General and Comparative Endocrinology 267 (2018) 172–182

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

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

D.E.K. Campbell[a], V.S. Langlois[a,b,c,*]

[a] Biology Department, Queen's University, Kingston, ON, Canada
[b] Institut national de la recherche scientifique (INRS) – Centre Eau Terre Environnement, Quebec City, QC, Canada
[c] Department of Chemistry and Chemical Engineering, Royal Military College of Canada, Kingston, ON, Canada

ARTICLE INFO

Keywords:
Thyroid hormones
Androgens
Crosstalk
Iopanoic acid
Real-time RT-qPCR
Silurana tropicalis

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 (i.e., steroid 5α-reductase type 2 (*srd5α2*) and *erα*) and production (i.e., 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 systems (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 TH-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 sexually mature *S. tropicalis*.

Understanding mechanisms of sex steroid-regulation of the TH axis

* Corresponding author at: Institut national de la recherche scientifique (INRS) – Centre Eau Terre Environnement, 490, de la Couronne, Quebec City (Quebec) G1K 9A9, Canada.
E-mail address: valerie.langlois@inrs.ca (V.S. Langlois).

https://doi.org/10.1016/j.ygcen.2018.07.001
Received 2 December 2017; Received in revised form 5 May 2018; Accepted 3 July 2018
Available online 07 July 2018

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, 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 transcripts and sex-steroid production were examined in testis and ovary tissue and from the surrounding media, respectively, 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 in and hormone production from 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 and 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 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 the 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 the 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.