General and Comparative Endocrinology 236 (2016) 63–69

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Research paper

Methyltestosterone alters sex determination in the American alligator (*Alligator mississippiensis*)

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ARTICLE INFO

Article history:
Received 11 February 2016
Revised 3 May 2016
Accepted 7 July 2016
Available online 9 July 2016

Keywords:
Methyltestosterone
Temperature-dependent sex determination
Androgen
Crocodilian

ABSTRACT

Effects of xenobiotics can be organizational, permanently affecting anatomy during embryonic development, and/or activational, influencing transitory actions during adulthood. The organizational influence of endocrine-disrupting contaminants (EDC's) produces a wide variety of reproductive abnormalities among vertebrates that exhibit temperature-dependent sex determination (TSD). Typically, such influences result in subsequent activational malfunction, some of which are beneficial in aquaculture. For example, 17-αmethyltestosterone (MT), a synthetic androgen, is utilized in tilapia farming to bias sex ratio towards males because they are more profitable. A heavily male-biased hatchling sex ratio is reported from a crocodile population near one such tilapia operation in Guanacaste, Costa Rica. In this study we test the effects of MT on sexual differentiation in American alligators, which we used as a surrogate for all crocodilians. Experimentally, alligators were exposed to MT *in ovo* at standard ecotoxicological concentrations. Sexual differentiation was determined by examination of primary and secondary sex organs post hatching. We find that MT is capable of producing male embryos at temperatures known to produce females and demonstrate a dose-dependent gradient of masculinization. Embryonic exposure to MT results in hermaphroditic primary sex organs, delayed renal development and masculinization of the clitero-penis (CTP).

1. Introduction

Recent theory maintains that most vertebrates exist somewhere along a continuum between strict genetic control of sex determination and strict environmental control (Sarre et al., 2004) and that placement along this continuum exists as a function of environmental influence on fitness (Charnov and Bull, 1977). Genotypic sex determination (GSD) is a preset mechanism of primary and secondary sexual structure differentiation based on the presence and subsequent expression of a single gene, or lack of that gene, resulting in production of a default sex (gene absent) or the alternate sex (gene present). In mammals, for example, the SRY (or SOX9) gene steers development away from a default female phenotype via promotion of a male reproductive tract and simultaneous suppression of a female tract (Kovacs and Ojeda, 2011). While GSD allows for a heritable sex, it can be influenced by gonadal sex disorders (Kovacs and Ojeda, 2011).

Environmental sex determination (ESD) is susceptible to many more external influences (Wibbels et al., 1994). Temperature dependent sex determination (TSD), a form of ESD, relies on specific thermal regimes to dictate the expression of aromatase and/or reductase, thus governing the sex steroid regime of the developing embryo and subsequent sex characteristics (Wibbels et al., 1994). Thermal thresholds or 'critical' temperatures, that serve as a thermal pivot between development of one sex or the other, are

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http://dx.doi.org/10.1016/j.ygcen.2016.07.007
0016-6480/

species-specific and vary widely among vertebrates with TSD (Janzen and Paukstis, 1991). TSD mechanisms also vary among taxa and three main mechanisms exist; male production at low temperatures and female at high, female production at low temperatures and male at high, and female at low, male at intermediate, and female at high temperatures (Valenzuela, 2004). A remarkable series of sex steroid and temperature manipulations have elucidated some constants among TSD mechanisms; 1) A critical period exists at which an embryo's sex determination is sensitive to both temperature and sex steroids; 2) feminization and masculinization are mediated by steroid-specific receptors; 3) aromatase and reductase inhibitors can manipulate sex regardless of temperature, however with unequal potency; 4) response to estrogens can produce female embryos at male-producing temperatures, but androgens can only masculinize embryos at threshold temperatures; and 5) mixed sex ratio clutches are produced at threshold temperatures rather than hermaphrodites (Wibbels et al., 1991, 1994; Wibbels and Crews, 1994; Crews et al., 1994; Wibbels and Crews, 1995; Crews; 1996).

Our understanding of how hormone sources, storage, and internal feedback mechanisms affect sexual differentiation mechanisms has greatly improved as a result of research in temperature-dependent steroid expression and utilization (Kamel and Kubajak, 1987; Janzen et al., 1998; Paitz and Bowden, 2011; Pfannkuche et al., 2011; Paitz et al., 2012). Unfortunately, so has our understanding of anthropogenic influences on such processes (Guillette et al., 1995). Numerous industrial and agricultural compounds, when introduced to natural systems, have endocrine-disrupting affects. Endocrine-disrupting compounds (EDCs) have negative activational effects on endocrine systems, but more daunting is the organizational role they play as hormone mimics during sexual differentiation and embryonic organization (Guillette et al., 1995). Because sexual differentiation mechanisms vary among reptiles, these taxa have become model indicators of EDC exposure. Among reptiles, contaminants that mimic estrogen are common (e.g. PCBs, Dioxin, Furans, DDE), while environmental androgens are far more rare, presumably as a result of the aromatizable nature of testosterone and the dominance of the estrogen pathway associated with TSD (Wibbels and Crews, 1995).

Among reptiles the most notable sentinel taxa for EDCs are crocodilians (Milnes and Guillette, 2008). Their popularity as 'model' organisms emerged because of the Lake Apopka superfund site, where dicofol, DDT and subsequent metabolites were discovered as contaminants in 1980 (Guillette et al., 1994). Such contaminants were deemed potent environmental estrogens after female alligators displayed unnaturally high 17β -estradiol plasma concentrations, polyovular follicles, polynuclear oocytes (Guillette et al., 1994), and reduced gonadal-adrenal mesonephros (GAM) aromatase activity (Crain et al., 1997). Males exhibited decreased plasma testosterone, reduced phallus size (Guillette et al., 1996) and poorly organized testes (Guillette et al., 1994). This case study exemplified the utility of crocodilians in understanding the activational and organizational effects of EDCs.

In Guanacaste, Costa Rica, three large tilapia farms utilize 17-αmethyltestosterone (MT) to produce all-male offspring that grow faster and reach larger maximum size than females. Preliminary data on MT persistence in water and soil was noted during initial testing of this fish farming practice, however, lipid persistence of the compound and its effects on vertebrates other than fish are unknown (Phelps and Popma, 2000; Gupta and Acosta, 2004). The nearby Tempisque Basin harbors a rapidly expanding population of American crocodiles (*Crocodylus acutus*) that exhibits a male-biased sex ratio (Bolaños-Montero, 2012; Murray et al., 2015). Hatchling sex ratios from this population do not match the ratios predicted by clutch thermal regimes and this sex ratio bias differs among clutches, with some clutches being male-biased and others not (Murray et al., 2016). Here, we test the potential for MT to produce male crocodilian embryos at female-producing temperatures and histologically analyze organizational effects of urogenital development from MT exposure during the experimental assay.

2. Materials and methods

2.1. Experimental assay

For this experiment, 108 American alligator (*Alligator mississippiensis*) eggs were collected from five clutches in June 2013 and 76 eggs from three clutches in June 2014. All eggs were collected at J. D. Murphree Wildlife Management Area, Port Arthur, TX, within five days of deposition, as assessed by daily nest monitoring and the width and length of banding (Masser, 1993). Eggs were transported to an Auburn University live animal facility and incubated at 28 °C, a female-producing temperature (Lang and Andrews, 1994). In 2013, eggs were misted with water daily in an incubator (Fisher Scientific, Isotemp model 655D) to maintain humidity. However, 50 eggs failed to complete development, likely because of dehydration. Therefore, eggs in 2014 were maintained at approximately 100% humidity using a vermiculite substrate and steam heating. Each year, four eggs were opened periodically to stage the embryos as described by Ferguson (1985), resulting in 126 experimental eggs in total. Prior to the temperature-sensitive period each year (stage 20, when sex determination occurs; Lang and Andrews, 1994), eggs were randomly assigned to one of five treatments using a random number generator. Eggs were randomly dispersed among plastic bins in the incubator with 10–14 eggs per treatment per year. Two treatment groups served as controls. One control received no treatment while the other received 5 μl of 95% ethanol (ETOH) to control for effects of the vehicle used to deliver MT to all treatment groups. Treatment groups received 4 μg/ml, 40 μg/ml, or 400 μg/ml of 17α- MT in 95% ETOH. These treatments exposed eggs to between 10 and 1000 times the natural amount of testosterone in alligator egg yolk (Conley et al., 1997), a range of doses standard for ecotoxicological dose-response assays with sex steroid hormones or related endocrine-disrupting compounds (Wibbels and Crews, 1995; Crain et al., 1997). Treatments were applied topically as 5 μl of solution deposited on the surface of an egg at stage 21, a technique that is used to transport compounds inside reptilian eggshells (Crews et al., 1991, Paitz et al., 2012). Using this method, Crews et al. (1991) found that at least 90% of applied compound was incorporated into the embryo. Additional eggs were incubated separately at male-producing temperatures (32 °C) to serve as control males for primary and secondary sex organ comparison.

2.2. Methyltestosterone quantification

Upon hatching, yolk samples were collected and frozen to quantify the concentration of 17α- MT that reached the embryo. Steroid hormones were extracted from egg yolk using a 3:2 volume solution of ethyl acetate and hexane, respectively. Samples were dried under vacuum at 25 °C and dissolved in 100 μL assay buffer supplemented with 10 μL of DMSO to encourage dissolution. 17α-methyltestosterone concentrations were quantified using a sandwich ELISA kit (MaxSignal® methyltestosterone kit, Bioo Scientific, Austin, TX; Rabbit; polyclonal). Cross-reactivity with testosterone was 0.3% and samples were not analyzed in duplicate as all other wells were occupied for another study. Optical density was determined using a Benchmark Plus microtiter plate spectrophotometer (Bio-Rad, Hercules, CA) at 450 nm. Hatchlings were individually marked via caudal scute removal, and snout-to-vent