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Molecular and morphological differentiation of testes and ovaries in relation to the thermosensitive period of gonad development in the snapping turtle, *Chelydra serpentina*

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ABSTRACT

Ambient temperatures during embryonic development determine gonadal sex in many reptiles. The temperature sensitive period for sex determination has been defined by shifting eggs between female- and male-producing temperatures in a few species. This phase spans 20–35% of embryogenesis in most species, which makes it difficult to define the mechanisms that transduce temperature into a signal for ovarian versus testicular development. We present an extensive set of studies that define a brief period when high temperature specifies, and then determines, ovarian fate in a northern population of snapping turtles, *Chelydra serpentina*. We shifted embryos from male to female temperatures, or vice versa, at various stages of development. Gonads in embryos incubated at female temperatures commit to ovarian fate earlier (by stage 18) than gonads in embryos incubated at male temperatures commit to testicular fate (by stages 19–21). In double shift studies, embryos were incubated at a female temperature, exposed to a male temperature for set times, and shifted back to the original temperature, or vice versa. The time required to induce ovarian development ($\leq$ 6 days at female temperatures) was much shorter than the time required to induce testicular formation ($>$ 20 days at male temperatures). Differentiation of the gonads at the histological level occurred after the sex-determining period. Nevertheless, we found that a change in temperature rapidly (within 24 h) influenced expression and splicing of *WT1* mRNA: the absolute abundance of *WT1* mRNA, the relative abundance of +KTS versus –KTS isoforms, as well as the ratio of +KTS:–KTS isoforms was higher in gonads at a male versus a female temperature. In conclusion, ovarian fate is more readily determined than testicular fate in snapping turtle embryos. The short sex-determining period in this species (6–8% of embryogenesis) will facilitate studies of molecular mechanisms for specification and determination of gonad fate by temperature.

1. Introduction

An intricate process of molecular and cellular differentiation results in morphological, physiological, and behavioral differences between males and females. Sexual differentiation results from cell autonomous interactions among a few key genes at the beginning of a complex gene regulatory network in fruit flies and nematodes (Cline and Meyer, 1996; Marín and Baker, 1998; Schütt and Nöthiger, 2000; Goodwin and Ellis, 2002). In the end, a large fraction of the genome is differentially expressed between male and female fruit flies and male and hermaphroditic nematodes (Jin et al., 2001; Ranz et al., 2003). There are also abundant sex differences in gene expression in mammals and birds (Ellegren and Parsch 2007; Mank et al., 2010; Naurin et al., 2011). In contrast to flies and nematodes, endocrine signals from the gonads control sexual differentiation of non-gonadal tissues in vertebrates (reviewed in Hughes, 2001; MacLaughlin and Donahoe, 2004). Thus, a defining event in vertebrate ontogeny occurs when the bipotential gonads commit to develop as ovaries or testes, a process commonly referred to as gonadal sex determination.

Sex-determining mechanisms are surprisingly labile in vertebrates. Sex chromosomes evolved independently in birds, mammals, lizards, snakes, and turtles (Lahn and Page, 1999; Pigozzi, 1999; Matsubara et al., 2006; Ezaz et al., 2010; Ellegren, 2011; Mank, 2012; Moghadam et al., 2012). Variation in sex determination is also significant within these groups (Whitfield et al., 1993; Vaiman and Pailhoux, 2000; Stiglec

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and Ezaz 2007). For instance, some lizards have sex chromosomes analogous to birds, mammals, or snakes, while others have temperature-dependent sex determination and no evidence of sexually dimorphic chromosomes (Viets et al., 1993; Harlow, 2004; Ezaz et al., 2010). Recent studies demonstrate that both genotypic and environmental mechanisms can coexist in the same species (Yamamoto et al., 2014). Temperature can override chromosomal sex determination in two lizard species: the eastern three-lined skink (*Bassiana duperreyi*) has male heterogamety (an XX/XY system) while the central bearded dragon (*Pogona vitticeps*) has female heterogamety (a ZW/ZZ system) (Quinn et al., 2007; Radder et al., 2008; Ezaz et al., 2010). Lastly, temperature determines sex in numerous turtles and all crocodilians (Lang and Andrews, 1994; Ewert et al., 2004; Deeming, 2004).

Although temperature-dependent sex determination (TSD) is often depicted as if it were strictly an environmental effect, several studies report broad-sense heritability, narrow-sense heritability and/or genotype–environment interactions for sex determination at temperatures that produce mixed sex ratios (Bull et al. 1982; Janzen 1992; Rhen and Lang 1998; Janes and Wayne 2006; McGaugh and Janzen, 2011; McGaugh et al. 2011; Rhen et al., 2011). In agreement with these studies, gonad pairs from single turtle embryos dissected and incubated separately in organ culture at a temperature that produces mixed sex ratios have a strong tendency to adopt the same fate, with both gonads becoming testes or both becoming ovaries (Mork et al., 2014). Taken together, these results imply that gene products mediate temperature effects on gonad development. Nevertheless we do not know the precise molecular genetic events that transduce temperature into a signal for ovarian versus testicular development.

We have been using a northern population of the snapping turtle, *Chelydra serpentina*, to study unique and conserved features of sex determination (reviewed in Rhen and Schroeder, 2010). In this population, low temperatures from 20 to 22.5 °C produce mostly males (60–90% males, respectively), exclusively males are produced between 23 and 27 °C, mixed sex ratios with increasing proportions of females are produced from 27 to 29.5 °C, and only females are produced above 29.5 °C (Rhen and Lang, 1998). Variation in sex ratio among clutches within this population (Rhen and Lang, 1998), along with a latitudinal cline in TSD pattern across North America (Ewert et al., 2005), suggests that the TSD pattern in snapping turtles is relatively free to evolve in response to local thermal regimes.

The snapping turtle has a long history as a TSD model and was the first species to have its sex-determining period defined by shifting eggs between male- and female-producing temperatures (Yntema, 1976, 1979). Subsequent research revealed that hormone manipulations during the temperature sensitive period of gonad development could override temperature effects: administration of exogenous estrogens induces ovarian development at male-producing temperatures while an aromatase inhibitor induces testicular development at a female-biased temperature (Gutzke and Chymiy, 1988; Crews et al., 1989; Rhen and Lang, 1994). The aromatase inhibitor presumably blocks conversion of endogenous androgens to estrogens, which appear necessary for normal ovarian development. Similar findings in other reptiles support the hypothesis that steroid signaling is involved in TSD (Merchant-Larios et al., 1997; Pieau and Dorizzi, 2004; Ramsey and Crews, 2009).

More recently, we used reverse transcription and quantitative PCR to measure transcript levels in snapping turtle gonads during the thermosensitive period (Rhen et al., 2007, 2009; Rhen and Schroeder, 2010). Incubation temperature had a significant effect on expression of genes known to play a role in sex determination or sexual differentiation in other vertebrates. Transferring eggs from a male-producing (26.5 °C) to a female-producing (31.0 °C) temperature increases androgen receptor (*AR*), aromatase (*CYP19A1*), forkhead box protein L2 (*FOXL2*), and steroidogenic factor 1 (*NR5A1*) mRNA expression in embryonic gonads, but decreases expression of estrogen receptor α (*ESR1*), doublesex and mab related transcript 1 (*DMRT1*), platelet-derived growth factor B (*PDGFB*), and Sry-box 9 (*SOX9*) mRNA. Most of these genes appear to be downstream in the sex-determining pathway (or network) because changes in their expression do not emerge until the third or fourth day of the temperature shift, which coincides with determination of ovarian fate. This leaves us with good markers for sex determination, but wanting for candidate genes that (1) respond quickly to temperature changes, (2) are involved in commitment to ovarian or testicular fate, and (3) regulate expression of downstream effectors like aromatase or *SOX9*.

Based on its role in sex determination in mammals, Wilms Tumor 1 (*WT1*) is a promising TSD candidate. *WT1* is required for initial formation of the kidney and urogenital ridge in mice (Kreidberg et al., 1993; Moore et al., 1999). Alternative splicing of *WT1* mRNA generates two isoforms (−KTS and +KTS), which play different roles in kidney versus gonad development. Ablation of the −KTS isoform results in kidney defects and streak gonads in mice of both sexes while deletion of the +KTS isoform decreases *Sry*, *Sox9*, and *Fgf9* expression and causes sex reversal in genetically male mice (Hammes et al., 2001; Bradford et al., 2009). Thus, the −KTS isoform is required for proper formation of the bipotential gonads, while the +KTS isoform is necessary for testicular development. Other research has shown that *WT1* regulates *Sox9* expression independently of *Sry* (Gao et al., 2006). Thus, *WT1* may have a role in sex determination that predates *Sry* evolution in the ancestor of therian mammals (Wallis et al., 2008; Tsend-Ayush et al., 2009).

Indeed, *WT1* transcripts have been detected in several TSD species (Spotila et al., 1998; Spotila and Hall, 1998; Western et al., 2000; Valenzuela, 2008; Valenzuela et al., 2013). *WT1* mRNA seems to be expressed at higher levels at male- vs. female-producing temperatures, but these studies are problematic because RNA was extracted from adrenal–kidney–gonad complexes rather than isolated gonads. Embryonic kidneys are much larger than gonads so variation in *WT1* mRNA expression in the kidneys, or lack thereof, could lead to erroneous conclusions about the potential role of *WT1* in the gonads (i.e., false positive or false negative results). In contrast, Schmahl et al. (2003) used immunohistochemistry to specifically localize WT1 protein expression in gonads of the red-eared slider turtle during the temperature sensitive period. These authors reported that more WT1 positive cells were proliferating (positive for WT1 and BrdU) at a male-producing temperature than at a female-producing temperature. This result is consistent with the idea that *WT1* plays a conserved role in testicular development, but the antibody used does not distinguish +KTS and –KTS isoforms.

To date, expression of +KTS and –KTS isoforms has not been measured in embryonic gonads of TSD species. Moreover, we do not know the temporal relationship between expression of different *WT1* isoforms or whether a particular isoform is associated with specification or determination of gonad fate. In this study, we used temperature shifts to (1) re-evaluate the thermosensitive period in snapping turtle embryos, (2) characterize histological changes that occur in gonads during and after the thermosensitive period, and (3) examine *WT1* expression in embryonic gonads isolated from the underlying mesonephros. We employed isoform specific TaqMan® probes and rigorous standard curves to precisely measure expression of +KTS and –KTS transcripts in embryonic gonads. We found that *WT1* mRNA expression changed within 24 h of a temperature shift, as did the ratio of +KTS and –KTS isoforms. The rapid change in isoform ratio shows that splicing of WT1 is temperature-dependent and suggests the +KTS isoform may be involved in specification of testicular fate, as is the case in mammals.

2. Results and discussion

2.1. Temperature sensitive period for sex determination

We used two types of temperature shifts to define the sex-determining period in snapping turtle embryos. In single shift