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Effects of thyroid endocrine manipulation on sex-related gene expression and population sex ratios in Zebrafish

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ABSTRACT

Thyroid hormone reportedly induces masculinization of genetic females and goitrogen treatment delays testicular differentiation (ovary-to-testis transformation) in genetic males of Zebrafish. This study explored potential molecular mechanisms of these phenomena. Zebrafish were treated with thyroxine (T4, 2 nM), goitrogen [methimazole (MZ), 0.15 mM], MZ (0.15 mM) and T4 (2 nM) (rescue treatment), or reconstituted water (control) from 3 to 33 days postfertilization (dpf) and maintained in control water until 45 dpf. Whole fish were collected during early (25 dpf) and late (45 dpf) testicular differentiation for transcript abundance analysis of selected male (*dmrt1*, *amh*, *ar*) and female (*cyp19a1a*, *esr1*, *esr2a*, *esr2b*) sex-related genes by quantitative RT-PCR, and fold-changes relative to control values were determined. Additional fish were sampled at 45 dpf for histological assessment of gonadal sex. The T4 and rescue treatments caused male-biased populations, and T4 alone induced precocious puberty in ~50% of males. Male-biased sex ratios were accompanied by increased expression of *amh* and *ar* and reduced expression of *cyp19a1a*, *esr1*, *esr2a*, and *esr2b* at 25 and 45 dpf and, unexpectedly, reduced expression of *dmrt1* at 45 dpf. Goitrogen exposure increased the proportion of individuals with ovaries (per previous studies interpreted as delay in testicular differentiation of genetic males), and at 25 and 45 dpf reduced the expression of *amh* and *ar* and increased the expression of *esr1* (only at 25 dpf), *esr2a*, and *esr2b*. Notably, *cyp19a1a* transcript was reduced but via non-thyroidal pathways (not restored by rescue treatment). In conclusion, the masculinizing activity of T4 at the population level may be due to its ability to inhibit female and stimulate male sex-related genes in larvae, while the inability of MZ to induce *cyp19a1a*, which is necessary for ovarian differentiation, may explain why its "feminizing" activity on gonadal sex is not permanent.

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1. Introduction

Genetic sex in most teleost fishes is chromosomally or polygenically determined and its phenotypic expression can be influenced by exogenous chemicals or ambient conditions (Patiño, 1997; Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002). While most studies of Zebrafish have concluded that its genetic sex is polygenically determined (Siegfried, 2010; Anderson et al., 2012; Liew et al., 2012), there is strong evidence for the existence of a WZ/ZZ sex determination system in natural (wild-type) Zebrafish populations (Sharma et al., 1998; Wilson et al., 2014) and for the loss of this system in intensively-selected laboratory strains (Wilson et al., 2014). Regardless of the genetic sex determination system or how strongly its expression is influenced by environmental conditions, key molecular mechanisms downstream of primary (master) sex-determining genes are likely to be shared among strains or species. For example, expression levels of the gonadal form of the aromatase gene (*cyp19a1a*), whose product catalyzes the conversion of androgens into estrogens, are generally higher at the onset of ovarian differentiation in putative females than in males of most teleosts (Guiguen et al., 2010), including Zebrafish (Orban et al., 2009; Siegfried, 2010).

Early gonads of genetic male Zebrafish pass through an ovarian-like phase containing meiotic oocytes, and subsequently transform into testes (Takahashi, 1977). It has been suggested that increased expression of *anti-Mullerian hormone* (*amh*) in genetic males suppresses the expression of *cyp19a1a* and redirects gonadal

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development towards testicular formation (Rodríguez-Marí et al., 2005; Wang and Orban, 2007). Another sex-related gene with dimorphic expression during the period of gonadal transformation in Zebrafish is *double-sex and MAB-3 related transcription factor* (*dmrt1*), which seems to increase in genetic males as testes begin to form (Orban et al., 2009; Siegfried, 2010). Relatively little information is available regarding the regulation and role of sex steroid hormone receptors during gonadal sex differentiation of teleosts. Some studies, however, have reported an increase in *androgen receptor* (*ar*) expression during testicular differentiation of Zebrafish (Hossain et al., 2008; Jørgensen et al., 2008). Also, sexually dimorphic expression of estrogen receptor β2 (*esr2a*) was observed during gonadal sex differentiation of Japanese Medaka (*Oryzias latipes*) (Chakraborty et al., 2011).

Sex steroids regulate or influence gonadal sex differentiation in most teleosts, including Zebrafish (Patiño, 1997; Strüssmann and Nakamura, 2002; Orban et al., 2009; Guiguen et al., 2010; Siegfried, 2010). There is also evidence implicating corticosteroid hormones in this process, especially in species where gonadal sex differentiation is influenced by environmental factors such as temperature (Hattori et al., 2009; Yamaguchi et al., 2010). Despite long-standing knowledge that thyroid hormones (TH) influence gonadal function in teleosts (Cyr and Eales, 1996; Carr and Patiño, 2011; Habibi et al., 2012), the possible contribution of the thyroid endocrine system to the regulation of gonadal sex differentiation has been largely ignored. Two earlier studies of Zebrafish found that manipulation of thyroid endocrine condition influences gonadal sex differentiation and may even cause sex-reversal. Namely, exposure to thyroxine (T4) during the larval phase causes male-biased populations (Mukhi et al., 2007; Sharma and Patiño, 2013) in a concentration-dependent manner (Sharma and Patiño, 2013). Conversely, goitrogen-induced hypothyroidism was associated with female-biased populations (Mukhi et al., 2007; Sharma and Patiño, 2013), but sex ratios reverted to control values as fish returned to euthyroid conditions following termination of the goitrogen treatment (Sharma and Patiño, 2013). Based on these observations, Sharma and Patiño (2013) concluded that hyperthyroidism during the larval phase causes permanent masculinization of genetic females but hypothyroidism only delays the ovary-to-testis transformation of genetic males. In the only other study available for teleosts, exposure to a TH-synthesis inhibitor (perchlorate) caused hermaphroditism in genetic females of Threespine Stickleback (*Gasterosteus aculeatus*) but did not induce full sex-reversal (Bernhardt et al., 2006). Among amphibians, responses to goitrogens are also species-dependent and include gonadal feminization (Hayes, 1998; Goleman et al., 2002) or masculinization (Hayes, 1998). The effect of TH on gonadal condition or sex ratio was not examined in the stickleback or amphibian studies. Furthermore, to our knowledge, sex-related gene expression profiles associated with thyroid-dependent changes in sex ratios have not been previously explored in any species.

The objective of this study is to determine the effects of thyroid endocrine manipulation on transcript levels of selected sex-related genes and their association with gonadal sex differentiation in Zebrafish. This study was conducted in parallel with a study of the association between thyroid condition and external body morphology that shared the same experimental fish populations (Sharma et al., 2016).

2. Methodology

2.1. Fish and general husbandry

Zebrafish broodstock were obtained from Aquatic Research Organisms, Inc. (Hampton, NH, USA). These fish are of unknown origin and originally supplied by commercial fish producers in Florida (USA). They are not deliberately bred for any traits and, according to terminology of Wilson et al. (2014), may be regarded as "natural" populations. Animal handling and experimental procedures were approved by the Texas Tech University Animal Care and Use Committee (protocol #09028-05C).

Broodstock were maintained and bred in the laboratory and fertilized eggs were obtained by group-spawning (4 males and 8 females per group) using previously described procedures (Sharma et al., 2016). Briefly, groups of 50 fertilized eggs, all derived from a single group-spawning batch, were initially placed in glass Petri dishes with 100 mL of reconstituted water consisting of 310 mg R/O Right salt mixture (Aquatic Eco-Systems, Inc., Apopka, FL, USA), 294 mg $CaCl_2{\cdot}2H_2O$, and 123.3 mg $MgSO_4{\cdot}7H_2O$ (Sigma-Aldrich, Saint Louis, MO, USA) per liter of reverse-osmosis water. At hatching (~72 h postfertilization, hpf), 40 individuals from each Petri dish were placed in respective 2-L beakers containing 800 mL of the appropriate treatment solutions. At 21 dpf, larvae were transferred to respective 20-L aquaria containing 15 L of the appropriate treatment solutions until 33 dpf, after which the solutions were replaced with reconstituted water through the end of the experiment (45 dpf). Temperature (28.5 °C) and photoperiod (12 h:12 h light:dark) were maintained constant throughout the experiment.

Husbandry procedures are described in Sharma et al. (2016). Briefly, fish were fed *Paramecium* spp. four times daily from 5 to 9 dpf; *Paramecium* in the morning and afternoon and Zeigler larval diet (150–250 μm) at noon from 10 to 11 dpf; and *Artemia* nauplii in the morning and afternoon and Ziegler diet (250–450 μm) at noon from 12 to 45 dpf. Approximately 80% of the rearing water was replaced with appropriate, pre-heated experimental solutions daily until 20 dpf, and from 21 to 45 dpf two thirds of the water was replaced every other day. Water quality was monitored to ensure maintenance within appropriate conditions.

2.2. Treatment solutions and experimental design

Treatments included a control (reconstituted water), T4 (CAS 51-48-9, Sigma-Aldrich), goitrogen (methimazole, MZ; CAS 60-56-0, Sigma-Aldrich), and rescue [MZ and T4] groups. Under this experimental design, a failure of co-treatment with MZ and T4 to rescue gene expression from the effect of MZ would suggest the existence of non-specific (non-thyroidal) mechanisms of MZ action.

Exposure concentrations of T4 (2 nM) and MZ (0.15 mM) were selected based on results of previous studies by our laboratory (Mukhi et al., 2007; Sharma and Patiño, 2013). Stock solutions of MZ were prepared in ultrapure water and T4 in dimethyl sulfoxide (DMSO; Sigma-Aldrich). To avoid the need for a solvent control, the same volume of DMSO carrier solution for T4 was added to all exposure solutions. The final concentration of DMSO in all test solutions was 0.00025% (v/v).

Each treatment was applied to 5 beakers/tank replicates and each replicate had an initial number of 40 individuals. This study followed the experimental design of earlier studies that reported a masculinizing effect of T4 in Zebrafish larvae (Mukhi et al., 2007; Sharma and Patiño, 2013); thus, exposures began at 72 hpf (yolk-sac larvae) and ended at 33 dpf (end of metamorphosis), for a total of 30 days. Samples were collected at 25 and 45 dpf to represent the early and late periods, respectively, of gonadal transformation from ovary-like structures to testes in genetic males (Uchida et al., 2002; Maack and Segner, 2003; Mukhi et al., 2007; Orban et al., 2009; Sharma and Patiño, 2013). At sampling, fish were euthanized by immersion in MS-222 (1 g/L tricaine methane sulfonate; Sigma-Aldrich) until opercula movement ceased. Two individuals from each replicate (total of 10 individuals per