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Are sex steroids essential for gonadal differentiation of the ornate frog, *Microhyla ornata*?

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ABSTRACT

Knowledge about sensitivities and responses of amphibian larvae to sex steroids and the chemicals alike is the first step towards understanding and assessing the effect of diverse chemicals that interfere with gonadal development and other endocrine functions. Herein, we used *Microhyla ornata* to determine the role of sex steroids on its gonad differentiation and sex ratio. Our results show that the exposure to increasing concentrations of estradiol-17β throughout larval development did not affect gonad differentiation resulting in 1:1 sex ratio at metamorphosis. But, females emerging from estradiol-17β treatment had larger ovaries with larger sized follicles. Further, testes of some males contained lumens, the number of which was dose dependent. Similarly, exposure to testosterone propionate had negligible effects on gonad differentiation. However, the mean diameter of the largest follicles was smaller in treated ovaries. Treatment of tadpoles with tamoxifen had no effect on gonad differentiation and ovary development while testicular development was accelerated at the highest concentration. Similarly, treatment of tadpoles with cyproterone acetate had little effect on gonad differentiation as well as development, hence the sex ratios at the end of metamorphosis. Further, in tadpoles exposed to increasing concentrations of formestane, gonad differentiation was normal resulting in 1:1 sex ratio. Thus, in *M. ornata*, both estradiol and testosterone are essential for the development of ovaries and testes respectively but, they are not critical to gonadal differentiation. Hence, the effects of sex steroids and other endocrine disrupting chemicals could be species-specific; different species may have differential sensitivities to such chemicals.

1. Introduction

Vertebrate sex differentiation is a highly coordinated and sequential process that is initiated at conception with the union of male and female gametes to establish genetic sex, progressing through the development of primary sex organs whose hormonal secretion in turn control the development of sex accessory structures and secondary sexual characters, and culminating in the development of sexual phenotypes. Two mechanisms of sex determination are prevalent in diverse taxonomic groups. In the majority of animal taxa with morphologically distinguishable heteromorphic sex chromosomes including birds and mammals, genetic factors play a critical role in primary sex determination (genetic sex determination, GSD) and gonad differentiation (Flament et al., 2011). However, the primary sex determination and gonad differentiation of fish, amphibians and reptiles are plastic to environmental factors (environmental sex determination, ESD), which can interfere with the genetic factors and even modify primary sex determination (Flament et al., 2011; Nakamura, 2009; Valenzuela and Lance, 2004).

Amphibian sex determination is primarily governed by genetic factors (GSD) but epigenetic factors such as temperature and sex steroids including steroid mimicking chemicals (endocrine disrupting chemicals; EDCs) of natural as well as anthropogenic origin can override GSD and induce sex reversal (Flament et al., 2011; Hayes, 1998; Nakamura, 2009, 2010; Piprek et al., 2012). Several studies have shown the importance of sex steroids in early gonadal development (Hayes, 1998; Nakamura, 2009, 2010; Piprek et al., 2012). Further, the significance of sex steroids in amphibian sex differentiation has also been shown using antiandrogens, antiestrogens and enzyme inhibitors of steroidogenesis (Cevasco et al., 2008; Chardard and Dournon, 1999; Hayes, 1998; Miyata et al., 1999; Miyata and Kubo, 2000; Petrini and Zaccanti, 1998). However, the role of sex steroids in controlling the primary sex determination and gonadal differentiation is still a matter of debate (Hayes, 1998; Nakamura, 2009). Moreover, their mechanism of action is not clear (Nakamura, 2010). Though, previous studies

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have implicated sex steroids as the key regulators of gonadal differentiation of amphibians, exposure of tadpoles to various sex steroids have yielded diverse results, raising doubts about the hormonal control of primary sex determination (Nakamura, 2010). For instance, sex steroids can induce complete, partial or no sex reversal depending on the species (Gyllenhammar et al., 2009; Hayes and Menendez, 1999; Hu et al., 2008; Miyata et al., 1999; Park and Kidd, 2005; Phuge and Gramapurohit, 2015; Saidapur et al., 2001). Surprisingly, sex steroids can have paradoxical effects on gonad differentiation depending on the species and the concentration used (Flament et al., 2011; Hayes, 1998; Hogan et al., 2008; Piprek et al., 2012). These diverse results could be attributed to species-specific differences in sensitivities and responses to various sex steroids (Flament et al., 2011; Piprek et al., 2012; Storrs and Semlitsch, 2008; Villalpando and Merchant-Larios, 1990). While this may be true to some extent, several other aspects of gonadogenesis such as the pattern of gonad differentiation, developmental stages of exposure and the rate of gonad development might also contribute to such variations (Wallace et al., 1999). The stage of exposure *per se* can significantly alter the final outcome of experiments (Hogan et al., 2008). Moreover, results of the hormonal control of sex differentiation in amphibians are compounded by the complex way of their gonadal development (Hayes, 1998; Nakamura, 2009, 2010). In the majority of amphibians with the undifferentiated type of gonad differentiation, testicular development is initiated during or after metamorphosis. Exposure to steroid hormones before or after these critical periods may immensely affect the results of experiments (Hogan et al., 2008). Similarly, the rate of ovarian development in relation to somatic development varies in amphibians (Ogielska and Kotusz, 2004; Storrs and Semlitsch, 2008). Exposure to sex steroids during non-sensitive stages might lead to erroneous results. Hence, while using amphibian tadpoles as model systems in understanding the hormonal control of gonadal differentiation, experiments should be carefully designed by considering these aspects.

In recent years, the decline of amphibian populations around the world is a major concern and many environmental contaminants acting as steroid mimics (thus interfering with normal gonadal differentiation/development) are implicated in their decline (Blaustein et al., 2011; Stuart et al., 2004). In this context, determining the effect of sex steroids on gonad differentiation in addition to accurate descriptions of gonadogenesis in species inhabiting diverse habitats and geographic regions is crucial. Such studies will help in determining species-specific differences in the sensitivities and responses to steroid hormones and accordingly prioritise conservation measures. Hence, in the present study, tadpoles of *Microhyla ornata* were used to assess the effect of sex steroids on gonadal differentiation and sex ratio at metamorphosis. The ornate frog, *M. ornata* was selected as a model system due to its abundance and wide distribution throughout India. The frog breeds in diverse habitats that are prone to various contaminants through agricultural runoff. Moreover, eggs, embryos and tadpoles are seen at the surface of water thus are prone to endocrine disruption by surface water contaminants.

2. Materials and methods

2.1. Collection and maintenance of animals

Two egg masses of *M. ornata* floating over the surface of water were collected from a semi-permanent pond situated on the Savitribai Phule Pune University campus (18° 55′N and 73° 82′E) on June 4, 2011 and quickly transported to the laboratory where they were maintained in a glass aquarium (60 cm × 45 cm × 15 cm) with aged tap water until hatching. Subsequently, hatchlings from both the egg masses were mixed thoroughly and maintained in another glass aquarium (60 cm × 45 cm × 15 cm) until used for experimentation. Developmental stages were identified according to Gosner (1960) and the tadpoles were used for experimentation on reaching stage 25.

To assess the effect of sex steroids on gonadal differentiation/development, different sex steroids, an antiestrogen, an antiandrogen and an aromatase inhibitor were used. Tadpoles of *M. ornata* were exposed to increasing concentrations of estradiol–17β (E_2), testosterone propionate (TP), tamoxifen (TAM), cyproterone acetate (CA) and formestane (FOR) at different developmental stages depending on the experimental design. Concentrations of chemicals chosen were based on the previous literature and size of the tadpoles (reviewed in Hayes, 1998; Phuge and Gramapurohit, 2015; Saidapur et al., 2001). All the chemicals used for experimentation were procured from Sigma (USA) except TP, which was obtained from FLUKA (Netherlands).

2.1.1. Experiment I

To determine the effect of sex steroids on gonad differentiation, the rate of development and sex ratio at metamorphosis, tadpoles were exposed to 0 (control), 12.5, 25 and 50 μg/L of E_2, TP, TAM, CA and FOR throughout larval development (stage 25–42). Tadpoles were reared in glass aquaria (45 cm × 30 cm × 10 cm) at a density of 5/L with 5 L of aged tap water. The aquaria were maintained at 24 ± 1 °C with 12:12 h light and dark cycle and the tadpoles were fed with zooplanktons, shredded spinach and egg albumin. All the treatments were replicated twice. In all, 50 tadpoles were used for each concentration of each chemical except for 25 μg/L TP group. The majority of tadpoles (80%) from one aquarium of 25 μg/L TP-treated groups died on the first day due to unknown reason. Hence, 20 more tadpoles were used for experimentation. Therefore, 70 tadpoles were exposed to 25 μg/L of TP. Freshly prepared steroids were added after the renewal of water on every alternate day. Stock solutions of the steroids were prepared by dissolving them in absolute ethanol (MERCK) to obtain the desired concentration. The stock solutions were prepared freshly after every 15 days and stored in the dark at 4 °C. The final concentration of ethanol in all treatments including the control group was 20 μl/L of water (0.002%). Duration of the treatment varied between 98 and 120 days depending on the chemicals.

2.1.2. Experiment II

In this experiment, tadpoles were exposed to 0, 50 μg/L of E_2, TP or TAM from stage 25–28 (during ovarian differentiation) to determine the existence of a sensitive window, if any, for gonadal sex reversal. Duration of exposure was 20–25 days. On reaching stage 29, the treatment was terminated and tadpoles were reared in steroid free water until stage 42.

2.1.3. Experiment III

To determine the role of androgens in controlling testicular differentiation, tadpoles were treated with 0, 50 μg/L E_2, TP or CA from stage 38–42 (during testicular differentiation) for a period of 16–20 days. Tadpoles from stage 25–37 were reared in steroid free water and then treated with different chemicals from stage 38–42.

On reaching stage 42 (metamorphic climax), tadpoles of all the experiments were transferred to plastic containers with little water to facilitate the completion of metamorphosis. Experimental units were monitored daily and mortality of tadpoles, if any, was recorded for each concentration of each experiment. At the end of metamorphosis, body mass, snout-vent length (SVL) and larval period were recorded for each individual of each experiment. Body