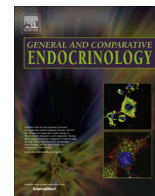




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Exogenous application of estradiol to eggs unexpectedly induces male development in two turtle species with temperature-dependent sex determination



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ABSTRACT

Steroid hormones affect sex determination in a variety of vertebrates. The feminizing effects of exposure to estradiol and the masculinizing effects of aromatase inhibition during development are well established in a broad range of vertebrate taxa, but paradoxical findings are occasionally reported. Four independent experiments were conducted on two turtle species with temperature-dependent sex determination (*Chrysemys picta* and *Chelydra serpentina*) to quantify the effects of egg incubation temperature, estradiol, and an aromatase inhibitor on offspring sex ratios. As expected, the warmer incubation temperatures induced female development and the cooler temperatures produced primarily males. However, application of an aromatase inhibitor had no effect on offspring sex ratios, and exogenous applications of estradiol to eggs produced male offspring across all incubation temperatures. These unexpected results were remarkably consistent across all four experiments and both study species. Elevated concentrations of estradiol could interact with androgen receptors or inhibit aromatase expression, which might result in relatively high testosterone concentrations that lead to testis development. These findings add to a short list of studies that report paradoxical effects of steroid hormones, which addresses the need for a more comprehensive understanding of the role of sex steroids in sexual development.

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1. Introduction

Whether an embryo develops into a male or female has important consequences on its morphology, physiology, behavior, and ultimately its fitness. Indeed, males and females differ in most aspects of their biology, and this observation has led to numerous questions about the underlying mechanisms that determine the trajectory of sexual development (Mittwoch, 1996, 2000). Research on this topic has revealed a remarkable diversity of sex-determining mechanisms (SDMs), ranging from SDMs completely dependent upon genetic factors that reside on sex chromosomes (genotypic sex determination; GSD) to those that are primarily dependent upon environmental factors experienced during embryogenesis (environmental sex determination; ESD). Even within SDMs,

numerous factors can interact to impact the sexual development of an individual (Kozielska et al., 2006; Radder et al., 2009; Warner et al., 2013). For example, in species that exhibit temperature-dependent sex determination (TSD, a form of ESD where temperature during development determines offspring sex), sex steroids can also influence the differentiation of gonads in ways that permanently affect an individual's sex (Wibbels and Crews, 1994; Bowden et al., 2000; Elf, 2004; Warner et al., 2009).

The hormonal environment surrounding embryos affects whether gonads differentiate into testes or ovaries, which has been repeatedly confirmed by experimental manipulation of the hormonal environment of developing embryos (e.g., Wibbels and Crews, 1992; Crews, 1996; Bowden et al., 2000). For example, application of 17 β estradiol to eggs almost always feminizes gonads, even at male-producing temperatures under TSD and in species that have sex chromosomes (Freedberg et al., 2006). Although this pattern is broadly consistent across taxa, estrogen-induction of male development has been reported (Hayes, 1998; Janes et al., 2007). In contrast, inducing male development with an application of testosterone to eggs has received less success, particularly when

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eggs are incubated at female-producing temperatures (Wibbels and Crews, 1992). This is likely because female-producing temperatures increase aromatase activity, thereby converting testosterone into estradiol (Wibbels and Crews, 1992; Rhen and Lang, 1994), and this hypothesis is supported by the induction of male sex determination using non-aromatizable androgen treatments (Wibbels et al., 1992). By converting androgens to estrogens, aromatase plays a key role in sex determination. This is exemplified by studies that block aromatase activity, which facilitates male development even under female-producing temperatures (reviewed in Warner, 2011). As a result, sex-reversed males have similar gonad morphology to normal males, exhibit normal male behaviors, and are capable of spermatogenesis as adults (Elbrecht and Smith, 1992; Piferrer et al., 1994; Wennestrom and Crews, 1995; Chardard and Dournon, 1999; Shine et al., 2007; Warner and Shine, 2008; Warner et al., 2010).

These hormonal manipulations not only have provided an important perspective on the role of steroids in sex determination, but also have yielded novel insights into the sex-specific effects of incubation temperature in many reptiles with TSD. For example, because incubation temperature and sex are naturally confounded under TSD, these sex manipulation approaches are useful in decoupling these factors to understand their independent effects on offspring fitness. This approach has been used on the common snapping turtle (*Chelydra serpentina*) and demonstrated that variation in offspring growth is influenced by incubation temperature and is not due to an innate difference between the sexes (Rhen and Lang, 1995). These hormonal manipulations have also been important in experimentally testing the leading model for the adaptive significance of TSD (Charnov and Bull, 1977), which states that incubation temperature has a differential effect on male and female fitness (i.e., TSD enables each sex to develop at its optimal incubation temperature). Indeed, by applying an aromatase inhibitor to eggs of the jacky dragon (*Amphibolurus muricatus*), Warner and Shine (2005, 2008) showed that males produced from eggs incubated at normal male temperatures have greater reproductive success than sex-reversed males produced under female temperatures (and likewise for females).

Although these hormonal manipulations have provided insights into the underlying physiological mechanisms of TSD and its adaptive significance, the generality of such endocrinological effects across species with TSD is still poorly studied. This is a valid concern because the pathways involved in sex determination under TSD could vary among taxa (Uller et al., 2007; Uller and Helantera, 2011), and therefore the impact of hormone application may diverge depending on a variety of factors (e.g., timing of application, genetic variation). In addition, different doses of chemicals to eggs could even have opposing effects on sexual differentiation (Hayes, 1998; Mori et al., 1998; Parmigiani et al., 2000), resulting in non-intuitive patterns. For example, prenatal exposure to small amounts of estradiol increases prostate size in mice, and excessive androgens can feminize fish (Mori et al., 1998; Devlin and Nagahama, 2002). In the frog *Rana pipiens*, estradiol can induce female development as expected, but relatively high doses result in 100% males (Richards and Nace, 1978; Hayes, 1998).

In this study, the effects of exogenous estradiol and an aromatase inhibitor on sex determination were quantified in two species with TSD, the painted turtle (*Chrysemys picta*) and the common snapping turtle (*C. serpentina*). Although both species have been studied extensively, hormone manipulation experiments have only previously been performed on *C. serpentina* and not on *C. picta*. This study was replicated four times (with independent, but similar experimental designs) due to paradoxical findings in the first experiment, and results demonstrate across all four studies that estradiol consistently induces male gonad development in offspring even at normally female-producing incubation temperatures.

2. Materials and methods

2.1. Study species

This work focused on two common turtle species that have been used extensively for research on TSD. The painted turtle (*C. picta*) ranges across most of the United States and southern Canada. At our study site (Thomson, Illinois), relatively warm constant incubation temperatures (>29 °C) produce female offspring and cool incubation temperatures (<27 °C) produce males. The pivotal temperature that produces a balanced sex ratio is about 28 °C (Paukstis and Janzen, 1990). The effects of exogenous application of estradiol or aromatase inhibitors have not previously been evaluated in this species.

The common snapping turtle (*C. serpentina*) also is distributed throughout much of the United States and southern Canada, as well as Central America and northern parts of South America. The effect of constant incubation temperature on primary sex ratios in *C. serpentina* differs considerably from that of *C. picta*, in that both cool and warm constant temperatures produce female offspring and intermediate temperatures produce males. At the study site in Illinois, constant temperatures <21 °C and >29 °C produce mostly females, and constant incubation at 24.5–26 °C produces males. Pivotal temperatures occur around 21.5 °C and 27.5 °C (Janzen, 2008). For this species, studies of a Minnesota population found that exogenous application of estradiol to eggs induces female development, whereas application of an aromatase inhibitor produces males (Rhen and Lang, 1994, 1995).

2.2. Collection of eggs

Four independent experiments were designed to evaluate the effect of exogenous applications of estradiol and an aromatase inhibitor on sex determination; these studies took place in 2008, 2010, and 2011 for *C. picta*, and in 2009 only for *C. serpentina*. For all years, known nesting areas were regularly searched for actively nesting females. When nesting females were observed, their location was marked and their eggs were retrieved later that day (typically within 3 h of oviposition). However, in 2008, 23 of the 44 *C. picta* clutches were obtained from females (captured during terrestrial nesting forays) by inducing oviposition with an injection of oxytocin; in later years all females were allowed to nest naturally. Nests were carefully excavated and single clutches were put into individual containers with moist sand. Eggs were gently cleaned of soil or sand, and then immediately weighed and individually labeled with a Sharpie marker. All eggs were kept cool prior to transport to Iowa State University where they were placed into incubation temperature treatments within 1–9 days of oviposition.

2.3. Experimental designs

Experimental designs were similar among years, but differed in some key aspects (Table 1). All experiments had a full factorial design to evaluate the effect of egg incubation temperature, chemical treatment (estradiol, aromatase inhibitor, control), and their interaction on offspring sex ratios.

2.3.1. *C. picta*

In 2008, eggs were randomly assigned to a position in a 4 × 5 matrix among 24 plastic shoeboxes and were half buried in moist vermiculite (–150 kPa). The shoeboxes were placed in one of three incubators (8 shoeboxes in each incubator) set at (1) a warm temperature (30 °C) known to produce females, (2) an intermediate temperature (28 °C) that produces a balanced sex ratio, and (3) a cool temperature (26 °C) that produces male offspring (Paukstis

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