α-androstane-3β, 17β-diol can bind to either *erα* or *erβ* significantly increasing transcription of both isoforms. Exposure to 17α-methyltestosterone significantly increased *cyp19* and *erα* expression in ovaries of immature female fish

(*Carassius auratus* of Pengze; Zheng et al., 2016). Exposure to 5α-DHT significantly increased *erα* expression in the prostate of male rats (Oliveira et al., 2007). Exposure to 5α-DHT has been shown to result in a rapid and consistent increase in E_2 production *in vitro* from ovarian explants of *P. promelas* (Ornostay et al., 2016; Ornostay et al., 2013). These 5α-DHT-mediated increases in E_2 production are also consistent with findings in fish species exposed to the androgenic compounds 17α-methyltestosterone or 17β-trenbolone (*C. auratus*: Zheng et al., 2016; *Gobiocypris rarus*: Gao et al., 2015; *P. promelas*: Ankley et al., 2008, 2003; *Gadus morhua*: Kortner and Arukwe, 2007). Plasma levels of E_2 were found to be unaffected by *in vivo* exposure to 5α-DHT in male and female adult frogs (*X. laevis*: Urbatzka et al., 2007; Coady et al., 2005); however, embryos lack hypophyseal-feedback mechanisms which could explain why expression differs. Moreover, Bissegger and Langlois (2016) demonstrated that androgens (T, 5α-DHT, and 5β-DHT) significantly decreased *srd5α1* and *srd5α3* transcripts in *S. tropicalis* ovary tissue *ex vivo*. Exposure to the potent androgen, 5α-DHT also modulated TH related gene expression in embryonic *S. tropicalis*. A decrease of approximately 50% in *trβ* expression was observed in NF stage 46 larvae exposed to 40 or 400 nM of 5α-DHT. In adult *S. tropicalis, trβ* expression significantly decreased by 50% in 5α-DHT treated ovaries *ex vivo* (Campbell and Langlois, *in preparation*). Exposure to finasteride (an inhibitor of srd5 activity) decreased TH-related gene expression (*dio1* and *dio2*) in NF stage 46 *S. tropicalis* (Langlois et al., 2010), which presumably was attributed to the accumulation of excess, aromatized androgens. In summary, the observed concomitant decrease in androgen- and TH-related expression indicates that exposure to exogenous androgens can (i) influence the sex-determining program in embryonic frogs and (ii) promote aspects of feminization.

This study is the first to have measured the presence of *sf1*, *dax-1*, and *sox9* transcripts in early amphibian development and further showed that two of these sex-determining genes (*sf1* and *dax-1*) can be regulated by THs. Indeed, direct exposure to T3 and peripheral *dio* activity inhibition by IOP significantly led to the increase in the expression of *sf1*, an essential mediator of gonadal ridge formation and differentiation, along with the testis-determining gene *dax-1* in NF stage 46 *S. tropicalis*. Exposure to exogenous 5α-DHT conversely decreased *sf1* and *dax-1* expression in addition to *sox9*, *ar*, *srd5α2,* and *trβ* transcript levels in embryos suggesting the activation of alternative metabolic pathways yielding estrogenic metabolites. Whether these changes are functionally important

post-embryogenesis remains to be elucidated. This study nonetheless provides a framework for future investigations to examine the role of THs in sex determination in amphibians.

Acknowledgements:

The authors would like to thank Dr. Sonja Bissegger, Nova Zhao, and Colin Campbell for their contributions to the project. This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada (RGPIN 418576-2012) and a Canada Research Chair (CRC) to VSL, and NSERC-PGS (PGS D2-460059-2014) to DEKC.

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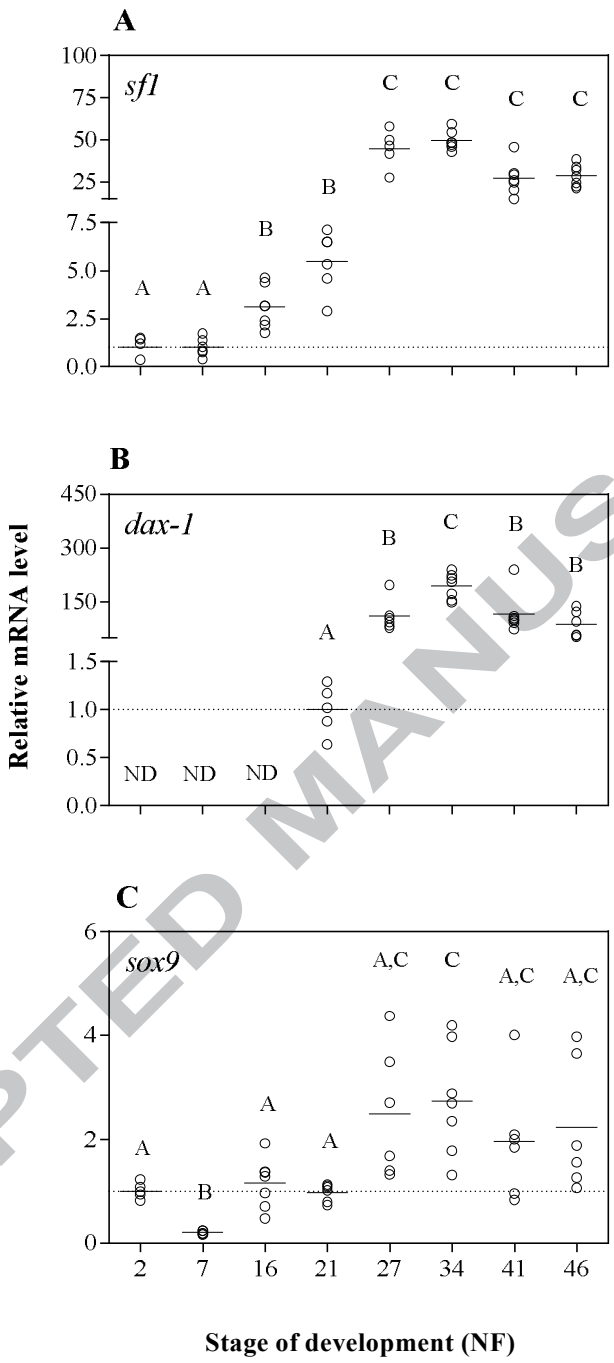

Figure 1. Developmental profiles of sex-determining genes during *S. tropicalis* embryogenesis and larval development. Transcript levels of *sf1* (A), *dax-1* (B), and *sox9* (C) were measured in whole embryos and larvae from NF stage 2 (two-cell stage) to NF stage 46 (beginning of feeding). Levels of mRNA are expressed relative to NF 2 except for dax-1 (relative to NF 21) and are normalized to RNA content. Symbols represent individual samples (n = 6–8 pools). Significant differences between treatments (indicated by different letters) were identified by one-way ANOVAs followed by post-hoc Tukey's tests ($p < 0.05$). Note that the scales of the y-axis vary between genes. ND represents not detected.

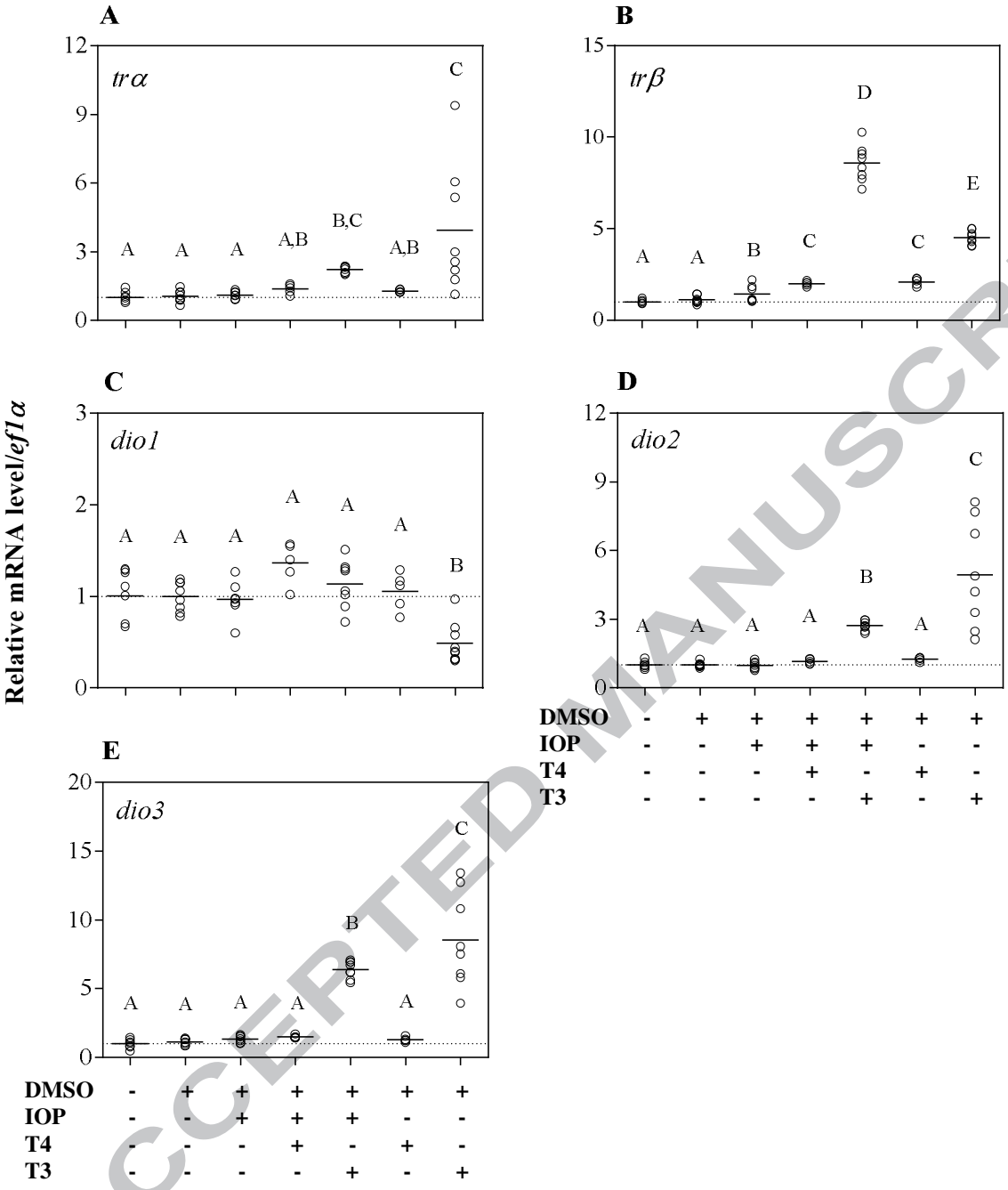

Figure 2. Expression of *trα* (A), *trβ* (B), *dio1* (C), *dio2* (D), and *dio3* (E) in whole *S. tropicalis* NF stage 46 larvae. Embryos were exposed to 10 µM of IOP, a co-treatment of 10 µM IOP + 5 nM T4, a co-treatment of 10 µM IOP + 50 nM T3, 5 nM T4 or 50 nM of T3 from NF stage 12 to NF stage 46. Symbols represent individual samples (n = 5–8). Gene expression data are normalized to *ef1α* and presented as fold changes relative to the control treatment. Significant differences between treatments (indicated by different letters) were identified by one-way ANOVAs followed by post-hoc Tukey's tests ($p < 0.05$). Note that the scales of the y-axis vary.

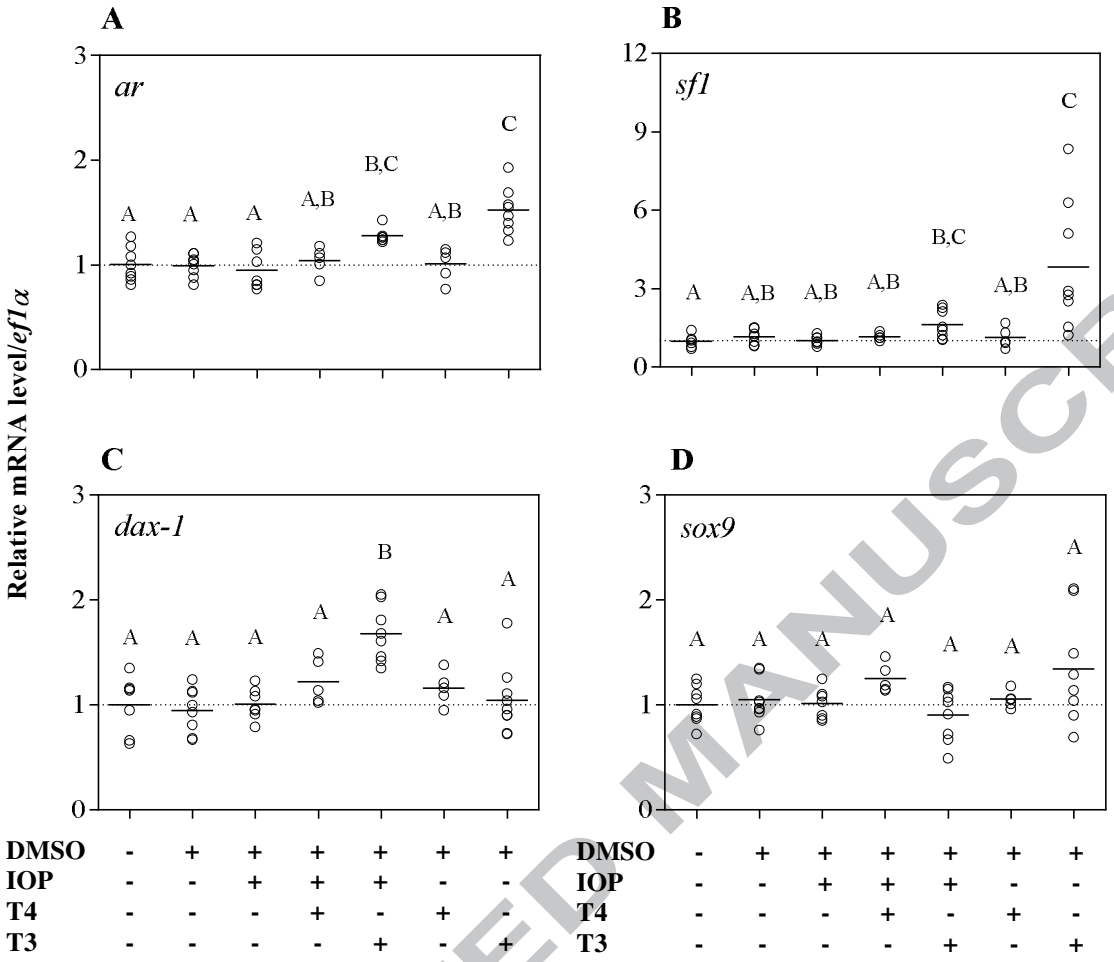

Figure 3. Expression of *ar* (A), *sf1* (B), *dax-1* (C), and *sox9* (D) in whole *S. tropicalis* NF stage 46 larvae. Embryos were exposed to 10 µM of IOP, a co-treatment of 10 µM IOP + 5 nM T4, a co-treatment of 10 µM IOP + 50 nM T3, 5 nM T4 or 50 nM of T3 from NF stage 12 to NF stage 46. Symbols represent individual samples (n = 5–8). Gene expression data are normalized to *ef1α* and presented as fold changes relative to the control treatment. Significant differences between treatments (indicated by different letters) were identified by one-way ANOVAs followed by post-hoc Tukey's tests ($p < 0.05$). Note that the scales of the y-axis vary.

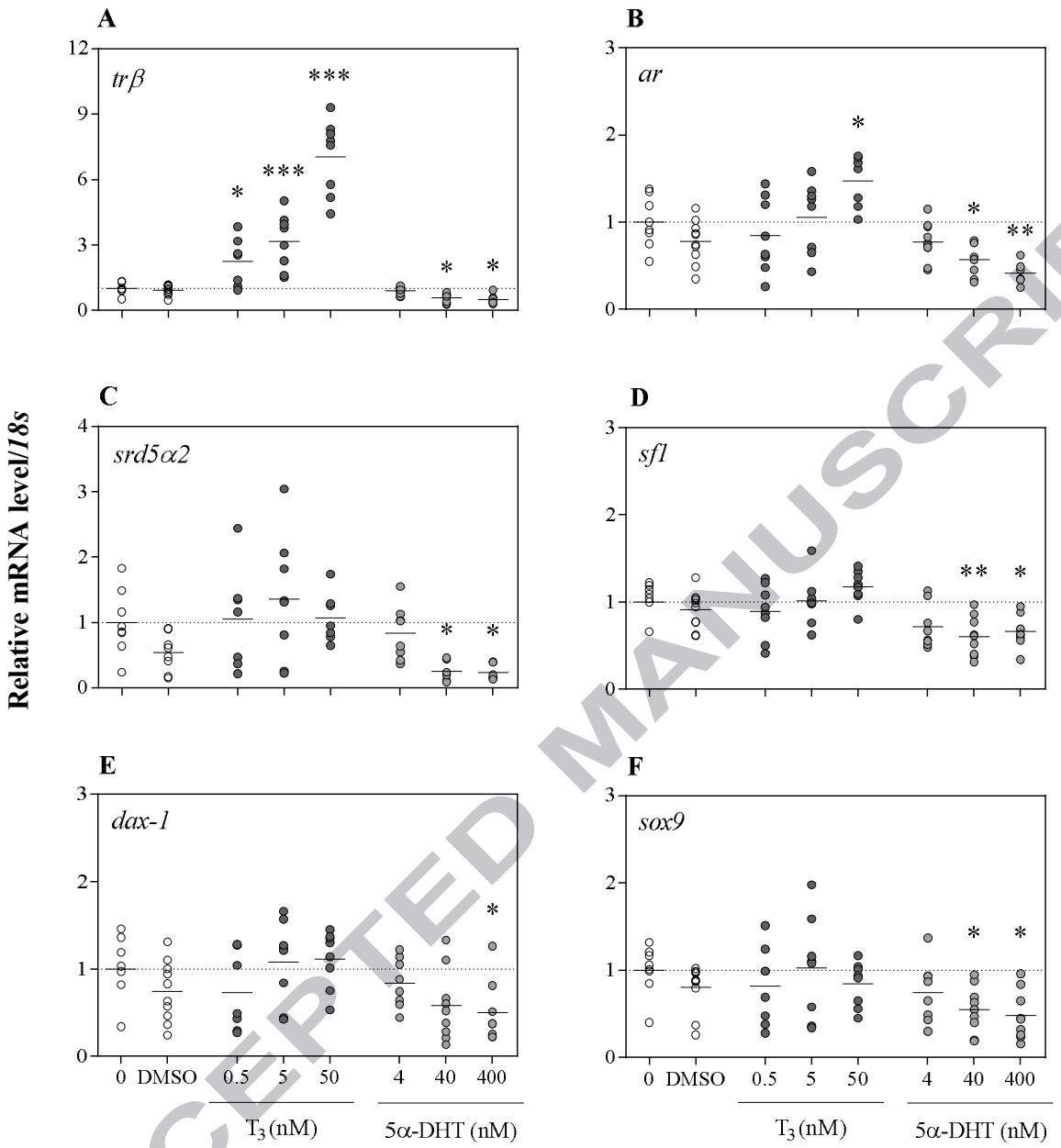

Figure 4. Expression of *trβ* (A), *ar* (B), srd5α2 (C), *sf1* (D), *dax-1* (E), and *sox9* (F) in whole *S. tropicalis* NF stage 46 larvae. Embryos were exposed to T3 (0.5, 5 or 50 nM) or 5α-DHT (4, 40, or 400 nM) from NF stage 12 to NF stage 46. Symbols represent individual samples (n = 5 – 8). Gene expression data are normalized to *18s* 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 test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Note that the scales of the y-axis vary. Control samples = open circles; T3-treatment = dark gray circles; 5α-DHT treatment = light gray circles.

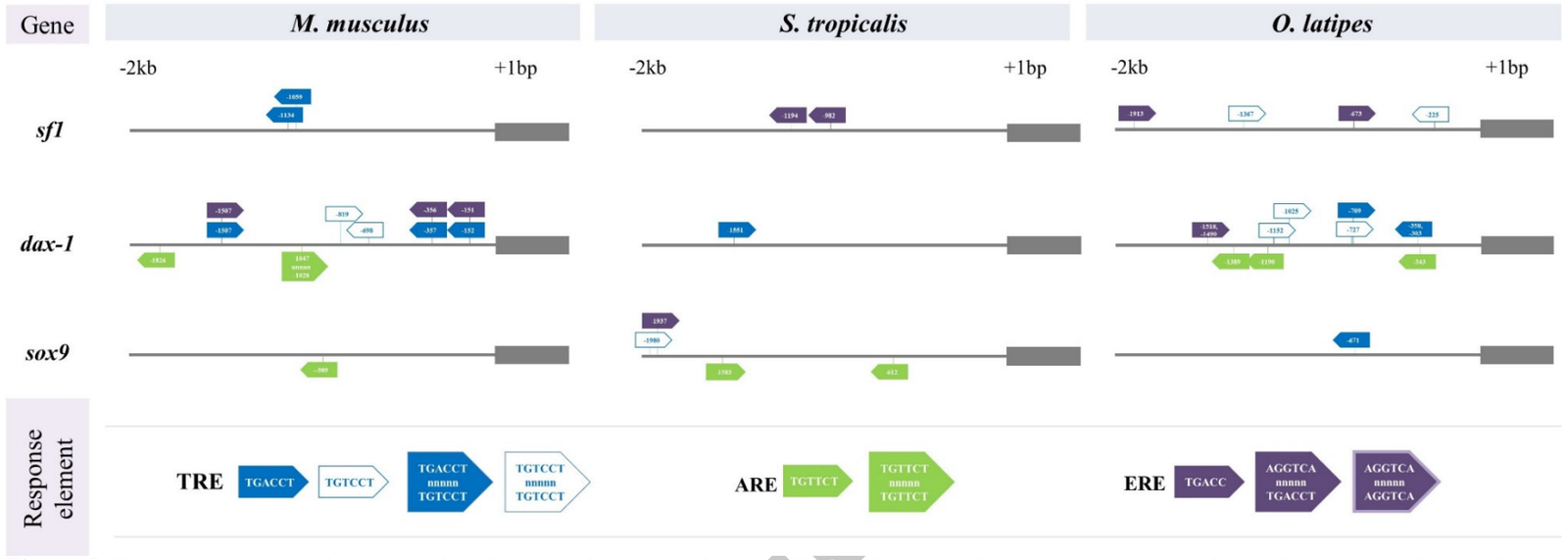

Figure 5. Promoter analysis of *M. musculus*, *S. tropicalis,* and *O. latipes sf1, dax-1*, and *sox9*. All sequences used for analysis were collected from the Ensembl Project (http://www.ensembl.org). Putative transcription factor binding sites within the putative promoter (-2000 to +1) were identified 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.

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Highlights

Expression of *sf1* and *dax-1* are regulated by thyroid hormones and androgens during *Silurana tropicalis* early development

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- Maternally transfered *sf1*, *sox9* transcripts detected in *Silurana tropicalis* embryos
- Expression in embryos exposed to co-treatments of iopanoic acid, T4, or T3
- IOP inhibited peripheral deiodinase activity with recovery by T3
- Exogenous 5α-DHT decreased *sf1* expression
- *In silico* analysis identified TH motifs in the putative promoter regions of *sf1* and *dax-1*