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Evolution of Developmental Control Mechanisms

A timecourse analysis of systemic and gonadal effects of temperature on sexual development of the red-eared slider turtle *Trachemys scripta elegans*

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ABSTRACT

Temperature dependent sex determination (TSD) is the process by which the environmental temperature experienced during embryogenesis influences the sex of an organism, as in the red-eared slider turtle *Trachemys scripta elegans*. In accord with current paradigms of vertebrate sex determination, temperature is believed to exert its effects on sexual development in *T. scripta* entirely within the middle third of development, when the gonad is forming. However, whether temperature regulates the transcriptome in *T. scripta* early embryos in a manner that could influence secondary sex characteristics or establish a pro-male or pro-female environment has not been investigated. In addition, apart from a handful of candidate genes, very little is known about potential similarities between the expression cascade during TSD and the genetic cascade that drives mammalian sex determination. Here, we conducted an unbiased transcriptome-wide analysis of the effects of male- and female-promoting temperatures on the turtle embryo prior to gonad formation, and on the gonad during the temperature sensitive period. We found sexually dimorphic expression reflecting differences in steroidogenic enzymes and brain development prior to gonad formation. Within the gonad, we mapped a cascade of differential expression similar to the genetic cascade established in mammals. Using a Hidden Markov Model based clustering approach, we identified groups of genes that show heterochronic shifts between *M. musculus* and *T. scripta*. We propose a model in which multiple factors influenced by temperature accumulate during early gonadogenesis, and converge on the antagonistic regulation of aromatase to canalize sex determination near the end of the temperature sensitive window of development.

1. Background and Introduction

Many reptiles, including crocodilians and turtles such as the red-eared slider turtle *Trachemys scripta elegans* (*T. scripta*), utilize temperature-dependent sex determination (TSD), in which sex is determined through a bimodal developmental response to egg incubation temperature. In *T. scripta*, ~100% males develop at the male producing temperature (MPT) of 26 °C, and ~100% females develop at the female producing temperature (FPT) of 31 °C (Fig. 1).

In general, vertebrate sex determination is believed to follow the paradigm established by Jost, who showed that removal of the testis from rabbit fetuses led to female secondary sex characteristics in XY animals (Jost et al., 1973; Jost, 1947). These experiments promoted the idea that differentiation of the gonad as a testis or ovary is the "primary" sex determination event that controls the development of all "secondary" sex characteristics such as genitalia, body shape and differences in brain development, through hormones and other secreted factors. Consistent with this paradigm, temperature is currently believed to exert its effects on sexual development in *T. scripta* entirely within the middle third of development, when the gonad is forming. Subsequently, sex is irrevocably determined and no longer responsive to thermal shifts (Bull and Vogt, 1979).

Evidence that the gonad can detect and respond to temperature by differentiating as a testis or ovary *ex vivo* comes from experiments in which gonads from *T. scripta* and the olive ridley sea turtle, *Lepidochelys olivacea*, were cultured in vitro at MPT or FPT

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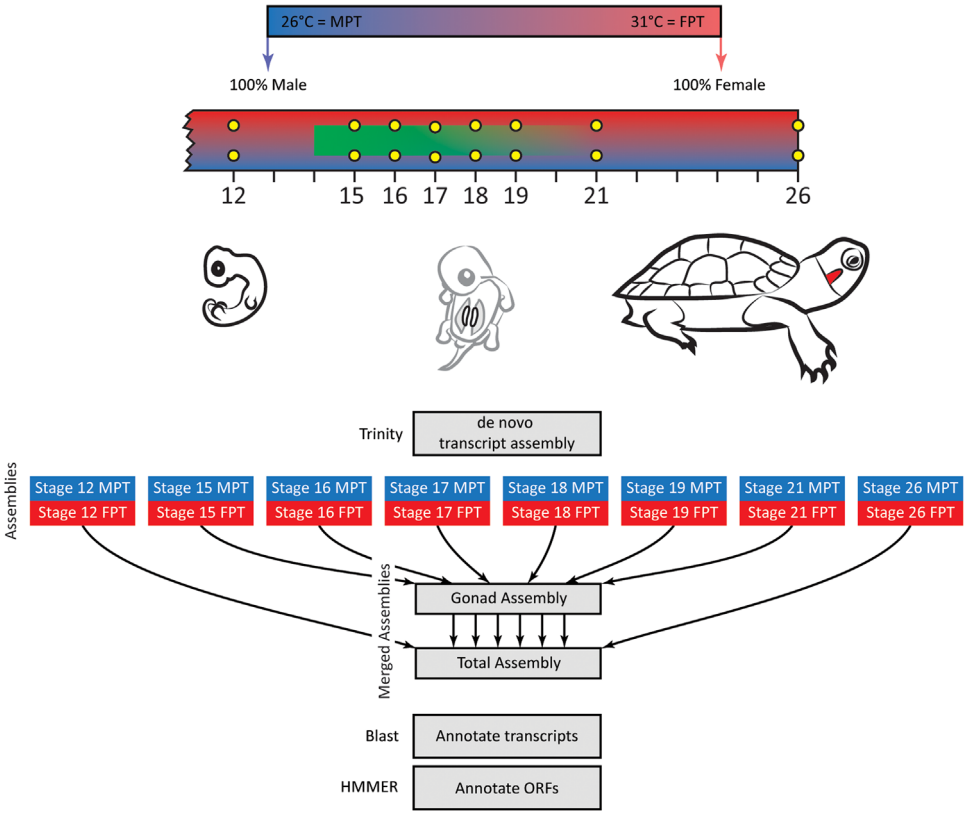

Fig. 1. Experimental design of RNA-seq and reference transcriptome generation. In *T. scripta*, incubation at 26 °C produces ~100% males and 31 °C produces ~100% females. We collected isolated gonad samples during the TSP in *T. scripta* (stages 15–19), and at a stage when gonadal fate is irrevocably set (stage 21). We also collected whole embryo data from stage 12 (prior to gonad formation), and a late stage to increase read coverage of all transcripts (stage 26). Each sample/stage combination was independently assembled by Trinity, for a total of 16 separate assemblies. These were merged into two reference assemblies for all gonad samples "allGonad" and all samples from both gonad and whole embryo "totalMerge". These two reference assemblies were annotated based on homologous mouse mRNA (BLAST) and peptide (HMMER) sequences in the Refseq database.

during the middle third of development (Merchant-Larios et al., 1997; Shoemaker-Daly et al., 2010). Nevertheless, these studies do not rule out the possibility that additional sources of in vivo non-gonadal temperature-based information influence primary or secondary sex determination. Whether temperature actually affects the transcriptome in *T. scripta* early embryos in a manner that creates a pro-male or pro-female environment has not been established, and an understanding of how temperature might influence sexual development beyond its influence on testis or ovary development is entirely lacking.

Despite the prevalence of TSD, very little is known about its molecular basis in any species. The primary advances in understanding TSD in *T. scripta* and related species have come through a candidate-based approach in which genes identified as important for mammalian genetic sex determination were studied in TSD species. While this approach provides a glimpse of transcriptional differences and similarities in some known elements of the pathways, it cannot uncover novel mechanisms or TSD-specific genes. In TSD species, temperature can function similar to inheritance or absence of the Y-linked gene *Sry*, which acts as the sex determination switch in mammals, but whether there are broad underlying similarities in male and female pathways is not known. What is known, however, is that the order of expression of some mammalian genes is not consistent with the order in *T. scripta* (C. Shoemaker et al., 2007, C.M. Shoemaker et al., 2007). In addition, it is clear that a strong divergence exists in the importance of sex hormone signaling in gonadal sex determination between GSD mammals and TSD reptiles, with this pathway being dispensable for the former and central to differentiation in the latter. However, the extent of conservation and how and when the two systems diverge are not understood.

Recently, next-generation sequencing has become a cost effective method to analyze transcriptome-wide gene expression even in non-model organisms that lack comprehensive genome assemblies. The first RNA-seq analysis to identify expression differences between the MPT and FPT developing gonad was recently completed in the American alligator (*Alligator mississippiensis*), a TSD species (Yatsu et al., 2016). This study was the first to identify a large number of candidate genes for the control of TSD. Changes in expression of signaling molecules such as *Wnt11* and epigenetic regulators such as *Kdm6b* were identified after shifting from FPT to MPT versus continual FPT incubation (Yatsu et al., 2016). This study also verified many known regulators of sex determination in mammals, similar to the historical homology-based candidate gene approach, but on a much larger scale.

To identify temperature responsive changes in the transcriptome of the *T. scripta* gonad, we conducted an RNA-seq analysis at six stages of gonad development at MPT and FPT. To uncover temperature-responsive transcriptional events occurring before or independent of gonad development, we also performed RNA-seq on MPT and FPT early embryo samples from a stage prior to gonad formation. We identified a strong pattern of differential expression in the early stage 12 embryo between the MPT and FPT temperatures. As expected, metabolic pathways differed at MPT and FPT. However, we also found gene expression differences in brain development represented in the data, and expression of sex hormone synthesis pathways with enrichment of some key regulators associated with either MPT or FPT. We identified several families of known temperature sensors, as well as genes whose transcript levels respond to temperature in the embryo and the