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Evolution of Developmental Control Mechanisms

Predetermination of sexual fate in a turtle with temperature-dependent sex determination

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ABSTRACT

Egg incubation temperature determines offspring sex in many reptilian species, including red-eared slider turtles, where embryos incubated at low temperatures during the initial stages of gonad formation develop as males, while those kept at higher temperatures develop as females. Incubation at the threshold, or pivotal, temperature (PvT) yields an even ratio of males and females. This strong susceptibility to temperature indicates that each embryo of this species is competent to develop as a male or a female. However, the mechanism that determines sexual fate at the PvT has not been identified. One possibility is that sexual fate is stochastic at the PvT, but coordinated by systemic signals within a single embryo. If this is the case, gonads explanted separately to culture should not coordinate their fate. Here we show that gonad pairs from embryos incubated at the PvT share a strong predisposition for one sex or the other when cultured in isolation, indicating that they were affected by shared genetic signals, maternally-deposited yolk hormones or other transient influences received prior to the stage of dissection. *In ovo* studies involving shifts from the male- or female-producing temperature to the PvT further indicate that embryos adopt a sexual differentiation trajectory many days prior to the onset of morphological differentiation into testes or ovaries and usually maintain this fate in the absence of an extreme temperature signal favoring the development of the other sex. Our findings therefore suggest that the outcome of sex determination in these reptiles is heavily influenced (i) by an inherent predisposition at the PvT and (ii) by the sexual differentiation trajectory established early in gonad development under male- or female-producing temperatures.

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Introduction

Unlike mammals and birds, which exhibit stable systems of genetic sex determination (GSD) and highly differentiated sex chromosomes, reptiles have evolved an extraordinary array of sex-determining mechanisms (Janzen and Phillips, 2006), with many species relying on environmental rather than genetic signals to determine offspring sex. Many different types of male (XX/XY) and female (ZZ/ZW) heterogamety have been reported among GSD reptiles, with different pairs of autosomes serving as the sex chromosomes and with varying levels of heterogametic differentiation (Pieau et al., 1999; Graves and Shetty, 2001; Kawai et al., 2007; Badenhorst et al., 2013). However, in all crocodilians so far tested and many turtles and lizards, embryonic sex is regulated by the incubation temperature of the egg during the middle third of development when the gonads are forming (temperature-dependent sex determination (TSD)) (reviewed by Pieau et al. (1999)).

Phylogenetic analyses indicate that the ancestral sex-determining mechanism in the vertebrate lineage was GSD, so a transition from GSD to TSD must have occurred at least once during reptilian evolution (Janzen and Krenz, 2004). Further evidence indicates that multiple independent transitions from TSD back to GSD subsequently took place as reptiles diverged (Janzen and Krenz, 2004; Janzen and Phillips, 2006; Pokorna and Kratochvil, 2009). Retention of some degree of thermosensitivity in GSD species may underlie these evolutionary transitions (Quinn et al., 2011). Indeed, in certain reptiles, egg incubation temperature can override a GSD mechanism, even when heteromorphic sex chromosomes are present. Such a system has been described in the dragon lizard *Pogona vitticeps*, which utilizes a ZZ/ZW GSD system (Ezaz et al., 2005) that can be overridden at high temperatures to produce ZZ females (Quinn et al., 2007). Similarly, the skink *Bassiana duperreyi* exhibits XX/XY heterogamety, but exposure to low temperatures causes XX females to develop as males (Shine et al., 2002; Radder et al., 2008; Quinn et al., 2009). Furthermore, in the GSD turtle *Apalone mutica*, the expression of several genes involved in gonad development is affected by egg

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incubation temperature, again supporting the idea that GSD systems may retain some sex-related thermosensitivity (Valenzuela, 2008). Collectively, these phenomena suggest that the border between TSD and GSD is blurred, and that both systems can, at least in part, stably exist simultaneously (Sarre et al., 2004; Barske and Capel, 2008).

The TSD phenomenon, first reported in a reptile 47 years ago (Charnier, 1966), typically manifests as one of three main patterns: MF or FM, in which one sex is produced at low temperatures and the other at high temperatures, or FMF, in which females develop at both high and low temperatures and males at intermediate temperatures (Ewert et al., 1994). Both sexes are produced at temperatures between the MPT and FPT(s), with the threshold, or pivotal, temperature (PvT) defined as the temperature(s) that produces an average sex ratio of 1:1 (Mrosovsky and Pieau, 1991; Ewert et al., 1994). Functional hermaphroditism has not been reported in reptiles, though in some species, intersex gonads are occasionally observed in hatchlings incubated at the PvT; these typically resolve into testes over time (Pieau et al., 1998). Because of this strong susceptibility to temperature, each embryo of a TSD species is theoretically competent to develop as male or female. However, the mechanism that determines whether an individual will develop as a male or a female at the PvT has not been elucidated.

Sex determination at the PvT could be stochastic. However, the fact that sexual outcomes at the PvT are concordant *in ovo*, i.e., individuals develop either two testes or two ovaries, indicates that development of the two bipotential gonads within a single embryo is coordinated. This could result from shared genetic information or from systemic signals originating from the yolk or the embryo itself. Indeed, it has been proposed that cryptic genetic mechanisms determine sex at the PvT, but are masked at more extreme temperatures (Zaborski et al., 1988; Pieau et al., 1998). Such mechanisms could potentially rely on the cumulative effects of multiple loci across the genome or within a pair of microscopically indistinguishable (homomorphic) sex chromosomes.

The limited evidence for cryptic heterogamety in TSD species largely derives from HY antigen studies performed in the 1980s. HY antigen is a minor histocompatibility marker linked to sex. In mammals, only males are positive for HY antigen, consistent with the fact that several components of the antigen map to the Y chromosome (reviewed by Wolf, 1998). Conversely, among non-mammalian vertebrates with differentiated sex chromosomes, the heterogametic sex tested positive for HY antigen in nearly all species analyzed (Nakamura et al., 1987), i.e., males in species with XX/XY heterogamety and females in species with ZZ/ZW heterogamety. While the nature of HY's involvement in sex determination or differentiation remains unresolved and controversial (Wolf, 1998), HY antigen status has provided a useful way of determining the heterogametic sex in GSD species with homomorphic sex chromosomes (Engel et al., 1981).

Differences in HY status between males and females are less straightforward in TSD species. In *Trachemys scripta*, a small sample of females tested positive for HY antigen in several tissues, suggestive of cryptic ZZ/ZW heterogamety (Engel et al., 1981; Nakamura et al., 1987). However, egg incubation temperature during the TSP was not known for the individuals assayed. More definitive experiments were performed in *Emys orbicularis*. Here, Zaborski and colleagues found that HY status in the gonad reflects phenotypic sex, whereas serological status may reflect genotypic sex (Zaborski et al., 1982, 1988). Under this hypothesis, HY-negative phenotypic females derived from FPT eggs represent thermally sex-reversed genotypic males, whereas HY-positive phenotypic males from MPT eggs represent sex-reversed genotypic females. The authors postulated the existence of a cryptic ZZ/ZW sex chromosome system in *E. orbicularis* (Zaborski et al., 1982), based on the assumption that HY positivity in blood is associated with the heterogametic sex in TSD turtles as in other vertebrates. However, no follow-up studies have been performed to substantiate these conclusions, and the genetic basis of these patterns remains unclear.

Such cryptic heterogamety or other potential polygenic mechanisms could, in theory, be influenced by temperature during or after an evolutionary transition to or from TSD. Indeed, the coexistence of functional TSD and GSD in a single organism, as observed in the dragon lizard (Quinn et al., 2007), provides an excellent evolutionary framework for transitions between the two systems. However, in *T. scripta*, the existence and/or nature of any genetic factors has remained elusive.

A possible alternative model is that intermediate temperatures result in moderate levels of aromatase expression/activity in the gonads and correspondingly intermediate serum estradiol levels (Pieau et al., 1998). As estrogen is a potent agonist of female development, its uniform global circulation could induce bilateral ovarian differentiation in embryos that meet a hormonal threshold, with male development occurring in those that fall below. Efforts to test the effects of exogenous estrogen on aromatase expression showed no effect at the MPT prior to stage 19, suggesting that the early coordination between left/right gonad development is not regulated by aromatase expression levels (Matsumoto et al., 2013). However, in theory, any circulating substance could serve the function of coordinating left/right gonad development.

In the present study, we explored the question of what underlies the development of males or females at the PvT in the red-eared slider turtle *T. scripta elegans*. We designed a simple experiment in which the two gonads of a given embryo were removed from the body early in the thermosensitive period (TSP) and cultured separately at the PvT. If genetic or systemic factors determine sexual fate prior to this time, then the two gonads should share the same information and therefore develop concordantly when cultured in isolation. If, conversely, the two gonads of a pair do not always adopt the same sexual fate, we could instead conclude that sex is not determined under these conditions by a shared predisposition but rather may require a systemic signal to coordinate the fates of the two gonads.

Materials and methods

Turtle eggs

Freshly laid red-eared slider turtle eggs were acquired from Robert Clark or the Kliebert Turtle & Alligator Farm (Hammond, LA) with the approval of the Louisiana Department of Agriculture and Forestry. In each shipment, we received approximately 500 eggs derived from mixed clutches laid within the same 24-h period. Clutches vary widely in size, but mature *T. scripta* females from different populations have been reported to lay an average of 6.6 to 9.4 eggs per clutch, with some reports of captive turtles laying 15–20, at 12–36 day intervals (Marlen and Fischer, 1999; Aresco, 2004). Assuming a conservative number of 20 eggs per clutch, a shipment of 500 eggs should include eggs from approximately 25 clutches from 25 different females. Based on our sampling simulation studies, in a group of 50 randomly selected eggs, we expect that eight or more would originate from the same clutch less than 1% of the time. We expect this to minimize potential distortions due to clutch variation.

Eggs were incubated in moist vermiculite in a humidified incubator at 26 °C (male-producing temperature, MPT), 31 °C (female-producing temperature, FPT) or 29.2 °C (PvT) with ambient CO_2. Based on experiments using *T. scripta* eggs obtained from

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