A Systems Level Analysis of Temperature-Dependent Sex Determination in the
Red-Eared Slider Turtle *Trachemys Scripta Elegans*

by

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Dissertation submitted in partial fulfillment of the requirements for the
degree of Doctor of Philosophy in the Department of Cell Biology
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ABSTRACT

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Sex determination is a critical biological process for all sexually reproducing animals. Despite its significance, evolution has provided a vast array of mechanisms by which sexual phenotype is determined and elaborated even within amniote vertebrates. The most prevalent systems of sex determination in this clade are genetic and temperature dependent sex determination. These two systems are sometimes consistent within large groups of species, such as the mammals who nearly ubiquitously utilize XY genetic sex determination, or they can be much more mixed as in reptiles that use genetic or temperature dependent systems and even both simultaneously. The turtles are a particularly diverse group in the way they determine sex with multiple different genetic and temperature based systems having been described. We investigated the nature of the temperature based sex determination system in *Trachemys scripta elegans* to ascertain whether it behaved as a purely temperature based system or if some other global source of sex determining information might be apparent within thermal regions insufficient to fully induce male or female development. These experiments found that sex determination in this species is much more complex and early acting than previously thought and that each gonad within an individual has the same sexual fate established enough that it can persist even without further communication between. We established a best practice for the assembly and annotation of de novo whole transcriptomes from *T. scripta* RNA-seq and utilized the technique to quantify the gene regulatory events that occur across the thermal sensitive period.

Evidence is entirely lacking on the resolution of TSD when eggs are incubated at the pivotal temperature in which equal numbers or males and females are produces. We have produced a timecourse data set that allowed for the elucidation of the gene expression events that occur at both the MPT and FPT over the course of the thermal sensitive period. Our data suggests that early establishment of a male or female fate is possible when temperature is sufficiently strong enough as at MPT and FPT. We see a strong pattern of mutually antagonistic gene expression patterns emerging early and expanding over time through the end of the period of gonad plasticity. In addition, we have identified a strong pattern of differential expression in the early embryo at stages prior to the formation of the gonad. Even without the known systemic signaling attributed to sex hormones emanating from the gonad, the early embryo has a clear male and female gene expression
pattern. We discuss how this early potential masculinization or feminization of the embryo may indicate that the influence of temperature may extend beyond the determination of gonadal sex or even metabolic adjustments and how this challenges the well-defined paradigm in which gonadal sex determines peripheral sexual characteristics.
DEDICATION

There was a lot that had to come together to make this research and this dissertation work. This document is dedicated to the most important people involved in it’s execution and production, my family. My wife Laura has been with me through the entire duration of my career, from my undergraduate, master’s and then doctoral research periods. She has given up a lot, sacrificed for many years, moved many miles and left behind too many people for each career transition to occur. Without her constant support none of this would have been possible and it certainly wouldn’t have been completed in such a polished and planned manner. Her strong mind and nimble fingers are as much present in this work as mine are and for that I am more thankful than she knows. I would also like to dedicate this work to my daughter Lena. She’s had to endure more of my absence in her early life than she or I would have liked, but always welcomed me home with hugs and excitement to keep me fueled for the days ahead. Both of you kept me sane and motivated to the best of your abilities throughout this entire process, and there is no doubt that your influence made this possible as much if not more than mine, thank you both from the bottom of my heart.
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CHAPTER I
Introduction to Sex Determination

Sex determination is the process by which a sexually reproducing organism begins to develop as one sex as opposed to the other. The process in more complex species, such as vertebrates, usually begins with the developing gonad. As the gonad forms, it has the ability to differentiate into either the male testis or the female ovary. The mechanism that directs an early “bipotential” gonad toward one of these two fates is termed “primary sex determination”. We know now that in the mouse, one of the most widely studied systems of sex determination, there are populations of cells within the bipotential gonad that adopt one of two possible fates as primary sex determination occurs (Figure 1, top). These cells belong to one of four lineages: germ cells, supporting cells, interstitial cells and endothelial cells (Figure 1).

As the gonad differentiates, the testis develops with germ cells inside seminiferous cords, surrounded by the supporting (Sox9 expressing) Sertoli cells with an interstitial population of specialized Leydig and fibroblastic smooth muscle cells (Figure 1). In the developing ovary, germ cells arrest in meiosis, while the supporting cell population from the cortex gives rise to granulosa cells, and the interstitial cells become the steroidogenic thecal cells (Figure 1). Each gonadal sexual fate also assumes a stereotypic vascular pattern that may be essential for the morphology and function of each organ (Figure 1). This pattern of divergent morphological and genetic differentiation has been heralded as an excellent developmental model for organogenesis. Due to the two distinct fates that can arise from a single bipotential organ primordium, the gonad has been widely utilized to study a range of developmental processes beyond sex determination.

Methods of sex determination throughout the animal kingdom are diverse, even within the vertebrate clade. In general, secondary sex characteristics are developed according to the primary sex determined by the gonad. In all vertebrates studied to date, the primary sex in the embryo is dictated by the differentiation of the primordial gonad into either a testis (male) or ovary (female) in accord with Jost’s paradigm of vertebrate sex determination based on his experimentation in rabbits (Josso, 2008). However, there are cases that challenge this paradigm, as in marsupials where
Figure 1. The Bipotential Gondad, Tesis and Ovary

The bipotential turtle gonad (top) can differentiate as a testis (bottom left) or ovary (bottom right). The primordial bipotential gonad is made up of two different tissue regions with different fates in males and females. In male turtles the medullary tissue (grey) expands and the primitive sex chords (Sox9+ cells, green) expand and elaborate while surrounding the germ cells (blue) that migrate internally from the cortex (white). In the female, the medullary tissue degenerates and Sox9 is no longer expressed in the primitive sex chord cells, the cortex expands and germ cells arrest in meiosis (pink).
sex chromosomes have been shown to dictate the sexual differentiation of genitalia prior to sexual specification of the gonad (Renfree, Chew, & Shaw, 2014).

Gonochorism, as opposed to hermaphroditism, is the condition in which each individual within a species is composed of a single sex. Gonochorism is widespread throughout the animal kingdom and is nearly ubiquitous within vertebrates. The method by which sex is determined during, or occasionally after, development varies widely between vertebrate clades. Within animals, multiple mechanisms of sex determination have been described over the years with the two most prevalent being genetic sex determination (GSD) and environmental sex determination (ESD). Genetic sex determination is defined by the inheritance of a genetic component at the time of conception. Mechanisms of GSD can range from a complex multigenic system, to a dosage-based system regulated by the number of sex chromosomes, to the simple genetic switch-based system observed in mammals.

Within ESD, sex-determining mechanisms vary wildly to produce individuals of a given sex, presumably under environmental conditions that maximize fitness and reproductive success for that sex. Some extreme cases of ESD include location-based sex determination, such as in the echiuroid worm *Bonellia*. In this species, the female differentiation occurs when larva settle on the ocean floor and male development only occurs when a larva settles near an existing female and migrates into the female's body where it differentiates into a sperm producing male symbiote (Wilczynski, 1960). Another extreme case exists in cichlid fish where sex is determined post-embryonically by social interactions and can even change as the social situation varies within a school. In this case, the largest and most dominant fish within a group will always be the functional male, while the rest of the school with be functional females or immature males. If the dominant male dies or is otherwise removed from the group, the next largest fish will take the place of the dominant male. Sometimes this transition involves the maturation of another male, and sometimes it involves the sex reversal of a functional female (Francis & Barlow, 1993).

While there have been a huge number of ESD mechanisms described throughout scientific literature, the most prevalent of these within amniote vertebrates is temperature-dependent sex determination (TSD) in which the environmental temperature experienced during embryonic development
dictates the sex of each individual. While GSD and TSD are by far the most common forms of sex determination within amniote vertebrates, it is not entirely clear which of these systems is ancestral to the other.

Within amniotes, mammals diverged the earliest from the other major groups, around 350 million years ago (Hedges, Dudley, & Kumar, 2006)(Figure 2). The hallmark characteristics of mammals, endothermy and internal development of offspring, may have been the driving factors in the development of the XY mode of sex determination that is endemic to the group. Outside mammals, the influence of sex hormones on determination of gonadal sex, as well as secondary sexual characteristics in amniotes is nearly absolute. However, given the intimate connection between the maternal and embryonic circulatory systems during gestation in placental mammals, the effects of female sex hormones originating from maternal blood would be problematic for the generation of male embryos. Considering this, it makes sense that mammals have evolved to exclude sex hormones from the process of gonadal sex determination. The evolution of endothermy, along with internal gestation, precludes mammals from utilizing environmental temperature as a primary source of sex determination as well. It seems likely that sex determining mechanisms diverged rapidly from sister amniote groups upon the evolution of these specific mammalian traits. The next major divergence in the amniote lineage was when the archosaurs split from the lepidosaurs about 270 million years ago (Figure 2).

The lepidosaurs (snakes and lizards) have a mix of sex determination (SD) mechanisms within the clade with snakes being entirely reliant on ZW genetic sex determination, a system in which the females contain the heteromorphic sex chromosome pair, and the lizards exhibiting TSD and GSD, and in some recently uncovered cases both TSD and GSD simultaneously (Holleley et al., 2015). The sphenodontian (tuatara lizards) are a unique group in that they, unlike any other lizard clade, are comprised entirely of TSD species. Two main groups from the archosaur lineage have become the main focus of TSD research over the past 50 years, the turtles and crocodilians. All crocodilians studied to date use TSD exclusively, while turtles are a mix with most species exhibiting TSD and others GSD. Within turtles that utilize GSD systems, there is no consistent evolutionary event that links them all together, and it appears that multiple XY and ZW systems have evolved separately and from multiple different autosomes. All birds examined so far have ZW sex determining systems. A
Figure 2. Tree of Life (Amniotes)

Major amniote clades diverged from 350 (Mammalia) to 220 (Birds/Crocodilia) million years ago. Mammals diverged the earliest from the rest of these groups. Turtles, Crocodilians and Birds are sister taxa within the Archosaur lineage. The Squamata and Sphenodontia are sister groups within the Lepidosaur lineage.
study comparing the evolutionary origin of the bird Z chromosome within archosaurs found that its origins can be traced to a single autosome in at least one crocodilian and one turtle (Kasai, O’Brien, Martin, & Ferguson-Smith, 2012). Many bird genomes have been sequenced and assembled, however genome drafts exist for only a few crocodilians and turtles and no further research on the autosomal origin of the bird Z or W chromosomes has been forthcoming.

Research on the chromosomal inheritance of sex was in its infancy at the turn of the 20th century. Original research in insects and invertebrates uncovered the first XY, ZW and XO GSD systems and as vertebrates were added to those species studied, it appeared cytogenetically that GSD was the predominant system employed by gonochoristic species. It wasn’t until the 1960’s that the first published observation of temperature-dependent sex determination came about. In 1966, Madeleine Charnier published findings that sex ratios of hatchling Agama lizards (Agama agama) could be skewed by temperature differences during egg incubation (Charnier, 1966). This research was published in the meeting records of the West African Society of Biology, and unfortunately was not widely read initially. No further studies on TSD were published for a number of years. It was in the early 1970’s that Claude Pieau from the Université Paris published the first reports of the effects of incubation temperature on the sexual development of one species of turtle (Emys orbicularis) and a tortoise (Testudo graeca) (Pieau, 1971). It was Pieau that first postulated that TSD might be an actual mechanism of sex determination apart from the well-recognized GSD systems, and not simply an observation due to differential mortality or aberrant development. Following the publications of Pieau on TSD in E. orbicularis, the phenomenon was verified in the snapping turtle Chelydra serpentine by C.L Yntema at the State University of New York in Syracuse, NY (Yntema, 1976). It was James Bull in the mid-to-late 1970’s that first did a systematic study of multiple turtle species to verify that TSD was widespread in turtles, and that TSD was not just an artifact restricted to laboratory incubation conditions but functioned under natural conditions in the wild (Bull & Vogt, 1979). At this point, TSD was recognized as a legitimate mechanism of sex determination, functional in many species, however the new theory was lacking in an evolutionary mechanism by which TSD would persist, or even benefit a species over time. James Bull and Eric Charnov at the University of Utah in Salt Lake City, UT, was the first to propose a viable mechanism by which TSD might be selected for in nature (Charnov & Bull, 1977). This new model, named the Charnov-Bull
model for evolution of temperature-dependent sex determination postulated that TSD could persist in a species in which environmental conditions strongly influence the fitness of an individual as either a male or female and the individual has little control over the environment it will experience. The main crux of this model, in so far as it affects reptiles such as turtles and crocodiles, is that there is no ability for the parent to predict the environment into which it’s offspring will be born. Since turtles have no (known) ability to predict the weather, it would be beneficial for the embryos to detect their environment and adjust their sex accordingly if that environment had a significant differential effect on fitness of males vs females. It wasn’t until 2008 that the first study was published in which this “differential fitness” of males and females of a TSD species was supported by exhaustive experimental analysis in an amniote vertebrate species. A study on the reproductive fitness of Jacky dragons *Amphibolurus muricatus*, by Warner and Shine found that reproductive success was indeed affected by incubation temperature when individuals were studied over their entire reproductive lifetime (Warner & Shine, 2008). What Warner and Shine found over the course of their 3.5-year study was that individual males or females incubated at the temperature that produces them naturally, produced more progeny over their lifetimes than did those that were experimentally sex reversed while incubated at the opposite temperature embryonically (Warner and Shine 2008). While a great number of publications on TSD were generated throughout the 1980’s, it was not until the 1990’s that the first major mechanistic step forward was made in understanding the molecular bases of TSD.

Though the observation that exogenous estrogen could sex reverse embryos incubated at a male producing temperature was made in the 1980’s, it wasn’t until 1994 that Crews and Bergeron first experimentally linked this phenomenon to the function of the Aromatase enzyme (Crews & Bergeron, 1994). David Crews would later go on to discover that the female producing temperature (FPT) had an effect on the expression of Aromatase and the first actual inroads to the temperate directed molecular mechanism of TSD were made. During the same study in 1994, it was discovered that adding exogenous testosterone to the egg of a red-eared slider *Trachemys scripta elegans*, could induce female development at male producing temperatures (MPT). Further analysis on the inhibition of both aromatase and 5-alpha reductase found that both enzymes seemed to be involved in the TSD process, if not necessarily directly in the case of 5 alpha reductase. Over the next decade,
the field was dominated by homology-based candidate gene analyses, in which a gene, previously linked to sex determination (usually in mammals), was cloned and its expression was correlated with either MPT or FPT development.

In *T. scripta*, one of the most common models for the study of TSD, a partial pathway for sex determination under the regulation of temperature has been outlined, primarily through these homology-based candidate gene analyses. In this turtle species, the MPT and FPT have been well defined as 26°C and 31°C respectively with the pivotal temperature, the temperature at which a 50:50 ratio of males to females is produced, being 29.2°C. Unlike many species uncovered during the earlier studies from Bull et al., *T. scripta* has only one MPT and one FPT, thus giving it an MF pattern of TSD in which low temperatures produce males and high temperatures produce females. This MF pattern is in contrast to many other species that produce females at low and high temperatures and males at middle temperatures in an FMF TSD pattern (Figure 3)(Kohno 2014).

Research in *T. scripta* benefits from its popularity for the study of TSD. Numerous labs have worked for years to establish protocols for husbandry and application of many standard molecular biology techniques for this species. Among the more useful tools was the establishment, by Eli Greenbaum in 2002, of a set of morphological embryo staging criteria that allows researchers to standardize the analysis of events over the course of development (E Greenbaum, 2002). Recent research utilized molecular genetics and techniques such as quantitative real-time polymerase-chain-reaction (qRT-PCR) to identify many genes (based on homology to mammalian homologs) that are expressed downstream of Aromatase activation, as well as male genes such as *Dmrt1* that are enriched in MPT gonads as early as Aromatase is enriched at FPT.

The gonad initially forms on the surface of the mesonephros around stage 14, at which point researchers believe the thermal sensitive period (TSP) begins. In contrast to mammalian GSD systems, *Sox9* expression in gonadal supporting cells is apparent at both the MPT and FPT through most of the TSP, before being down regulated at the FPT around Greenbaum stage 19. At MPT, the first male enriched genes identified by homology-based candidate gene analysis in *T. scripta* are *Dmrt1*, *Amh* and *Sf1*. Enrichment of these three genes begins around stage 16, shortly after gonadogenesis has begun, and the degree of enrichment in MPT gonads compared to FPT generally
Figure 3. TSD has Multiple Modes of Sex Determination

Two main modes of TSD are seen in reptiles. Most of the species studied early in TSD research exhibited a female-to-male-to-female pattern (FMF) with females produced at low or high temperatures and males in the intermediate region (Macrolems temminckii (red line), and Alligator mississippiensis (black line)). Trachemys scripta exhibits a less common male-to-female pattern (MF) of TSD (blue line).

Adapted from Gilbert, S (Developmental Biology. 6th edition)
increases over time through the end of the TSP. At FPT the first gene known to be enriched is Aromatase (Cyp19a1), which is followed by Rsps1 and then Foxl2 between stages 17 and 18 (Kohno et al., 2014; Matsumoto & Crews, 2012). The verification of this handful of genes, identified by their homology to mammalian sex determining genes, comprised the majority of what was known of the genetic network underlying TSD through the first decade of the 21st century. This is the point at which next-generation high-throughput sequencing technology had advanced sufficiently that its analytical power was great enough, and cost low enough to open genome/transcriptome-wide analysis to the study of the non-model organisms that dominate TSD research.

By the end of 2013, three different turtle genomes had been sequenced, assembled, annotated and released to the public. The turtle genomes were from the painted turtle (Chrysemys picta bellii), the green sea turtle (Chelonia mydas), both among the most widely studied TSD models throughout the world, and the Chinese soft-shelled turtle (Pelodiscus sinensis), a GSD turtle species. These, along with a few crocodilian genomes, opened the doors to a wide range of genomic analyses on archosauria, and TSD in particular. While no genomic analysis has yielded a TSD specific locus shared among any species, there has been some progress made in understanding the genome dynamics of TSD. One of the initial genomic discoveries related to TSD was the identification of temperature specific DNA methylation events at the promoter of Aromatase in multiple species. This phenomenon was first observed in the European sea bass Dicentrarchus labrax, a fish in which temperature affects offspring sex ratios (Navarro-Martín et al., 2011). Later, the same phenomenon was observed in T. scripta (Matsumoto, Buemio, Chu, Vafaee, & Crews, 2013). At this promoter, methylation has significant implications for TSD because of the position of Aromatase near the top of the TSD gene expression cascade and its importance in the initiation of the female fate. However, whether methylation at this locus is causative or simply correlated with expression activation is not known. Less targeted unbiased RNA-seq based approaches are in their infancy for the analysis of TSD.

One study performed an RNA-seq do novo assembly based on total transcripts from T. scripta whole embryos, combining stage 14 and stage 17, a period when the shell is forming. This study provided the first large assembly of T. scripta sequences to the public, and was useful to the TSD community, and the T. scripta research community specifically. More recently, the first RNA-seq analysis to identify expression differences between the MPT and FPT developing gonad was completed in the
American alligator (*Alligator mississippiensis*), a TSD species. This study was the first to identify a large number of candidate genes for the control of TSD through exposure to MPT and FPT. 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. This study also verified many known regulators of sex determination in mammals, similar to the historical homology-based candidate gene approach but at a much larger scale. Recently, a study used next-gen sequencing to identify a single nucleotide polymorphism (SNP) in a single gene that correlated with sexual phenotype in the common snapping turtle (*Chelydra serpentina*). This gene, cold-inducible RNA binding protein (CIRBP) contains a single A to C SNP that correlates with sex when eggs are raised at the pivotal temperature (Schroeder, Metzger, Miller, & Rhen, 2016). This study also defined the gonadal expression pattern of CIRBP and suggested that the gene itself may be the causal variant at this locus that mediates gonadal fate.

Although major strides have been made utilizing modern techniques, such as next-gen sequencing, more refined and comprehensive studies are needed to provide an understanding of TSD on par with our current grasp of highly studied mammalian systems of GSD.
CHAPTER II
Predetermination of Sexual Fate in a Turtle with Temperature-Dependent Sex Determination

The research presented in this chapter was conducted in collaboration with Lindsey Mork and Blanche Capel (Mork, Czerwinski, & Capel, 2014).

2.1 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 & 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 (Badenhorst, Stanyon, Engstrom, & Valenzuela, 2013; Graves & Shetty, 2001; Kawai et al., 2007; Pieau, Dorizzi, & Richard-Mercier, 1999). However, in all crocodilians tested so far, 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 & Krenz, 2004). Further evidence indicates that multiple independent transitions from TSD back to GSD subsequently took place as reptiles diverged (Janzen & Phillips, 2006; Pokorna & Kratochvil, 2009). Retention of some degree of thermosensitivity in GSD species may underlie these evolutionary transitions (Quinn, Sarre, Ezaz, Marshall Graves, & Georges, 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 (Quinn et al., 2009; Radder, Quinn, Georges, Sarre, & Shine, 2008;
Shine, Elphick, & Donnellan, 2002). Furthermore, in the GSD turtle *Apalone mutica*, the expression of several genes involved in gonad development is affected by egg 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 (Barske & Capel, 2008; Sarre, Georges, & Quinn, 2004).

The TSD phenomenon 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, Jackson, & Nelson, 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 (Ewert et al., 1994; Mrosovsky & Pieau, 1991). 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, Dorizzi, Richard-Mercier, & Desvages, 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 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 (Pieau et al., 1998; Zaborski, Dorizzi, & Pieau, 1988). Such mechanisms could potentially rely on the cumulative effects of multiple loci across the genome or within a pair of microscopically indistinguishable (homomorphous) sex chromosomes. These types of genetic mechanisms could, in theory, be influenced by temperature during or after an evolutionary transition to or from TSD. However, in *T. scripta*, the existence and/or nature of any genetic factors has remained elusive. 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. 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 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 what is thought to be a pure TSD species, the red-eared slider turtle Trachemys 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.

2.2 RESULTS

2.2.1 Synchronization of Sexual fate in Turtle Gonad Pairs Cultured Separately Ex Ovo

To investigate whether a shared systemic signal or a cryptic genetic mechanism determines sex at the PvT, where the effect of temperature is effectively null, we determined whether the two gonads of a given PvT embryo adopt the same fate when cultured in isolation. The cultures were initiated at stage 17. At this stage, gonads from embryos incubated at the MPT and FPT are morphologically indistinguishable (Wibbels, Bull, & Crews, 1991), and can be cultured to a point where sexual differentiation into a testis or an ovary is evident (Mork & Capel, 2013; Shoemaker-Daly, Jackson, Yatsu, Matsumoto, & Crews, 2010). In addition, embryos shifted from MPT to FPT at stage 17 all
develop as female, and the majority shifted from FPT to MPT develop as males (Wibbels et al., 1991), implying the persistence of sexual plasticity at this stage.

The gonads of embryos incubated in ovo at the PvT were removed at stage 17 and cultured separately at the PvT for eight days (Figure 4). Sexual fate was evaluated at the end of the culture period by immunostaining for SOX9 and β-catenin. In gonads that adopt male fate, nuclear SOX9 expression is retained, and β-catenin is enriched in the medullary testis cords. In gonads that adopt female fate, SOX9 expression is downregulated, and β-catenin is reduced in the medulla and enriched in the cortex (Mork & Capel, 2013). Gonad pairs cultured in isolation at the PvT consistently adopted the same sexual fate (Figure 4). Strong SOX9 expression was detected in approximately one third of the pairs, indicative of testis fate (Figure 4B, E), another third exhibited little to no SOX9 expression, indicative of ovary fate (Figure 4D, E), and the remainder showed intermediate levels (Figure 4C, E). Of this last group, most gonads contained only a few SOX9-positive nuclei or prevalent cytoplasmic staining, suggesting that most would have developed as ovaries given a longer culture period, though cortical enrichment of β-catenin was not yet apparent in most cases (Figure 4C). The correlation in SOX9 staining intensity between gonad pairs was highly significant (r = 0.894; p < 0.0001). Synchronization between gonad pairs indicates that their sexual differentiation trajectory was either fixed by a common environmental/yolk-derived signal before stage 17, or determined by a shared genetic predisposition. However, this result argues against an environmental mechanism that relies on systemic communication after stage 17 to coordinate gonad fate.

These conclusions were supported by a second experiment performed in parallel with the PvT cultures. Here, embryos were incubated at the PvT until stage 17, at which point the gonad pair was dissected from each embryo, and the gonads were divided into two culture dishes, one of which was cultured at the MPT and the other at the FPT (Figure 5A). Previous studies indicated that gonads isolated from stage 16.5 MPT or FPT embryos and cultured at the opposite temperature undergo sex reversal, similar to intact eggs shifted at stage 17 (Shoemaker-Daly et al., 2010; Wibbels et al., 1991). We therefore predicted that the gonad shifted from PvT to MPT would develop as a testis, whereas the gonad shifted to the FPT would develop as an ovary. Interestingly, in half of the pairs, the gonad shifted to the MPT showed high SOX9 expression, as expected, but intermediate SOX9
Figure 4. Sexual Fate is Synchronized Between PvT Gonad Pairs Cultured in Isolation

(A) Design of the PvT culture experiment. ((B)–(D)) Pairs of stage 17 gonads cultured individually at the PvT for eight days and then immunostained for SOX9 (green) and β-catenin (magenta). Nuclei were stained with Hoechst (blue). The two gonads of each pair showed very similar staining patterns and morphology. ((B), (B′)) Example of a 'male' pair, with high SOX9 expression and clear medullary cord structures enriched for β-catenin. ((C), (C′)) Example of an 'intermediate' pair, with low/cytoplasmic SOX9 staining and persistent β-catenin staining in the medulla. ((D), (D′)) Example of a 'female' pair, with no apparent SOX9 expression and cortical enrichment of β-catenin. (E) Quantification of SOX9 staining intensity in the 19 surviving gonad pairs cultured at the PvT, ranked according to the value of the first gonad imaged of each pair (i.e., gonad A). The examples shown in ((B)–(D)) are marked with asterisks. Scale bars represent 20 μm.
Figure 5. A Possible Sexual Bias was Evident in Gonads Subjected to Temperature Shifts

(A) Gonads from stage 17 PvT embryos were split up so that one was cultured at the MPT and the other at the FPT for eight days. The samples were immunostained for SOX9 (green) and β-catenin (magenta), and nuclei were stained with Hoechst (blue). In approximately half of the pairs, the samples appeared predisposed to the male fate, such that the gonad shifted to the MPT had high SOX9 expression (B) and the gonad shifted to the FPT presented intermediate SOX9 levels (B’). Some of the other pairs appeared predisposed towards the female face, such that SOX9 was undetectable in the gonad shifted to the FPT (C’) and very weak in the gonad cultured at the MPT (C). The data from the seven surviving gonad pairs are summarized in (D), ranked according to increasing SOX9 intensity in the MPT sample. The examples shown in (B) and (C) are marked with asterisks. Scale bars represent 20 µm.
levels were observed in the gonad shifted to the FPT (Figure 5B, D). In the other half of the pairs, the gonad shifted to the FPT showed high cortical β-catenin and no SOX9 expression, whereas very weak SOX9 staining was observed in its partner at the MPT (Figure 5C, D). We propose that the first group was predisposed towards the male fate and the second group towards the female fate. This interpretation could explain why it was difficult to shift gonads from the first group to the female fate and gonads from the second group to the male fate. We predict that a longer culture duration would have resulted in full sex reversal, as previously observed in gonads shifted from FPT to MPT or MPT to FPT and cultured for up to 20 days (Moreno-Mendoza, Harley, & Merchant-Larios, 2001; Shoemaker-Daly et al., 2010).

2.2.2 Establishment of Sexual Fate Early in the TSP

One interpretation of our finding that sexual fate is coordinated in the absence of systemic communication between the gonads is that sex was determined prior to the beginning of the culture period – by either an early-acting environmental signal or a genetic mechanism – and maintained along the same trajectory thereafter. Therefore, in a third experiment, we used an in ovo temperature shift assay to further test whether sexual fate established by temperature prior to stage 17 is maintained in the absence of a strong ongoing temperature signal. Here, we incubated cohorts of 50 eggs at the MPT or FPT up to stage 17 and then shifted them in ovo to PvT until just prior to hatching (stage 26). Because the PvT is equally permissive and unbiased to male and female development, we predicted that if sexual fate were stably determined by an environmental influence prior to stage 17, the initial incubation at the extreme temperature would decide the sex of the embryos, such that the MPT→PvT and FPT→PvT embryos would develop as males and females, respectively. We observed a strong difference between groups pre-incubated at MPT and FPT in their tendency to sex-reverse. Among the surviving hatchlings incubated at the FPT prior to stage 17, ~72% (n = 21/29) remained female, ~17% (n = 5/29) sex-reversed to male, and ~10% (3/29) were indistinguishable as either male or female. In contrast, the eggs that were incubated at the MPT until stage 17 all developed as males (n = 22/22), without a single sex reversal (Table 1).
Table 1. Sexual Fate is Generally Maintained at the PvT Once Established at the MPT or FPT During Early Gonad Development

<table>
<thead>
<tr>
<th>Gonadal sex</th>
<th>MPT preincubation</th>
<th>FPT preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>100.00% (22/22)</td>
<td>17.24% (5/29)</td>
</tr>
<tr>
<td>Female</td>
<td>0.00% (0/22)</td>
<td>72.41% (21/29)</td>
</tr>
<tr>
<td>Intersex</td>
<td>0.00% (0/22)</td>
<td>10.34% (3/29)</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>0.0241</td>
</tr>
</tbody>
</table>
2.3 DISCUSSION

How sex is determined at the pivotal temperature in a TSD species represents a potential intersection between environmental and genetic mechanisms of sex determination. Some authors, notably Claude Pieau’s group in the 1980s and 90s, argued that because the PvT is equally permissive to male and female development, genetic influences likely determine sex under these conditions (e.g., Pieau et al., 1998; Zaborski et al., 1988). Our finding that paired gonads cultured in isolation at the PvT adopt the same sexual fate supports the existence of cryptic genetic mechanisms operating at the PvT. However, our evidence is not entirely conclusive, as the shared sexual predisposition could also be potentially attributed to a non-temperature-related environmental signal, such as a maternally-deposited factor in the yolk or stochastic variation in neuronal or other embryo-derived signals, to which the two nascent gonads were equally exposed prior to dissection (Janzen, Wilson, Tucker, & Ford, 1998; Radder, Pike, Quinn, & Shine, 2009). Although most evidence available to date argues against a significant role for maternally-derived yolk hormones in determining sex in TSD species (reviewed in Radder, 2007), other non-steroidal factors remain a possibility. Our in ovo experiments involving pre-incubation at the MPT or FPT prior to shifting to the PvT revealed a discrepancy in the stability of the male and female programs. Although pre-incubation at the MPT prior to stage 17 seems to be sufficient to canalize the male pathway when eggs are shifted to the PvT, this was not true for about 1/3 of embryos pre-incubated at the FPT, which did not maintain female fate but instead developed as males when shifted to the PvT at the same stage. Furthermore, experiments in which one gonad of a PvT embryo was shifted to the FPT and the other to the MPT also revealed a sexual predisposition shared by both gonads of the pair. These results argue for an inherent bias stemming either from genetic differences or the influence of other maternal or environmental factors acting on the embryo prior to stage 17.

2.3.1 Cryptic Genetic Mechanisms Operating in TSD Species

If genetic factors do determine sex at the PvT, they would likely have weaker effects than the dominant (e.g., Sry in mammals) or dosage-linked (DMRT1 in birds) determinants present in GSD systems, as they can so easily be overridden by temperature. Interestingly, in the TSD European pond turtle Emys orbicularis, (Pieau et al. (1998) found that gonads of embryos incubated at the PvT showed the
first signs of morphological differentiation later than stage-matched MPT/FPT embryos, indicating that without the driving influence of temperature, the sex-specific transcriptional programs may take longer to manifest. Indeed, if we consider sex as a threshold trait in which a male- or female-promoting signal must reach a particular level to sway the system towards one of two dichotomous outcomes (male vs. female) (Mittwoch, 2006; Quinn et al., 2011), only a slight genetic predisposition in favor of the male or female program might be sufficient to determine sex in the absence of a strong temperature signal. This predisposition could potentially be polygenic and consist of genetic variants at multiple loci that collectively determine whether the male or female signal meets the required threshold. There is evidence for such a mechanism operating in zebrafish (Liew et al., 2012).

Another possibility is that TSD species harbor cryptic, undifferentiated sex chromosomes carrying dominant or dosage-dependent regulators. While karyotyping analyses have not distinguished sex chromosomes in *T. scripta* (Bickham & Baker, 1976; Stock, 1972), many reptiles with GSD have homomorphic or micro sex chromosomes that cannot easily be distinguished by standard methods (Badenhorst et al., 2013; Ezaz et al., 2005; Ezaz et al., 2006; Martinez, Ezaz, Valenzuela, Georges, & Marshall Graves, 2008). 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, Wachtel, Lance, & Becak, 1987), i.e., in the male in species with XX/XY heterogamety and in the female 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, Klemme, & Schmid, 1981). Differences in HY status between males and females are less straightforward in TSD species. In *T. 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 *E. orbicularis*. Here, Patrick Zaborski and
colleagues found that HY status in the gonad reflects phenotypic sex, whereas serological status may reflect genotypic sex (Zaborski, Dorizzi, & Pieau, 1982; Zaborski et al., 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.

If cryptic heterogamety is indeed present in TSD species, populations of TSD species could potentially contain many individuals in which the sexual genotype (e.g., ZZ or ZW) is discordant with the sexual phenotype (ovaries or testes). If such individuals suffered a significant fitness detriment (as they do in mammals, where similarly sex-reversed individuals are often sterile), we presume that the species would evolve away from temperature sensitivity and towards a GSD mechanism. As TSD is clearly a successful strategy, we should instead conclude that the potential genotype-phenotype discordance – if it exists – does not carry a fitness cost. This is consistent with the Charnov-Bull model for TSD (Charnov & Bull, 1977), which predicts that there must be a fitness benefit to producing a given sex at a particular temperature (i.e., females at high temperatures). To be maintained in the population, this benefit would likely have to supersede any potential detriment derived from genotype-phenotype incongruities.

### 2.3.2 Timing of Sex Determination

Our culture experiments indicate that sexual fate is coordinated between the two gonads of a PvT embryo through the use of shared genetic (or potentially yolk-derived) information rather than by a systemic signal acting during the bipotential period of gonad development. In addition, our temperature-shift studies suggest that sexual trajectory is set well before the end of the TSP many days prior to the onset of morphological differentiation into testes or ovaries. Male fate is more strongly canalized by stage 17 at the MPT than is the female fate at the same stage at the FPT. However, in both cases, the sexual trajectories established prior to stage 17 were usually perpetuated thereafter unless the gonads/eggs are shifted to an extreme temperature. Even in cases where the two gonads of a pair were incubated at two different temperatures after stage 17, they showed signs of a lingering predisposition towards a single fate, at least for the first week of the culture period (Figure 5).
A handful of candidate sex-determining genes have been shown to be differentially expressed at the MPT and FPT as early as stage 16 (Ramsey & Crews, 2007; Ramsey, Shoemaker, & Crews, 2007; Rhen, Metzger, Schroeder, & Woodward, 2007; C. Shoemaker, Ramsey, Queen, & Crews, 2007; C. M. Shoemaker, Queen, & Crews, 2007; Smith et al., 2008), but the degree to which these differences reflect sexual differentiation trajectory or sex determination has been difficult to evaluate, given the ease with which embryos can be sex-reversed until the end of the TSP (Wibbels et al., 1991). Our findings indicate that these early gene expression differences likely reflect the adoption of a strong sexual differentiation trajectory early in the TSP. Ongoing studies in our lab are using RNA-seq analysis to examine the full extent of the differences between the male and female transcriptional programs as they operate at MPT, FPT and PvT during the TSP. In addition, we are investigating genomic diversity in *T. scripta* males and females to identify potential regions involved in generating sexual bias in the absence of a strong thermal influence. This work, coupled with other emerging genomic resources for multiple TSD species (Kaplinsky et al., 2013; Shaffer et al., 2013; St John et al., 2012; Wang et al., 2013), will substantially help in understanding TSD at a mechanistic, as well as an evolutionary level.

### 2.4 MATERIALS AND METHODS

#### 2.4.1 Turtle Eggs

Freshly laid red-eared slider turtle eggs from multiple clutches were acquired from Robert Clark or the Kliebert Turtle & Alligator Farm (Hammond, LA) with the approval of the Louisiana Department of Agriculture and Forestry. 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 CO₂. In this species, incubation at 29.2°C temperature yields an average of 50% male and 50% female embryos across clutches (Wibbels & Crews, 1995), whereas incubation at 26°C or 31°C yields 100% males or 100% females, respectively (Bull & Vogt, 1981; Wibbels, Cowan, & LeBoeuf, 1998). Embryos were staged by criteria established by Greenbaum (E. Greenbaum, 2002). At stages 17 or 26, they were removed from their eggshells, quickly euthanized and placed into phosphate-buffered saline (PBS) for dissection.
2.4.2 Gonad Culture

The pair of gonads from each stage 17 PvT embryo were labeled with a unique identifying number and cultured in separate dishes (Figures 4A and 5A) using a previously published technique (Mork & Capel, 2013). In brief, gonads were dissected away from the neighboring mesonephros and laid in thin wells shaped in strips of agar gel (1.5% agar in Leibovitz’s L-15 medium (Gibco)) placed in 35-mm tissue culture dishes. Each strip was immersed in 0.3 ml of culture medium comprised of 10% charcoal-stripped FBS in Leibovitz with 50 μg/ml ampicillin and 1.25 μg/ml Fungizone (Gibco). Pairs in which one or both of the explants developed fungal infections were discarded and not included in the analysis. One group of samples was cultured at the PvT in ambient CO₂ for eight days (Figure 4A). In the second group, one gonad from each pair was shifted to the FPT (31°C), the other was shifted to the MPT (26°C) and both were cultured for eight days (Figure 5A). On each day of the culture period, the gonads were moistened with 5 μl of medium. The culture medium was changed every other day. On the eighth day, the gonads were removed from the wells, washed in PBS and fixed in 4% paraformaldehyde overnight at 4C.

2.4.3 In Ovo Temperature Shift Experiment

Eggs were incubated at either the MPT or FPT in groups of 50 until they reached stage 17, as determined by assaying age-matched eggs grown at the same temperature. These eggs were then shifted to an incubator maintained at the PvT, where they remained until the hatching stage (26). At this point, each surviving pre-hatchling staged embryo was sexed based on gonadal morphology.

2.4.4 Immunocytochemistry

Cultured gonads were processed for whole-mount immunocytochemistry as previously described (Barske & Capel, 2010). The samples were incubated with primary antibodies targeting SOX9 (Chemicon AB5535; 1:1000) and β-catenin (Sigma C7207, 1:200) and then with Cy3-conjugated donkey anti-mouse (Jackson Immunoresearch) and AlexaFluor 647 donkey anti-rabbit (Invitrogen) secondary antibodies. Nuclei were stained with Hoechst 333420 (Invitrogen). The samples were imaged in the longitudinal plane with a LSM710 Meta confocal microscope and the affiliated Zen software (Carl Zeiss, Inc.).
2.4.5 Quantification of SOX9 Staining Intensity and Data Analysis

SOX9 staining was quantified in representative 40x images of each cultured gonad using the CellProfiler program (Carpenter et al., 2006). The software was trained to recognize nuclei, the light intensity of each cell was calculated, and a mean intensity value was determined across all nuclei in the full image. To evaluate the relationship between the gonad pairs, Pearson's correlation coefficient was calculated using JMP10 software (SAS Institute). A p-value ≤ 0.05 was considered significant.
CHAPTER III
Optimized Assembly Methods for the Gonadal Transcriptome of the Red-Eared Slider Turtle Trachemys Scripta Elegans Under Male- and Female-Producing Temperature Influences

The research presented in this chapter was conducted in collaboration with Anirudh Natarajan, Lindsey Mork, Loren Looger and Blanche Capel

3.1 INTRODUCTION

Temperature can affect the expression of many genes and the activity of many proteins, including heat shock proteins (Lindquist & Craig, 1988) and TRP channels (Clapham, 2003; Seebacher & Murray, 2007). Temperature-dependent effects on developmental pathways in exothermic vertebrate species have not been well studied. For most developing tissues, transcriptional differences must be effectively compensated or buffered such that organs and tissues, such as hearts and limbs, develop similarly across the viable thermal range. However, this is not true for the gonad, which can respond to different incubation temperatures by developing as a testis (male) or an ovary (female). Many reptiles, including alligators, crocodiles and the red-eared slider turtle Trachemys scripta elegans (T. scripta), show temperature-dependent sex determination (TSD) in which sex is determined through a bimodal 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 (Figure 6A). As in other vertebrates, development of the gonad as a testis or ovary controls the physiological sex of the organism through hormones and other secreted factors (Jost, Vigier, Prepin, & Perchellet, 1973). Consistent with this paradigm, temperature exerts its effects on sexual development during the middle third of development, when the gonad is forming. Experiments isolating the gonad of T. scripta as well as the olive ridley sea turtle (Lepidochelys olivacea), and culturing it at MPT or FPT during this period, led to testis or ovary development, suggesting that molecular pathways within the gonad are directly responsive to temperature (Merchant-Larios, Ruiz-Ramirez, Moreno-Mendoza, & Marmolejo-Valencia, 1997; Shoemaker-Daly et al., 2010). After the middle third of development, sex is irrevocably determined and no longer responsive to thermal shifts (Wibbels et al., 1991).
Figure 6. Diagram of TSD, Sample Collection and Workflow

(A) Diagram of TSD. Eggs incubated at 26°C yield ~100% males, whereas eggs incubated at 31°C yield ~100% females. (B) Tissue samples from MPT and FPT were collected from gonads immediately after the close of the temperature-sensitive window (stage 21). Twelve pairs of gonads were collected at each temperature and pooled. The two pooled samples (red or blue boxes) were sequenced separately, trimmed, filtered, and assembled separately. We also merged MPT and FPT transcriptomes (red+blue box, St21All) prior to assembly to test whether merging improved annotation. (C) Trinity assemblies and annotation were performed for the 3 different read pools (M21, F21, and combined M21/F21). (D) As an alternative approach, reads from each of the 3 pools were mapped to the C. Picta genome with Bowtie2, then submitted to Trinity.
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 has identified transcriptional differences in some known elements of the pathways (C. M. Shoemaker et al., 2007), it cannot uncover novel mechanisms or TSD-specific genes. Recently, next-generation sequencing has become a cost effective method to analyze transcriptome-wide gene expression even in non-model organisms without a comprehensive genome assembly. After testing multiple assemblers we settled on Trinity (Haas et al., 2013) to generate transcriptome assemblies for the developing testis and ovary from embryos incubated at MPT and FPT. These transcriptomes represent an important resource to facilitate the study of embryonic development and TSD at male- or female-producing temperatures, and will serve as a scaffold for rapid alignment, interpretation and referencing of data from future experiments.

### 3.2 RESULTS

#### 3.2.1 Sample Collection and Processing

Eggs were incubated at MPT or FPT from the time of arrival (day 2 after laying) until they reached stage 21, the point at which continued thermal influence no longer has any effect on sexual phenotype. At stage 21, embryos were collected, gonads were dissected away from the mesonephros, and RNA was collected from four pools of 12 pairs of gonads from embryos incubated at each temperature to produce two pooled MPT gonad and two pooled FPT gonad samples. Due to the unknown level of genetic diversity within this population of *T. scripta*, pooling of multiple individuals serves the dual purpose of increasing RNA yield and averaging expression from genetic variants in the population. RNA was isolated from each of these samples, barcoded to keep reads separate for each temperature and tissue type, and sequenced (Figure 6B).

After trimming and filtering to remove low quality sequences, the reads from each sample were mapped to the genome of a related species, the painted turtle *Chrysemys picta* (*C. picta*). The mapped reads were separated by temperature so that mapping results contained only MPT reads (M21), FPT reads (F21), or combined as all reads from both temperatures (St21All) (Figure 6C). Through
systematic testing we found that the Trinity software suite reliably provided the best assembly results of the packages we tested, and was subsequently used for all assemblies. To assess the difference between a genome-guided approach and a de novo assembly, we ran Trinity on the three sets of reads (M21, F21, St21All) as standard de novo assemblies without using mapping to cluster reads by genomic position, and compared to Trinity run in genome-guided mode on the same three read sets mapped to the C. picta genome (Figure 6D,E). The resulting assemblies were annotated through a pipeline comprising BLAST searches against the “vertebrate other”, “vertebrate mammal” and “invertebrate” Refseq mRNA collections. Further annotation was derived from phmmer (Eddy, 2011), which searches for all translated long open reading frames (ORFs) against sequences of the “vertebrate other”, “vertebrate mammal” and “invertebrate” Refseq peptide databases.

### 3.2.2 Overall Features of Assemblies Comparing Multiple Methods

In general, the de novo assemblies (e.g. without utilizing genomic mapping data to C. picta; noted as “DN”) (Figure 6D) produced fewer contigs in all cases when compared to the genome-guided assemblies (noted as “GG”) (Figure 6E) for the equivalent read sets (Figure 6A). In both sets of assemblies, DN and GG, the combined temperature groups (St21All) yielded the most contigs at 175,558 (166,333,608bp total length) and 241,641 (185,066,847bp total length), respectively. The M21 samples yielded 22% and 26% (135,793 vs 175,558 and 178,283 vs 241,641) fewer contigs than the combined assemblies, and 28% and 20% (105,548 vs 135,793 and 147,990 vs 241,641) more contigs than the DN and GG F21 assemblies. When comparing the two assembly methods for each read set, the difference in contig number was similar, ranging from a 24% difference in M21, to 29% in F21 and 27% in the St21All group.

To assess assembly quality, we mapped the assembled contigs to the C. picta genome using Blat (Kent, 2002) to compare them to similar C. picta transcripts at each mapping position. Using stringent cutoffs of 95% sequence similarity along 70% of the contig length, and discarding all contigs that satisfied these criteria in more than one genomic location, we determined how many contigs from each assembly mapped with high confidence to the C. picta genome. Given the significant level of sequence divergence between these two turtle species, successful stringent genomic mapping of a contig is a good indicator of contig quality. In all cases, >80% of the assembled contigs reliably mapped to the C. picta genome. However, unexpectedly, a 3-5% lower percentage of contigs in the
GG assemblies reliably mapped to the *C. picta* genome using Blat. The St21All-GG assembly had the lowest Blat mapping rate in this assay at 81%, as compared to 84% of M21-GG and 83% of F21-GG contigs (Figure 7A). As all reads used in the GG assemblies mapped to the *C. picta* genome independently, it seems odd that the assembled contigs might be less likely to do so. This result is likely do to the exclusion of many reads from less conserved regions (often UTRs) of certain mRNAs leading to assembly of some shorter and/or less unique contigs that could not be mapped back to a single genomic position.

The results of the BLAST and phmmer annotation were much more variable between assembly and sample types than the mapping results. The St21All samples from DN and GG methods both yielded the most BLAST-annotated contigs, which is not surprising considering they contain the most total contigs. However, as a percentage of total contigs, the St21All BLAST-annotated contigs was the lowest for both methods. Since these assemblies are comprised of mixed reads from both testis and ovary samples, the complexity of the reads is likely much higher, and could make misassemblies more likely. The rate of contigs mapping to multiple genomic locations was relatively constant across all DN assemblies at 6.73, 6.76 and 6.77 percent for M21-DN, F21-DN and St21All-DN respectively. Due to these relatively consistent genome mapping results across the DN assemblies, the lower percentage of annotated contigs in the combined St21All-DN assembly is likely not due to generation of chimeric contigs (contigs generated by merging reads from multiple loci), but the greater complexity of the RNA pool leads to generation of more contigs for which an existing homologous Refseq sequence cannot be identified. The DN group had higher proportions of BLAST-annotated transcripts in each assembly than in GG assemblies from the same read pool. This may be due to exclusion of sequences from GG assemblies derived from low homology stretches near intron/exon boundaries or UTRs. This led to shorter contiguous assemblies in the GG group, which resulted in lower BLAST scores. In contrast, even though the DN group had fewer contigs, they were longer assemblies and gave higher blast scores. The F21 sample had the greatest proportion of annotated transcripts from both methods with 40% annotation in GG and 52% in DN. There was a consistent trend in the phmmer annotation rate between DN and GG methods with DN being nearly 2x higher in all cases (Figure 7A)
Figure 7. Assembly Quality was Improved in De Novo Samples

Three de novo (M21-DN, F21-DN, and St21All-DN) and three genome guided (M21-GG, F21-GG, and St21All-GG) assemblies were assessed for quality by genome mapping and identification of homologous sequences at the nucleotide and amino acid levels. (A) The total number of contigs per assembly are shown for comparison to total contigs mapped to the *C. picta* genome with Blat, nucleotide annotations from Blast, and peptide annotations by HMMER. All contigs, annotated by sequence homology, as Sox9 or Foxl2 and mapping by Blat to the Sox9 and Foxl2 loci, are provided from the de novo assemblies as examples. (B) Coverage data at the Sox9 locus are compared among all three DN assemblies. Read coverage for the M21 sample (blue) was much higher across the entire locus than F21 (red). M21 contigs (blue) and St21All contigs (green) contained an extended 5’ region of the transcript absent in the F21 (red) and reference *C. Picta* mRNA “XM_005282966.2” and peptide “XP_005283023.1” sequences (dark grey). Gaps in the *C. picta* genome (light grey regions) appear as introns in the M21 and St21All Sox9 transcripts, since calls for non-coding regions depended on proximate localization in the C. picta genomic landscape. The *C. picta* SOX9 peptide “XP_005283023.1” position is displayed to allow for identification of the 3’ and 5’ UTRs. (C) Foxl2 contigs covering the entire reference mRNA “XM_005282516.1” sequence were assembled for F21 (red) and St21All (green) with no detectable differences in their alignments, but were absent from the M21 sample. The *C. picta* FOX12 peptide “XP_005282573.1” position is displayed to allow for identification of the 3’ and 5’ UTRs.
Due to the nature of the Trinity assembly process, multiple “isoforms” are generated for each expressed transcript. These isoforms are comprised of transcripts with variations in exon composition or length. The additive effect of assembling all “likely” combinations of exon compositions with the variability in either exon or untranslated region (UTR)/unspliced intron lengths tends to generate more isoforms at any given locus as the complexity of the input reads increases. In an RNA sample from a genetically diverse population such as this one, the different alleles at a genomic locus present in the transcript pool increase the combinatorial possibilities for connecting each polymorphism to all others. Increasing sample complexity in the St21All dataset increased the numbers of “possible” isoforms assembled for a given gene.

Using the same method for assessment of assembly quality on a transcriptome-wide scale, we inspected individual transcript assemblies using Blat to map assembled transcripts to the *C. picta* genome. Using the UCSC genome browser or Integrated Genomics Viewer (IGV), we visualized the mapped contigs in the context of the genome and compared their structure to the homologous *C. picta* transcripts at each locus (Kent et al., 2002; Robinson et al., 2011). We visualized multiple assemblies simultaneously using the Blat mapping results, and compared samples of the DN and GG assemblies in their genomic context. The GG method produced transcript sequences that were often truncated in comparison to those derived from the DN method. Since sequence conservation declines rapidly outside of protein coding regions, it is likely that mapping to UTRs or non-coding regions was very inefficient between the *T. scripta* reads and the *C. picta* genome, leading to assembly of truncated contigs. Coverage and annotation data for two genes, *Sox9* and *Foxl2*, illustrate these points. Using *Sox9* as an example of a well-studied male gene, reads from different samples with different expression levels and regulatory status affected the assembly of transcript isoforms. Since *Sox9*, which eventually becomes a male-specific gene, is expressed in both MPT and FPT samples at stage 21, we see transcripts assembled in MPT, FPT and St21All samples (Figure 7B). Comparing the assemblies containing MPT reads (where *Sox9* is most highly expressed), it is evident that the MPT and St21All assemblies contained much longer transcripts (Figure 6B). Using the *C. picta* mRNA and peptide sequence for comparison, noted by their Refseq accession/version as XM_005282966.2 and XP_005283023.1, respectively, the longer M21 and St21All transcripts contained a very long 5’ UTR compared to the predicted *C. picta* mRNA. The appearance of introns in this 5’ region (grey
columns with red arrowheads) is due to gaps in the *C. picta* genome at 3 long and 1 short position upstream of the *Sox9* transcriptional start site (TSS). The read coverage of the elongated 5’ UTR was low, and was all but lost in comparison to the much higher levels of the ORF and 3’UTR. It may be that a small subset of *Sox9* transcripts contain this 5’UTR, or simply that transcription occurs at low levels along the promoter region, compared to the sharp increase at the previously identified mRNA 5’ UTR, and the resulting reads were incorporated into the transcript *in silico*. The truncated isoform derived from the F21 assembly may also be an artifact of the assembly process, resulting from low read coverage in the FPT sample. Combining the MPT and FPT reads in the St21All assembly resulted in increased complexity, leading to the generation of 3 transcripts that differ slightly in the long 5’UTR region. In this example of a gene that is expressed in both MPT and FPT samples, the assemblies from both temperatures generated transcripts encompassing the entire length of the reference *C. picta* transcript. In contrast, in the case of a female gene whose expression is sex-specific like *Foxl2*, the difference between the single temperature and the combined assemblies was nearly eliminated. *Foxl2*, unlike *Sox9*, does not have a period of transient expression at both MPT and FPT, thus was only present in assemblies including FPT reads. The FPT and St21All assemblies were identical and both contained a transcript that encompasses the entire *C. picta* mRNA, including its entire ORF. Both assemblies also contained a 5’ truncated transcript that began halfway through the ORF, which may be due to low read coverage, although though we cannot rule out the existence of this transcript *in vivo*.

Considering the combined analysis for assessing assembly quality based on identification of homologous sequences for each contig for each sample type and assembly method, the *de novo* assembly method was superior in all aspects. The genome-guided assemblies contained more contigs in each case (M21, F21 and St21All); however, even though all reads used in these assemblies individually mapped to the *C. picta* genome, a smaller percentage of the assembled contigs could be mapped back to the *C. picta* genome post-assembly. By the same criteria, these analyses also suggest that the M21 and F21 assemblies were quantifiably superior to the St21All using either the *de novo* or genome-guided method. Despite this fact, even in the assembly with the highest annotation rate (F21-DN), we found that only about half the total assembled contigs could be annotated with either BLAST or phmmer. This result was inconsistent with the genome mapping results against *C.*
C. picta that showed high similarity positions for 81-87% of contigs, despite the fact that the C. picta genome is still incomplete and derived from a different, though closely related species. By directly visualizing, via IGV and the UCSC Genome Browser, the mapping positions of contigs along the C. picta genome, and identifying the intronic regions based on the gaps between exons, we identified the reason for this inconsistency. Many of the small, unannotated contigs in our assemblies aligned to regions that were not part of the mature mRNAs and thus did not annotate to known transcripts or encoded peptides.

3.2.3 Assembly of Intronic and 3' UTR Sequences

In each of the 6 assemblies we identified “peri-genic” contigs that were associated with introns or 3’ UTRs of annotated contigs, but were assembled as independent units. To quantify this association, we used the C. picta genome to characterize the locations of introns in each annotated contig. The alignment positions of each annotated contig were uploaded to the UCSC genome browser separately for each assembly. Using the UCSC table browser, we extracted the intronic positions by generating a bed file for each assembly track reporting only introns. Similarly, we defined all 3’ UTRs of each contig by generating another bed file for each assembly, reporting only the 50 bases immediately downstream of the 3’ end of each transcript to avoid overlap with neighboring genes. Using Bedtools, we identified each unannotated transcript that overlapped either an intron or 3’ UTR of a coding transcript by at least 80% of its length. Most often a cluster of contigs mapped within one or more introns of a transcript (e.g. Dmrt1) or the 3’UTR of a transcript (e.g. Cyp19a1 “Aromatase”), or more rarely, in both locations (Figure 8A-B). Although transcription was also observed in the 5’ UTR of many genes, these sequences were usually contiguous with the coding region of the transcript, thus did not fall into the unannotated cohort (see Figure 7B).

At the Dmrt1 locus, a high density of peri-genic contigs, unannotated by BLAST or phmmer, mapped to the 3’ end of the last intron (Figure 6A). The coverage plot for the Dmrt1 locus showed a high read density in the 3’ end of the last intron compared to all other introns, but much lower coverage than exonic positions of the gene (Figure 8A). The same mapping results could be seen in the St21All assembly and to a lesser extent the F21 assembly. Coverage of the Dmrt1 locus was lower for both exons and introns in the F21 sample compared to M21, as one would expect since Dmrt1 is considered a testis-specific gene in this and many other species, but multiple Dmrt1 transcript
isoforms were still assembled. The *Dmrt1* transcripts for F21 took two forms, one that was roughly full-length and another that was truncated and contained a transcription start site within the second intron of the -full-length transcript. The St21All assembly also contained full-length and truncated isoforms of *Dmrt1*; however, the truncated form in this case was missing the first 2 exons and contained no additional exons compared to the -full-length sequence (Figure 8A).

At the *Aromatase* locus, a similar mapping result could be seen with the difference that the extra-exonic reads and peri-genic contigs mapped to the 3’UTR instead of an intron as seen in *Dmrt1* (Figure 8B). As Aromatase is not expressed in males, there were no transcripts assembled for *Aromatase* in the M21 assembly, as expected, and there were no reads mapping to the gene or the 3’UTR region of this locus, so the F21 and St21All assemblies were nearly identical. These samples showed a single, nearly full-length transcript with no additional isoforms; however, there were small fragments of the 3’UTR that overlapped enough of a Refseq *Aromatase* sequence to be annotated by BLAST. Read coverage of the region immediately 3’ to the *Aromatase* coding sequence was relatively high even compared to the exonic regions, but had many peaks and valleys across its length (Figure 8B). The identification of a clear difference in intron versus 3’UTR mapping of a “male” gene (*Dmrt1*) and a “female” (*Aromatase*) gene led us to investigate whether large scale differences exist between M21 and F21 assemblies.

Comparison of the proportion of peri-genic contigs that map to either an intron or 3’UTR of an annotated transcript indeed revealed a general difference between the M21 and F21 assembly mapping data. The number of 3’UTR peri-genic contigs was relatively constant across all three assemblies with 9% in St21All, 10% in M21 and 12% in F21 (Figure 8C). The difference between assemblies became apparent when looking at the proportion of peri-genic contigs that mapped to introns within each assembly. Of the peri-genic contigs mapping to introns there were roughly 17% more in the M21 when compared to the F21 assembly (Figure 8C). Because contig counts do not account for differences in sequence length, we also compared peri-genic mapping by total length of contigs mapped per category. The trend was very consistent between these two measurement methods. The proportion of UTR-mapped peri-genic contigs was nearly identical, but the M21 assembly had a much greater total length of intronic peri-genic contigs compared to F21 (Figure 8C).
Figure 8. Contigs Lacking Blast/HMMER Annotation Map to Introns and 3’UTRs of Annotated Transcripts

All contigs mapped by Blat to the Dmrt1 and Foxl2 loci, whether annotated based on sequence homology by Blast or HMMER (M21 Annotated and F21 Annotated), or lacking homology based annotation (M21 Unannotated, and F21 Unannotated) are provided from the de novo assemblies as examples. (A) Dmrt1 is expressed, though differentially, in both M21 and F21 samples as depicted by mapped RNA-seq read coverage for the Dmrt1 locus for the M21 (blue) and F21 (red) samples. Dmrt1 is encoded in 5 exons (filled boxes). Transcripts were assembled in both M21 (blue) and F21 (red) samples, and in the merged St21All sample. A large number of intronic sequences were assembled from reads originating from the last intron of Dmrt1 with significantly more in M21.
samples than in F21. (B) No reads from M21 samples mapped to the Aromatase locus, so mapped RNA-seq read coverage is shown for only the F21 (red) samples. Aromatase is encoded in 11 exons (filled boxes). Expression is limited to F21 samples, and it is also assembled in the St21All sample. Female-specific non-coding transcripts map to the 3’UTR of Aromatase. (C) Overall quantification of intronic (green), intergenic (red), and 3’UTR (blue) mapping contigs showed a very similar profile between the M21 and St21All samples, but an enrichment of intronic sequences in the M21 assembly relative to F21, which showed a higher proportion of intergenic sequences.
3.3 DISCUSSION

Research in the field of TSD has been hampered by the lack of genetic/genomic resources available in more widely studied organisms such as mice or zebrafish. However, the rapid advances in genome and transcriptome sequencing coupled with falling costs have the potential to dramatically change the field. Here we present the first transcriptome assembly of all genes expressed in the differentiating testis and ovary in the TSD model *T. scripta*. An optimized pipeline of both protein- and nucleic acid-homology searches was used to achieve a high level of transcript annotation along with *in silico* validation of nearly all assembled sequences by mapping to the genome of the related species *C. picta*.

Previous transcriptomes generated for *T. scripta* were from unspecified incubation temperatures and were derived from adult brain tissue or mixed stage whole embryo samples (Kaplinsky et al., 2013). Our use of 70 million high-quality strand-specific paired-end Illumina reads followed by *de novo* transcript assembly resulted in the identification of 348,621 annotated mRNAs as well as ORFs for 227,005 annotated peptides in stage 21 gonad samples from a total of $3.9 \times 10^8$ base pairs of sequence. The majority of contigs without BLAST or phmmer annotations from homologous sequences were peri-genic contigs mapping primarily to introns or 3'UTRs of known transcripts within the *C. picta* genome. The explanation for the abundance of transcripts from introns is unclear, but we note that alternative splicing is a driver of sex determination in other widely studied sex-determination systems such as *Drosophila* (Baker, 1989; Hodgkin, 1989). Evidence also exists in plants and yeast for temperature effects on highly conserved components of the cellular splicing machinery leading to the failure to properly release excised introns (Campodonico & Schwer, 2002; Schlaen et al., 2015). A similar process in exothermic *T. scripta* embryos could conceivably lead to accumulation of intronic sequence at MPT and may be a direct result of lower temperatures compared to FPT. This may be a simple correlation based on temperature independent of sex determination; however, the existence of such a sexually dimorphic phenomenon could conceivably act as a splicing based mechanism by which temperature might directly influence gene expression.
3.4 CONCLUSIONS

Our assessment of de novo assembly using different combinations of samples and tissues suggests that reducing tissue complexity within the sample improves the quality of the resulting assembly. While it is not yet possible to isolate cell types from turtle tissue, by generating separate assemblies from gonads developed at either MPT or FPT, we have minimized the complexity of the tissue sample to the extent possible, while still generating a comprehensive set of gonad-expressed sequences under the influence of FPT and MPT. This assembly represents the first transcriptome-wide data set for a TSD species in which MPT and FPT expression can be differentiated. These assemblies will provide an excellent resource for further study of TSD in T. scripta, and our methods can be easily repeated to expand this analysis to additional developmental stages as well as other species or tissues.

3.5 METHODS

3.5.1 Turtle Egg Husbandry

Freshly laid T. scripta eggs from multiple clutches were acquired in 2014 from Robert Clark (Hammond, LA) and The Kliebert Turtle & Alligator Farm (Hammond, LA), with the approval of the Louisiana Department of Agriculture and Forestry. Eggs were incubated continuously in moist vermiculite in a humidified incubator with ambient CO₂ at either 26°C (MPT) or 31°C (FPT). Embryos were staged by criteria established by Greenbaum (E. Greenbaum, 2002). At stage 21, embryos were removed from their eggs, quickly euthanized by decapitation, and placed into phosphate-buffered saline for dissection.

3.5.2 Sample Collection, RNA Preparation and Sequencing

Gonads were dissected from embryos held at MPT or FPT. Twelve pairs of stage 21 embryonic gonads/temperature were pooled. Total RNA for each sample group was isolated using the Qiagen RNeasy Micro kit, tested for quality using an Agilent Bioanalyzer, and submitted to the Molecular Biology Shared Resource at HHMI Janelia Research Campus. Libraries were prepared with the Illumina TruSeq Stranded prep kit then sequenced on an Illumina HiSeq 2000 instrument with 100-bp paired end reads.
3.5.3 Transcript Assembly

After generating quality metrics with FastQC (Andrews, 2010) for each set of reads, the first 12 bases were trimmed with Trimmomatic (Bolger, Lohse, & Usadel, 2014) due to poor quality and abnormal base and k-mer distribution. Read pairs were then filtered again with Trimmomatic to remove all pairs in which at least one member had an average quality score below 20. Workflows were tested for pooled MPT gonad and FPT gonad reads (see Results for a full treatment), but we found that optimal results were achieved by keeping MPT gonad and FPT gonad reads separate. Genome mapping for the genome-guided assemblies was done using Bowtie2 (Langmead & Salzberg, 2012) and the *C. picta* genome “v3.0.1/chrPic1” from the UCSC Genome Bioinformatics website. Bowtie2 was run in strand-specific, paired-end mode for each sample (F21 or M21) independently. The resulting mapping results in SAM format were sorted then converted to BAM format before passing to Trinity for assembly. For the GG_St21All assembly, the M21 and F21 BAM files containing the mapped reads were merged with Samtools (Li et al., 2009) and submitted to Trinity as a single file. All trimmed, strand-specific read pairs from the MPT and FPT gonad samples that survived filtering were passed to Trinity (Haas et al., 2013) for individual M21 or F21 assemblies, then all reads together for a single St21All run in strand-specific, paired-end mode on eight cores of a high-memory server with all other options set to default. Contigs 200bp or longer were retained.

3.5.4 Transcript Annotation

Annotation for all assemblies was done through homologue identification at both the nucleic acid and amino acid levels. BLAST (Camacho et al., 2009) was used to find matches to highly similar reference sequences in three different Refseq databases. All assembled transcripts were queried against the “vertebrate other”, “vertebrate mammalian” and “invertebrate” databases with only hits containing ≥90% base level identity reported. BLAST hits were then filtered to remove those that did not align >50% of the query sequence length. From the filtered set of BLAST hits, the hit with the highest bitscore was kept for each transcript in each assembly. To further improve the annotation through identification of more divergent gene homologues, phmmer (Eddy, 2011) was run on all open reading frames (ORFs) over 100aa in length. Peptide sequences from the same three Refseq databases (vertebrate other, vertebrate mammalian and invertebrate) were used as the target sequences, and hits with an E-value below 0.01 were reported.
Mapping of transcripts to the *C. picta* genome was performed using Blat against the *C. picta* genome v 3.0.1 downloaded from the UCSC genome browser server (Kent et al., 2002). Blat searches were restricted to reporting hits with 95% sequence identity along 70% of the query length and a minimum score of 100. In order to reduce noise due to repeat sequences in transcripts, the raw Blat (Kent, 2002) output was filtered to remove any transcripts that had reportable alignments to more than one genomic locus. To obtain intronic positions of all annotated transcripts in the gonad assembly, transcripts were mapped to the *C. picta* genome. The mapped transcripts were applied as a custom track to the *C. picta* v3.0.1 genome, then using the table browser, all intron positions were downloaded in bed format using the “introns plus” option set to 0. The same method was used to isolate 50bp regions immediately downstream of each annotated transcript by selecting the “downstream by” option while downloading the positions in bed format. Unannotated transcripts overlapping annotated introns and/or UTRs were identified using the BEDTools (Quinlan & Hall, 2010) “intersect” function to intersect intron or UTR positions, with all unannotated transcripts requiring a minimum of 80% of the unannotated transcript overlapping either an intron or UTR for reporting.
CHAPTER IV
A Timecourse Analysis of Systemic and Gonadal Effects of Temperature on Sexual Development of the Red-Eared Slider Turtle *Trachemys Scripta Elegans*

The research presented in this chapter was conducted in collaboration with Anirudh Natarajan, Lindsey Mork, Loren Looger and Blanche Capel (Czerwinski et al., 2016, In revision).

4.1 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 (Figure 9).

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, 1947; Jost et al., 1973). 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 & 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 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
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.
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 gonad. Utilizing our timecourse, we identified the order of sequential, mutually antagonistic gene activation/repression events for all genes associated with the final MPT and FPT expression pattern at the close of the gonadal temperature sensitive period (TSP). We also identified groups of genes that are activated in a heterochronic manner relative to the mouse. Despite the fact that sex determination can still be reversed in the turtle prior to stage 19 (Bull & Vogt, 1979), many transcriptional differences accumulate at MPT vs. FPT, culminating with the establishment of gonadal sex hormone signaling at stage 18.

4.2 RESULTS

4.2.1 Sample Collection

To identify genes whose expression varies with temperature, we collected embryos for RNA extraction at specific developmental stages (based on Greenbaum’s staging criteria (E Greenbaum, 2002)) before, during and after the TSP. To investigate the effect of temperature on the embryonic transcriptome prior to formation and signaling influence of the gonad, four stage 12 MPT and four stage 12 FPT embryos were collected and combined into two MPT and two FPT pooled pairs for RNA extraction. At stage 12, many embryonic tissues, including the liver, the nephric system and the brain are observable. However, the gonad has not initiated development at this stage. To investigate the MPT and FPT transcriptome in the gonad, the gonad was isolated from the mesonephric complex, which was dissected from embryos throughout the TSP: stage 15, stage 16, stage 17, stage 18, stage 19 and at stage 21, a stage post-TSP.
4.2.2 Temperature Affects Transcript Levels in Multiple Organs and Steroid Hormone Synthesis Pathways Prior to Gonad Formation

More than 500 annotated genes are significantly differentially expressed in stage 12 embryos incubated at MPT vs. FPT. In an exothermic organism, we expected that a large number of genes related to metabolism would be differentially expressed with temperature variability; this is indeed true. Within the “Up Tissue” gene ontology analysis we performed via DAVID (Huang da, Sherman, & Lempicki, 2009), transcripts associated with liver and kidney were enriched in both MPT and FPT embryos (Figure 10A). GO analysis revealed that kidney-associated genes were primarily enriched for biosynthetic and metabolic processes including response to “nutrient level” and “starvation.” The list of liver-associated genes yielded similar results but was biased toward metabolic processes. Surprisingly, the largest proportion of differentially regulated genes was assigned to the brain. In embryos incubating at FPT, the only sub-region of the brain that showed enrichment was the cortex, while many sub-regions were enriched in the MPT samples (Figure 10B). At least three of the enriched regions in the MPT samples, the pituitary, hypothalamus and olfactory bulb, from which gonadotropin-releasing hormone (GnRH) neurons arise in mammals, represent brain structures important in the regulation of reproduction. For example, we detected temperature specific differential expression of members of the Reelin signaling pathway (Figure 10C), which is important for the development of the hypothalamus, a brain structure that is widely recognized for its stereotypical sexual dimorphism in many vertebrates, including TSD reptiles (Cariboni et al., 2005; Crews, Coomber, Baldwin, Azad, & Gonzalez-Lima, 1996).

Aside from differences in the development of whole tissues, there were also striking differences in signaling pathways between MPT and FPT in early embryos. We were surprised to see differential expression of genes encoding steroid hormone synthesis enzymes in stage 12 embryos, prior to gonad formation (Figure 10D, Detailed depiction in Figure 15). Previous studies have detected both testosterone and estrogen in the yolks of turtle eggs (Elf, Lang, & Fivizzani, 2002; Janzen et al., 1998). Levels of these steroids in the yolk can vary significantly by clutch and possibly even between eggs in a single clutch. We found that the embryo itself expresses hormone synthesis enzymes that differ based on incubation temperature. Although many steroid pathway genes are common to MPT and FPT embryos, expression of Akr1d1 (5β-reductase) is enriched at MPT. This enzyme functions to shuttle testosterone to 5β-DHT, a non-aromatizable androgen, reducing the pool of
Figure 10. Prior to Gonadogenesis, Brain, Liver and Kidney Genes, As Well As Enzymes in the Steroid Synthesis Pathway Are Differentially Expressed

(A) Genes annotated as enriched in brain, liver or kidney make up the majority of temperature-based differentially expressed genes in the stage 12 embryo, with brain genes making up the largest proportion. (B) Brain cortex genes are enriched within this set at FPT, whereas transcripts generally ascribed to multiple different regions of the brain are represented in the MPT brain-enriched set. (C) Components of the Reelin signaling pathway are differentially expressed in the stage 12 embryo. MPT-enriched genes Tbr1, Reelin and Dab1 and shown in blue, with FPT-enriched Apoe, an inhibitor of Reelin signaling, shown in red. The receptors Vldr and ApoER2 are expressed, but not specifically enriched at either incubation temperature. (D) Different steroid synthesis pathway members are enriched in MPT and FPT. The key regulator Akr1d1, responsible for the irreversible conversion of precursors testosterone, cortisol and cortisone to their 5β-reduced forms, is specific to MPT.
testosterone that can be aromatized to form estrogens (Kondo et al., 1994). In birds, 5β-DHT has also been reported to inhibit Aromatase function in the brain which represents another potential masculinization mechanism for Akr1d1 expression at MPT (Schumacher, Hutchison, & Hutchison, 1991). Another major trend is the over-representation of ion-binding genes based on molecular function ontologies. Among the ion-binding genes, cation-binding and specifically calcium binding, shows differential MPT vs. FPT enrichment in the early embryo.

4.2.3 Identification of Differentially Expressed Temperature Responsive Genes

Among gene products known to respond to temperature stimuli, two classes, the heat shock proteins (HSP) and transient receptor potential (TRP) channels, are the best-studied examples. Although a few candidate genes in each of these categories have previously been investigated (Kohno et al., 2010; Yatsu et al., 2015), no unbiased catalogue of these two classes of potential regulators has yet been reported in relation to TSD. We first investigated expression of all genes encoding proteins known to sense temperature based on Mus musculus annotation (Figure 11A). We found 17 of these genes expressed in the turtle: two (Arbb2 and Nr2f6) are expressed only in the gonad, one (Calca) is expressed only in the stage 12 embryo, and 14 are expressed in both embryo and gonad. Also represented in the data are 16 TRP channels, as well as 35 Hsp40 family chaperone protein (DNAJ) and 28 other HSP temperature responsive genes. Expression levels of some of these genes show a significant difference at MPT and FPT, but this need not be true for the gene to function as a thermal sensor in TSD. We note that no MPT or FPT expression bias is consistent between the embryo and gonad for any gene in this set.

Assuming that genes with inherently temperature sensitive transcript levels behave similarly in the embryo as in the gonad, we identified a set of genes showing the same pattern of MPT or FPT enrichment in the early embryo as in all stages of gonad development (stage 15 - stage 21) (Figure 11B). There are a total of seven genes that are significantly differentially expressed and enriched at FPT in all samples, and four that show an MPT pattern in both the embryo and all gonad samples (Figure 11B). When we loosened the requirement to identify MPT or FPT enrichment from stage 12 only through stage 18 (just prior to the end of the TSP), we identified an additional four genes that are FPT-enriched, and an additional three genes that are enriched at MPT during the same period. Of these 18 genes, Jarid2 is of particular interest because it encodes a histone methyltransferase with
Figure 11. Both Temperature-Sensing Proteins and Genes Whose Transcript Level Responds to Temperature Are Expressed in the T. Scripta Embryo and Gonad

(A) Genes expressed in the whole stage 12 embryo and/or gonad that encode proteins sensing temperature, including the two major temperature-sensing gene families, Transient Receptor Potential channels (TRPs) and heat shock proteins (HSPs and DNAjs). White boxes indicate non-differential expression, hatched boxes indicate lack of expression, blue boxes indicate expression at MPT, and red boxes indicate expression at FPT in the embryo (left column “E”) or the gonad (right column “G”). Genes are assigned as differentially expressed in the gonad if they are consistent between at least two stages of the timecourse and never enriched at both temperatures. (B) Genes whose expression level is affected by temperature in both the embryo and the gonad. Blue hues indicate MPT enrichment and red hues indicate FPT enrichment. Only genes that were significantly different and consistently enriched at the same temperature from stage 12 through stage 18 are shown.
the potential to act as a master regulator of gene expression at MPT. Further evidence supporting a role for Jarid2 in sex determination comes from its close relative Jmjd1a, which regulates Sry expression in mammals and when knocked-out induces male-to-female sex reversal (Kuroki et al., 2013).

### 4.2.4 A Small Group of Genes Not Known to Be Causal in Mammalian Sex Determination Systems Show Constant MPT or FPT Gonad Enrichment From Stage 15

Prior candidate-based analyses of TSD identified many major regulators that were differentially expressed early in gonadogenesis. Among these, Dmrt1 (doublesex and mab-3 related transcription factor 1) and Aromatase (Cyp19a1) were the earliest differentially expressed MPT and FPT genes previously reported (Shoemaker-Daly et al., 2010). Our analysis identified six MPT-enriched (Vwa2, Fdxr, Nov, Kdm6b, Rbm20 and Pcsk6) and four FPT-enriched (Fank1, Avil, Twist1 and Hspb6) genes that are differentially expressed prior to Dmrt1 and Aromatase (Figure 12A,B). These genes differ from the previous group because they are not differentially expressed in stage 12 embryos, but initiate their differential pattern in the stage 15 gonad, then retain MPT or FPT enrichment across the entire temperature sensitive period. These 10 genes suggest that a gonad-specific regulatory mechanism exists that establishes MPT and FPT specific expression of certain genes much earlier than previously known, and preceding the differential pattern of the candidate regulators of sex determination, Dmrt1 and Aromatase. Of these 10 genes, Pcsk6 has been previously associated with mouse sex determination, in that it showed male gonadal enrichment and correlated with a key male sex determinant, Fgf9 (Munger et al., 2009). Twist1 inhibits the trans-activator function of Sox9 (Sry-related HMG box gene 9) (Gu, Boyer, & Naski, 2012), and thus is potentially involved in female specification in TSD vertebrates as well. Another of these genes, Kdm6b (Lysine (K)-Specific Demethylase 6B), recently implicated in alligator sex determination (Yatsu et al., 2016), shows constant enrichment at MPT, and has the potential to regulate many other genes epigenetically through histone demethylation. Fdxr is critical for cytochrome p450 function; this MPT-enriched activity could help to establish androgen precursors before the later expression of downstream enzymes.

### 4.2.5 A Cascade of Gene expression in the Gonad at MPT or FPT Indicates Mutually Antagonistic Sex Determination Programs

To determine how sexually dimorphic expression leading to the induction of functional testis or ovarian differentiation is acquired, we mapped the expression of each gene at MPT and FPT across
Figure 12. A Program of Sequentially Activated and Repressed Genes is Apparent Across the Temperature Sensitive Period of Gonad Development

Six genes maintain constant male-enrichment (A) and another 5 maintain constant female-enrichment (B) across the timecourse. (C,D,E) Sequential activation of differential expression occurs from stage 15 through stage 21. (C) *Dmrt1* and *Amh* acquire differential expression primarily through up-regulation at MPT, while *Csrnp1* and *Sox9* acquire dimorphic expression mainly through down-regulation at FPT. (D) The sequential cascade of dimorphic expression within the gonad is depicted, with one gene per row, based on log₂ fold difference between MPT and FPT with blue hues indicating MPT enrichment, and red FPT enrichment (For gene names in panel D. Log₂ fold change for each gene from the time point prior to sexual dimorphism to the first time dimorphism is reached is represented on a purple (down-regulation) to green (up-regulation) plot in the MPT (left) and FPT (right) gonads. (E) The predominant mode of FPT enrichment is up-regulation at FPT as seen in *Gldn, Cyp19a1* and *Dpt*, but with other examples of concurrent FPT up-regulation and MPT down-regulation are also evident as in *Mertk*. All expression plots are shown with normalized FPKM values.
each stage of our timecourse. We then binned the group of genes that acquire statistically different expression at each stage of gonad development (Figure 12B-D). Differential expression of each gene could result from up-regulation in one sex or down-regulation in the other (or both). To find the global pattern of changes of MPT- and FPT-enriched genes, we calculated the fold-change from the stage just prior to differential expression to the stage at which differential expression was first obtained in both MPT and FPT gonads.

In general, the pattern of mutual antagonism between the male and female pathways seems to prevail within this group of sequentially differentially expressed genes. Male enriched genes often become dimorphic through both up-regulation at MPT and down-regulation at FPT. A typical pattern is shown, for example, by Dmrt1, a gene tightly linked with maleness in most species (Figure 12C). Differential expression of Dmrt1 is achieved mainly by up-regulation at MPT, with a contribution from down-regulation in the FPT gonad between stages 19-20. Differential expression of Amh (Anti-Müllerian hormone) occurs one stage later primarily by up-regulation at MPT, whereas Csrnp1 (cysteine and serine rich nuclear protein 1) and Sox9 obtain MPT enrichment by down-regulation at FPT. Similar MPT up-regulation of Amh was seen in the alligator (Yatsu et al., 2016).

Many FPT-enriched genes show strong up-regulation at stages 15-16 with little contribution from MPT down-regulation (Figure 12C,D). A typical example of this pattern is Gldn (gliomedin). This period is followed by stages where the contribution from down-regulation at MPT is significant as in Mertk (c-mer proto-oncogene tyrosine kinase). Some FPT genes like Aromatase, and Dpt (dermatopontin) are activated de novo at stages 18-19, and never expressed at MPT (Figure 12E).

Given the large group of genes that are MPT (127)- or FPT (217)-enriched by stage 18, it is surprising that temperature shifts can still result in sex reversal of some turtles until at least stage 19 (Lance, 2009; Wibbels et al., 1991). A strong pattern of down-regulation of the alternative fate is evident around stage 18 at both MPT and FPT. Silencing of the alternative fate is completed slightly later at MPT than at FPT, which may explain why sex can be reversed at a later stage when shifting eggs from MPT to FPT, than it can when shifting eggs from FPT to MPT.
4.2.6 A Cross-Species Hidden Markov Model Analysis Compares the Timecourse of Gene Expression Between Turtle and Mouse.

Many previous studies investigated expression of small groups of mammalian sex-determining genes in TSD species (Crews, 1996; Shoemaker-Daly et al., 2010; Wibbels et al., 1991). To compare the gene expression events that occur during TSD and GSD on a broader scale, we trained and applied a Hidden Markov Model (HMM) to our turtle differential expression timecourse data (Detailed depiction in Figure 16). Similar to the technique used in Munger et al., this HMM reduced noise and increased sensitivity to detect male or female enrichment across the timecourse. It also provided a common scale to compare the turtle timecourse data to our previously published data from the mouse gonadal expression timecourse (Munger, Natarajan, Looger, Ohler, & Capel, 2013).

We limited this analysis by selecting only (1) genes for which a state path was calculated from both the turtle and mouse data, and (2) genes specific to either MPT/Male or FPT/Female for the turtle and mouse data sets. These genes were sorted by the time of initial differential expression in the turtle for both the MPT/Male and FPT/Female genes (Figure 13A-B). We found a total of 65 genes that were sexually enriched in both species, and for which a state path could be determined in both the mouse and turtle data sets. There were 43 genes male-enriched and 22 female-enriched in both species.

It is evident that the timecourse in the mouse (E11.0-E12.0) captured the earliest sexually dimorphic changes in the gonad, with Sox9 appearing as the earliest dimorphic gene at E11.2. In contrast, dimorphic expression is established earlier than expected in the turtle; 21 genes were already dimorphic at the first stage of the turtle timecourse (stage 15). Among the cross-species male-enriched genes were well known regulators of male fate such as Sox9 and Amh. The cohort of shared female-enriched genes lacked hallmark genes of female development such as Foxl2, due to its relatively late female enrichment in the mouse data set, and Aromatase, due to the lack of conservation of its role in GSD compared to TSD. However, it is worth noting that 43 male and 22 female genes are conserved in association with the initiation of testis or ovary development between turtle and mouse, and some patterns/timing of expression are highly conserved. For example, several genes become enriched and then lose that enrichment at the end of the timecourse in both species (e.g. Ednrb, Lgr5, Hapln1 and Snx18) (Figure 13A). The maintenance of strong sex-

Figure 13. Some Groups of Genes Show a Heterochronic Shift in Dimorphic Expression Onset in the Turtle vs the Mouse

(A-B) The results of HMM analysis are depicted by color based on state path Viterbi calls from two HMMs trained on *T. scripta* or *M. musculus* timecourse data. Time points are depicted from left to right for turtle stages 15-21 and mouse stages E11.0-E12.0, and aligned based on earliest dimorphic expression in *T. scripta*. (A) Male genes (blue dots) enriched in MPT *T. scripta* (left columns) and XY *M. musculus* (right columns). (B) Female genes (red dots) enriched in FPT *T. scripta* (left columns) and XX *M. musculus* (right columns). In both plots, non-dimorphic expression is indicated by empty dots. (C-D) Genes are ordered based on their earliest acquisition of differential expression in the turtle (left) and mouse (right). Groups of genes that cluster together are colored the same. The position of each gene cluster is noted for both turtle and mouse indicated by a diagonal colored box connecting the species. (C) Male-enriched genes ranging from turtle stages 15-18 (left) and mouse stages E11.2-E11.8 (right). (D) Female-enriched genes ranging from turtle stages 15-19 (left) and mouse stages E11.4-E11.8 (right).
linked expression patterns of these genes across hundreds of millions of years of evolution strongly suggests functional conservation. However, the exact regulatory timing of their expression for each species is different.

4.2.7 Groups of Genes Show a Heterochronic Shift in Expression Onset in Turtle vs Mouse

To investigate the similarities and differences in the timing of dimorphic expression of these 65 genes, we aligned male (MPT) and female (FPT) genes based on dimorphism onset in turtle and mouse (Figure 13C,D). Within each stage of the timecourse, we re-ordered genes to reveal subsets of genes that shifted together between species. For many genes, the sequential relationship in both male and female pathways is maintained between species. However, there are some exceptions. Within the male cohort (Figure 13C) two genes, Espn and Nedd9, which are dimorphic shortly after Sox9 at the onset of the mouse pathway, shift with Sox9 to stages 16-17 in the turtle. Another group of genes expressed toward the end of the timecourse in the mouse, Dtna, Elovl6, Hapln3, Scap, Sclo3a1 and Sox8, are shifted to precede Sox9 differential expression in the turtle. The genes Sox13 and Shisa2 behave individually, shifting over significant distances in the cascade.

There are significantly fewer conserved female genes identified in this analysis, and they behave more similarly between species, except for a few small groups (Figure 13D). Acss1 and Ndg1, among the earliest genes to show dimorphic female expression in the mouse, are shifted to the boundary between stages 16-17 in the turtle. Similarly, Lef1 and Slitrk1 are shifted from the earliest group in the mouse to stage 18-19 in the turtle. Trpm2, Grik5 and Nfatc4 are among the earliest genes to show female dimorphism in the turtle, but only become dimorphic between E11.6-E11.8 in the mouse. The Myl1, Axin2, Cdkn1c, Ltbp1 group has a conserved early position in both turtle and mouse pathways, but most of the mouse genes that become dimorphic at E11.8 are distributed across stages 15-19 in the turtle, although their overall order of expression is conserved.

We note that some highly studied mammalian sex-determination genes lack transcriptional sexual dimorphism in the turtle. The two most notable are the key mammalian signaling molecules, Fgf9 and Wnt4. Both of these genes were detected in turtle gonads at low levels, but were lacking in differential expression at all stages of the analysis. It is possible that (1) these genes are expressed
earlier than the stages we analyzed in the turtle, (2) the involvement of these signaling pathways in 
sex determination is specific to mammals, or (3) other Wnt and Fgf family members differentially 
expressed during the turtle timecourse assume the roles played by Fgf9 and Wnt4 in mouse.

4.3 DISCUSSION

This is the first transcriptome-wide gene expression analysis in a TSD organism in which the effects 
of temperature on the embryo-wide transcriptome prior to gonad formation are investigated in 
parallel with the effects of temperature on gonadal sex determination. Our results suggest that in T. 
scripta, both brain development and the expression of several sex-specific steroidogenic enzymes 
are regulated by temperature prior to gonad formation. We define a large group of temperature-
sensing and -responding genes in the embryo and gonad that may influence TSD. Within the gonad, 
we identify a group of MPT- and FPT-specific genes, up-regulated from the earliest stage of our 
timecourse, and we define the downstream cascade of gene expression for both male and female 
pathways. Using an HMM to compare turtle and mouse cascades, we show that heterochronic shifts 
have occurred involving groups of shared genes. The existence of antagonistic signals and strongly 
canalized pathways is conserved between turtles and mammals, but in T. scripta many antagonistic 
elements converge on the activation or inhibition of aromatase at stage 18.

4.3.1 Extra-Gonadal Differential Expression Occurs Prior to Gonadogenesis and 
Includes Stereotypical Sexually Dimorphic Genes

One of the striking findings of this study is that temperature-dependent gene expression differences 
associated with brain development exist between MPT and FPT embryos prior to gonadal 
development. Pioneering studies by Alfred Jost showed that removal of the fetal testis in rabbits led 
to development of all female secondary sexual characteristics, establishing the paradigm that global 
secondary sexual development is dictated by the gonad (Jost, 1947; Jost et al., 1973). However, 
there are contradictory examples, even in mammals. In marsupials, development of the genitalia 
is controlled by sex chromosome dosage and occurs prior to hormone synthesis in the gonad 
(Renfree et al., 2014). In TSD species, it is possible that sexual differentiation of the gonad and 
secondary sex characteristics both utilize temperature in their regulation, but one need not be 
dependent on the other.
Regions of the brain in TSD reptiles that exhibit sexual dimorphism were identified in previous studies, and it had been speculated that these arise directly through egg incubation temperature rather than by signals from the gonad (Crews et al., 1996; Flores & Crews, 1995). Here, we show directly that a key signaling pathway in brain development, the Reelin pathway, is affected by incubation temperature very early in embryonic development, prior to gonad formation. The differences in the sex hormone synthesis pathway in stage 12 embryos observed in this study match those previously observed in the adult male and female turtle brain. This suggests that early temperature-biased brain development eventually gives rise to sexually dimorphic adult brain phenotype and behavior, such that cooler temperatures lead to masculine, and warmer temperatures to feminine brains and behavior, consistent with thermal influences on the gonad. If sexually dimorphic brain development is normally tied to temperature, independently but in parallel with gonadal sex determination, then the requirement for a strong sex-specific fitness advantage postulated by the Charnov-Bull model would be met, matching reproductive behavior and gonadal sex at each temperature (Charnov & Bull, 1977). The reduction in reproductive fitness observed in hormonally sex-reversed Jacky dragons (Warner & Shine, 2008) could be explained by a mismatch between sexual physiology and brain development/reproductive behavior, effectively producing a transgendered individual.

Our studies also suggest that non-gonadal regulation of systemic signaling factors such as sex hormones or calcium could have a direct impact on gonadal differentiation in vivo. It was previously shown that the gonad senses temperature directly when dissected and cultured in vitro (Moreno-Mendoza et al., 2001; Wibbels et al., 1991). However, this does not mean that the embryonic environment does not influence the process of sex determination in ovo. It is clear that sex hormone signaling is a key regulator in gonadal sex determination in most, if not all, TSD species (Crews, 1996). There is evidence that androgen and estrogen content differs between the yolks of eggs within and between clutches (Elf et al., 2002). Our data suggests that extra-gonadal sources of hormone synthesis exist prior to the development of the gonad. Nearly the entire sex hormone synthesis pathway is in place within the stage 12 embryo, with expression dimorphisms that could produce or exacerbate existing intra-egg variation in androgens/estrogens through production of masculinizing non-aromatizable androgens such as 3β-DHT via Akr1d1. Small differences in global sex hormone signals could influence sex determination of the gonad as suggested for yolk hormones, as well as
secondary sex characteristics such as brain development. This is the first direct evidence in a TSD organism that environmental contributions to sex determination may originate or coexist outside the gonad, and that the process could begin at developmental stages preceding gonadogenesis. If some global mechanism of temperature sensation and response does exist, it is possible that the gonad is never completely naive to environmental stimuli and could arise at stage 14 in the presence of cumulative signals biasing development towards one pathway.

Inherently temperature-responsive genes could also contribute to TSD. This category includes genes whose transcription level is responsive to temperature, and genes encoding proteins with temperature-dependent activity. The group of genes whose transcription level is likely to be directly thermosensitive has been difficult to identify based only on gonad data because it is impossible to know whether a gene in the gonad responds directly to temperature, or is activated downstream of other local temperature induced signals. Reasoning that a gene whose transcription was inherently responsive to temperature would show consistent up- or down-regulation in the embryo and gonad, we assembled a list of genes that behave in this manner at FPT and MPT. In this study, we identified many genes expressed in both embryo and gonad that encode proteins annotated as temperature responders. Some of these show dimorphic expression, although this is not a requisite for acting as a temperature sensor. Prominent among these genes are TRP channels that transduce temperature stimuli into cation (usually Ca\textsuperscript{2+}) flux. Calcitonin, whose expression is excluded from the gonad, is a known temperature regulator and hormone involved in the regulation of serum calcium in mammals (Yuzurihara et al., 2003). These findings raise the possibility that temperature-induced calcium regulation plays a direct role in altering the embryonic environment prior to and during gonadogenesis.

4.3.2 Small Sets of Genes are Dimorphic Prior to Stage 15

There are 6 MPT- and 4 FPT-enriched genes that are strongly differentially expressed by stage 15, and remain so across the entire gonadal timecourse, with relatively constant expression fold-difference between MPT and FPT. Although all of these genes are expressed in the stage 12 embryo, they show no sexual dimorphism prior to their specific activation in the gonad. This suggests that there is a gonad-specific mechanism that initiates their differential expression. One or more of these genes could act as the initial environmental-responsive sensor that relays or translates temperature
information to the downstream testis and/or ovarian differentiation cascade. Further studies are required to determine whether these genes rapidly change under fluctuating temperature conditions during the TSP in the turtle.

4.3.3 Gene Expression Differences Accumulate and Converge on Antagonistic Regulation of Aromatase at Stage 18

The mammalian gonad arises as a bipotential primordium with an ovarian bias at E10.5 and remains plastic for approximately 24 hours, until after Sry expression reaches its peak in XY gonads at E11.5 (Jameson et al., 2012). After that stage, morphological differentiation occurs as the result of mutually antagonistic gene expression cascades in which male and female pathways progressively activate more sex-specific genes while repressing those associated with the other sex (Munger et al., 2013).

In T. scripta, we identified genes that are strongly up-regulated at FPT over the duration of the timecourse with virtually no MPT expression such as Cyp19a1, Dpt and Gldn. This is a much less common pattern at MPT. Even Amh, which is strongly up-regulated early in the MPT gene network, still shows some minimal expression at FPT. This non-reciprocal pattern may hint at a default male state. Until stage 18, the turtle gonad develops morphologically like a testis, with primitive SOX9-positive sex cords that later degenerate at FPT when Sox9 expression is lost (Barske & Capel, 2010; Moreno-Mendoza et al., 2001; C. Shoemaker et al., 2007). This may also be true at the transcriptional level: the ovarian fate may be induced from an initially male-like state, the converse of the situation in Mus musculus (Jameson et al.).

In the turtle, the gonad undergoes a much longer period of plasticity (1-2 weeks, depending on temperature), remaining morphologically undifferentiated until after stage 18, despite an accumulating cascade of sex-specific gene expression. One possibility is that sex determination in T. scripta is the result of a “parliamentary decision” as suggested by Georges (Sarre, Ezaz, & Georges, 2011), in which expression differences that accumulate between stages 12-18 bias the gonad in one direction or the other. The “parliament” may include temperature-responsive genes, chromatin regulators, and morphological determinants (Figure 14). Despite these differences, the gonad remains undifferentiated and open to sex reversal due to temperature shifts until the stage at which canalizing signals converge on the activation or repression of estrogen production (Lance,
Figure 14. Model for TSD in *T. Scripta*

Cumulative inputs during the TSP may establish a bias that converges on aromatase activity. (A) Between stages 15-18 dimorphic gene expression patterns accumulate at MPT and FPT. After stage 18, accumulated differences may tip the balance toward male or female fate when a specific activating signal is triggered or a signal that blocks differentiation disappears. (B) Signals regulate aromatase function in an antagonistic manner. At MPT, AMH inhibits aromatase expression, while at FPT, high aromatase expression produces estrogen, which inhibits *Amh* expression. At MPT, high calcium levels inhibit aromatase, while at FPT, CRISP blocks intracellular Ca\(^{2+}\) release. At MPT testosterone is irreversibly converted to 5β-DHT by Akr1d1, which effectively reduces estrogen production. Akr1d1 may operate in the embryo, prior to gonad formation. E2: estrogen.
2009). It is unclear why canalization is initiated at stage 18, and not earlier. This could be due to the gradual accumulation of signals that create a significant bias, a specific activating signal, or the disappearance of some “bipotential” factor that blocks sex-specific development prior to this stage.

After stage 18, antagonistic signals converge on the regulation of aromatase. At MPT, Anti-Müllerian Hormone (AMH) shows an increase in expression between stages 16-17, and strong up-regulation at stage 18. AMH has been shown to inhibit expression of aromatase in human cells (Chang, Klausen, & Leung, 2013); a similar mechanism could serve here as a buffer against estrogen production and feminization. At FPT, aromatase shows a surge in expression around stage 18, perhaps as a result of cumulative signals at higher temperature. Coincident with aromatase up-regulation is up-regulation of a CRISP venom-like protein in the FPT gonad, which functions to inhibit calcium release in muscle cells (Yamazaki & Morita, 2004). Intracellular calcium levels can block aromatase function through multiple phosphorylation events (Balthazart, Baillien, Charlier, & Ball, 2003). We propose that CRISP acts as a buffer against the effects of intracellular calcium concentration on phosphorylation and inhibition of aromatase function, similar to the way AMH buffers masculinization by inhibiting aromatase expression. Production of estrogen by aromatase both drives aromatase expression and inhibits AMH expression. Prior to stage 18, the gonad remains plastic; however, after stage 18, the canalization of male or female pathways is driven by strong feedback loops, and antagonism of the alternative pathway.

4.3.4 Heterochronic Gene Expression Events Occur in TSD Compared to Mammals

The sex determination cascade in *T. scripta* shares similarities with the one in mammals, but there are significant differences in timing between groups of genes. In mammals, *Sox9* expression in males occurs prior to induction of *Amh* up-regulation. *Amh* is differentially expressed by stage 17 in our dataset, consistent with reports in other reptiles and birds (Oreal et al., 1998; C. Shoemaker et al., 2007; Yatsu et al., 2016). We have identified a number of other genes that acquire sexually dimorphic expression in a different order between *T. scripta* and mammalian GSD systems. Some of these could shift in accord with morphological differences in development, suggesting functional relationships. For example, the heterochronic shift in *Sox9*, *Espn* and *Nedd9* could be related to the existence of primitive sex cords in both MPT and FPT gonads in *T. scripta*, but not in mouse primordial gonads. These differences in the timing of differential expression of male and female SD genes could also
suggest that there are multiple entry points into the cascade that controls sex determination. The strong canalization of the system ensures that once a significant bias exists, feedback loops amplify one fate while the alternative pathway is silenced. Work in systems that are amenable to genetic manipulations or well-designed pharmacological studies in turtles may unravel the significance of these shifts in the timing of gene regulation through the male and female regulatory cascades.

4.4 METHODS

4.4.1 Turtle Egg Husbandry

Freshly laid Trachemys scripta elegans eggs from multiple clutches were acquired from Tangi Turtle Farm (Hammond, LA) and The Klebert Turtle & Alligator Farm (Hammond, LA), with the approval of the Louisiana Department of Agriculture and Forestry over a 3-year period from 2013-2015. Eggs were collected and shipped overnight within one day of being laid. Upon arrival all eggs were incubated continuously in moist perlite in a humidified incubator with ambient CO₂ at either 26°C (MPT) or 31°C (FPT). Embryos were staged by criteria established by Greenbaum (Greenbaum, 2002). Embryos were removed from their eggs, quickly euthanized by decapitation, and placed into phosphate-buffered saline for dissection. Stage 12 embryos were removed from their eggs, quickly rinsed three times in clean phosphate-buffered saline to remove all traces of yolk or other contaminants, and then were snap frozen in liquid nitrogen in individual microcentrifuge tubes.

4.4.2 Sample Collection, RNA Preparation and Sequencing

Gonads were dissected from embryos continually incubated at MPT or FPT and removed from the mesonephros prior to RNA isolation. On average, 20 pairs of embryonic gonads/temperature/stage were pooled to yield adequate RNA for sequencing of each replicate, for either two or three biological replicates per stage. For the stage 12 whole embryo samples, two embryos were pooled prior to RNA extraction for each of two replicates per temperature. Stage 26 embryo data was derived from a pool of all soft tissues excluding the gonad for both MPT and FPT. Total RNA for each sample group was isolated using the Qiagen RNeasy Micro kit, tested for quality using an Agilent Bioanalyzer, and submitted to the Molecular Biology Shared Resource at HHMI Janelia Research Campus. Libraries were prepared with the Illumina TruSeq Stranded prep kit, and then sequenced on an Illumina HiSeq 2000 instrument with 100-bp paired end reads. All sequence reads are deposited with NCBI SRA under BioProject PRJNA331105 with SRA accession SRP079664.
4.4.3 Transcript Assembly

After generating quality metrics with FastQC for each set of reads, the first 12 bases were trimmed with Trimmomatic (Bolger et al., 2014) due to poor quality and abnormal base and k-mer distribution. Read pairs were then filtered again with Trimmomatic to remove all pairs in which at least one member had an average quality score below 20. Reads for all replicates from each combination of stage and temperature were submitted to Trinity [http://www.nature.com/nbt/journal/v29/n7/abs/nbt.1883.html] for separate de novo assembly runs. Contigs 200bp or longer were retained and used for downstream reference transcriptome generation. Each of the 16 resulting de novo assemblies were merged and submitted to Evidential Gene for comparison and merger to generate a single assembly with minimal redundancy while maximizing the maintenance of long ORFs in each contig. This process was completed once with all assemblies resulting from gonad samples only (stages 15, 16, 17, 18, 19 and 21) to generate the allGonad assembly, then again with all 16 assemblies at the same time to generate a second more inclusive reference containing gonadal, as well as non-gonadal genes, referred to as totalMerge. Using the transcript annotation from BLAST and HMMER, transcripts were grouped into gene isoforms based on the homologous gene by which each was annotated. All downstream analysis was done at the gene level based on these two assemblies (allGonad and/or totalMerge) with gene isoform relationships inferred from the annotation. All assembled sequences are deposited with NCBI TSA under BioProject PRJNA331105.

4.4.4 Transcript Annotation

Annotation for all assemblies was done through homologue identification at both the nucleic acid and amino acid levels. BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) was used to find matches to highly similar reference sequences in the Mus musculus Refseq databases. All assembled transcripts were queried with Blast+ against the Mus musculus mRNA database with only hits containing ≥90% base level identity reported. BLAST hits were then filtered to remove those that did not align >50% of the query sequence length. From the filtered set of BLAST hits, the hit with the highest bitscore was kept for each transcript in each assembly. To further improve the annotation through identification of more divergent gene homologues, phmmer (Eddy, 2011) was run on all open reading frames (ORFs) over 100aa in length. Peptide sequences from the Mus musculus peptide Refseq databases were used as the query sequences, and hits with an E-value
below 0.01 were reported. The annotation used for the primary analysis was based off *Mus musculus* specifically to ease the comparison between this new *T. scripta* data and existing data from mouse sex determination.

### 4.4.5 Differential Expression Analysis

The reads from each replicate were mapped to the allGonad assembly for quantification in all cases except for those in which the stage 12 embryo was being compared with Bowtie2 (Langmead & Salzberg, 2012). Thresholds were set to allow for reporting all valid alignments for each read pair up to 10, discarding all reads with greater than 10 valid mappings. Only genes with a summed read count of at least 10 reads across all timepoints were used for downstream analysis; the rest were discarded. RSEM gene level read counts were summed for all isoforms of each gene and then rounded to the nearest integer and submitted to DESeq2 for calculation of differential expression. Differential expression was calculated for MPT vs FPT for all stages and tissues as well as all adjacent timepoints within each temperature group.

### 4.4.6 Hidden Markov Model

Similar to the previously reported technique from Munger et al., a 3 state Hidden Markov Model (HMM) was defined in which the states represented a gene being differentially expressed and male-enriched (M), female-enriched (F) or not differentially expressed (0) at each stage of the gonadal RNA-seq timecourse. Fold difference from the comparison of MPT vs FPT at each stage was used to train the HMM after conversion to a discrete scale of symbols. Each fold difference was represented by one of six symbols (f3, f2, f1, s, m1, m2, m3) ranging from “f3” with the highest degree of FPT enrichment, to “m3” with the highest degree of MPT enrichment, and “s” in the middle representing not significantly different or “similar”. The HMM was trained with symbolized fold differences from all genes that were significantly different between MPT and FPT at one or more stages, or were significantly different between two or more adjacent stages at either MPT or FPT. After training, the model was used to predict a Viterbi path for all genes with a minimum count of 30 reads across the entire timecourse from MPT or FPT. The 6-symbol scale was then collapsed into simple male (M), female (F) or not different (0) for each stage to generate a state path for each gene. Using the *Mus musculus* based annotation of the *T. scripta* reference transcriptome, the state path for each gene
was compared to the previously reported state path for that gene during mouse sex determination between embryonic stages 11.0 and 12.0.
Figure 15. Steroid Hormone Synthesis Network with MPT or FPT Expression Enrichment of Nodes from Embryo or Gonad RNA-seq Data

Figure adapted from KEGG pathways[43] graph rendered by Pathview[44]. Enzymes are represented by boxes with white boxes representing lack of detection in any RNA-seq sample. Each enzymatic product is represented by a circle with accompanying text label. Enzymes with enrichment in the stage 12 embryo are highlighted with light blue (MPT enrichment) light red (FPT enrichment) and light green (expressed without temperature enrichment). If a gene was not expressed in the stage 12 embryo but was in expressed the gonad at any time point it is highlighted in dark blue (MPT specific enrichment) dark red (FPT specific enrichment) or dark green (expressed without temperature enrichment). Cyp19a1: aromatase.
Figure 16. Hidden Markov Model Statistics Before and After Training

(A). Transition probabilities between male, similar and female stages are shown for stages 16 through 21 before (after) training. Start state is represented by row with male, similar and female from top to bottom than the transition state within the three groups again as male, similar and female from top to bottom. Each stage is represented horizontally so that transitions occur between adjacent stages from left to right. (B) Emission probabilities for male enriched emissions (m3, m2, m1) on top, similar state in the middle, and female enriched emissions (f1, f2, f3) on the bottom. Colors represent the degree of fold difference with blue hues representing higher male emission and red female emission probabilities.
CHAPTER V
Turtle Egg Husbandry and Derivation of Turtle Cell Lines

5.1 The Genetics of Farmed Turtle Eggs are Complex and Not Well Understood

The red eared slider turtle, *Trachemys scripta elegans* is an aquatic turtle that is native to North America, but now has established wild populations around the globe likely do to escaped or released pets. Many American researchers acquire *T. scripta* eggs from turtle farms in southern Louisiana where captive adults are bred and their eggs are collected daily during the summer months. These farms have been in place for multiple generations, yet little to no information exists regarding the genetics of the breeding populations. Discussions with Keith Boudreaux, owner of one particular farm in Hammond LA, Tangi Turtle Farm, have provided some minor information as to what might be expected from his farm's genetic pool. Turtles are often captured as adults from wild populations in the local area. These new adults vary in age and are retained in the breeding pool for an unknown number of years. The *T. scripta* population at Tangi Turtle Farm has been maintained in this fashion since 1953 so that any given breeding adult could have been acquired from the wild at any time in the last 30 years up to the current season due to the long lifespan of captive *T. scripta* (Gibbons 1987). The practice, in general, is to not introduce locally bred offspring back into the breeding pool. However, since females lay their nests in the same housing space in which they live and breed, it is likely that nests are sometimes missed and the offspring do indeed enter the breeding pool to some degree. Therefore, the eggs acquired from a turtle farm can be expected to be generally outbred with an unknown degree of inbreeding. Another important point to note is that these farms usually breed and sell multiple species of turtles with overlapping breeding seasons and habits. This is an important aspect of acquiring eggs since it is not only likely, but proven that eggs from other species will be, to a small degree, included in shipments of eggs that are intended to be entirely composed of *T. scripta*. When turtle eggs are allowed to develop to more advanced stages of embryogenesis, the species-specific distinguishing characteristics, such as shell pattern and head/limb morphology, can be identified. We observed at least a few cases per year in which an egg, once opened, was found to contain a Mississippi map turtle *Graptemys pseudogeographica kohni* embryo. The very small percentage of contamination from these stray eggs of the wrong species is generally insignificant for most experimental designs, but must be considered in experiments that require drawing conclusions.
from limited numbers of embryos. All farms from which we have acquired eggs collect their eggs each morning so that no nests remain uncollected at the end of each day, and each egg collected can be assumed to have been laid the previous night. The eggs are then immediately packed in moist vermiculite, perlite or peat moss and shipped overnight by a commercial parcel service to arrive early the next morning. This shipping method has proven to be generally successful and eggs arrive in good health assuming no unforeseen delay in delivery.

Information and protocols for the husbandry of turtle eggs are lacking in published scientific literature. The general protocol cited in scientific papers consists of incubating the eggs in moist vermiculite with no more than a 1.5 inch layer covering them at the surface. Often, protocols will describe the amount of water that is added to the initial substrate used for incubation as well as the water added to maintain moisture to account for evaporation or when replacing the substrate. This simple protocol is insufficient to support successful incubation of a high percentage of viable healthy embryos and can lead to high rates of embryo death throughout the duration of incubation. By identifying alternative sources of information on husbandry and multiple seasons of trial and error, I have generated an improved and detailed protocol that increases embryo viability greatly, and maintains consistency of embryonic development between seasons and batches of eggs.

5.2 There are Two Major Types of Incubation Substrate Used in Laboratory Rearing of Turtle Eggs

The most important variable in the successful rearing of turtle eggs is the water used for maintaining egg hydration: both the quality and quantity of water is important. A *T. scripta* egg that is properly hydrated and incubated within a viable physiological range will have a distinct shape and rigidity, and departures from these temperature and moisture ranges will be easily noticeable in both egg shape and feel. One important clarification to any published protocol is to differentiate between the two main types of substrate used for lining egg incubation containers and covering the eggs during incubation. Most protocols tend to list vermiculite as the substrate used in their egg incubation; however, perlite is often used instead and often mistaken for actual vermiculite. Both these minerals are lightweight porous materials that are naturally occurring but heated in order to expand and increase the size and number of the internal voids that act to hold and slowly release water. Both vermiculite and perlite are excellent substrates for incubating turtle eggs of all stages, but each
has pros and cons depending on the experimental application intended for the eggs. In general, vermiculite retains moisture much better and requires much less monitoring to maintain the proper hydration of eggs. Depending on the source of vermiculite and the degree of thermal expansion it’s been subjected to, it can tend to be more fragmented or “dirty” than perlite. The smaller fragments of vermiculite allow the material to pack more densely around eggs, leaving less air exchange and a greater amount of the eggshell in direct contact with the surrounding substrate. Vermiculite, due to its smaller particles, tends to stick more readily to the eggshell and is more difficult to rinse off the egg leading to more debris contaminating embryos while removing them. Perlite on the other hand generally remains in larger rounded fragments or crumbles entirely to dust, all forms being very easy to rinse away. Moisture content of perlite tends to change more rapidly from evaporation. The larger fragment size leads to more airspace surrounding the eggs but tends to maintain the water content of the eggs as well as vermiculite if hydrated appropriately. The main difference in perlite’s ability to maintain proper egg moisture is that evaporation tends to proceed much more rapidly. The effect is that eggs incubated in perlite need to be tended much more often than eggs held in vermiculite, and the exposure of each egg to external moisture is much less constant. It is unknown whether there is any significant effect on the embryo when external moisture varies more or less, but exposure to substrate that is too wet or too dry for extended periods of time (days) does have a significant impact on embryo mortality.

5.3 Source and Treatment of Water is Critical for Egg Survival

Historically, the lab has used simple autoclaved tap water for the purposes of keeping eggs. The autoclaving process is thought to be sufficient to cause the evaporation of all harmful water additives, or at least to the degree that they are no longer concentrated enough to harm the eggs. This process seemed sufficient for a number of years of research and egg survival was adequate. Egg survival started to decline during the course of my research, from historically 80-90% survival within the first 2 weeks, survival dropped down to 40-60%, and there was some indication that water quality may have been the cause. It was suggested that a change in the treatment of Durham city water occurred at some point of time when chloramine was substituted for traditional chlorine, among other changes. Chloramine is more stable in water than chlorine so that it does not evaporate as readily and will be retained in water even after heating. Because our turtle eggs have water applied to the egg boxes every
day to offset evaporation, there was a possibility that chloramine was accumulating in the perlite as well as the eggs over time. In an attempt to improve the water quality for egg husbandry, I began to pre-treat the water with a water conditioner (Prime Water Conditioner, SeaChem) designed for use in aquariums. The tap water was treated in 4L volumes then left at room temperature over night before autoclaving. Upon adopting this protocol, egg survival immediately went back up to 90%. No exhaustive study of chloramine, or other water additives, was done on the eggs or water prior to or after this alteration in our water treatment protocol, but the increase in egg viability has persisted.

5.4 Egg Hydration Within a Specific Range is Critical for Proper Embryonic Development and Viability

Aside from water quality, water quantity is also essential for embryo viability. When eggs of *T. scripta* are first laid, they are an oblong and roughly elliptical. Over the course of development, the length of the egg does not increase appreciably, but it gradually becomes more spherical as the narrower circumference of the egg expands. This is the case for an egg that is not over- or under-hydrated. When eggs are over-hydrated for an extended period of time by adding too much water to their substrate repeatedly for multiple days, the egg will swell and the shell becomes turgid and stretched. Eggs that are grown in these conditions for a long period of time (weeks) will produce embryos that are malformed or non-viable. It is unknown if this over-hydration effects sex determination, but there have been some reports of over-hydration biasing eggs toward male development (CITE). On the other end of the spectrum, under-hydrated eggs will lose their turgidity over time and tend to die more often than over-hydrated eggs. Eggs in under-hydrated conditions are easy to identify because they will have concave “dents” in the eggshell. Both under- and over-hydration can cause an embryo to die in ovo, but if improper hydration is identified early enough, eggs can often be recused by transferring them to substrate with proper hydration levels. Under-hydration can also be confused for egg death or infertility during the early stages of development. These two conditions can be differentiated by both the look and feel of the egg. Eggs containing dead embryos tend to have more ridged eggshells that, when compressed, feel as though the shell is thicker or more leathery than the thinner more pliable shell of a living egg. Eggshells in general let some degree of light through them when the egg is alive. When held closely in front of a bright light (candeling), the line where the yolk on the bottom meets the albumin on the top can be seen. A living egg will have a clear distinction between these two layers that can be seen as a shadow through the shell, and a gentle rocking
motion of the egg will cause the yolk to move slightly as it is somewhat fluid. An egg containing an embryo that has died will lose the separation of the yolk and albumin layer eventually. The eggshell of a dead egg is also thicker and more opaque, and if the shadow of the edge of the yolk can be seen through the shell, the yolk will be thicker and not fluid enough to move readily as the egg is rocked. It is important to be careful when rocking the eggs to observe this phenomenon however as turtle embryos are attached to the underside of the egg shell by the vascular bed of the yolk sac and too much or too rapid movement can tear the membranes securing them, causing the embryo to die.

5.5 Egg Orientation is Fixed During Development
In bird eggs, the embryo sits on top of the yolk with the vascular bed of the yolk sac surrounding the yolk below it. This allows the yolk and the embryo on top of it to rotate, keeping the embryo always upright. In contrast, turtle eggs, cannot be rotated the way bird eggs often are during incubation as the fixation of the embryo to the eggshell restricts which side of the egg must remain up. After an egg is laid and early embryogenesis has occurred, the embryo becomes fixed to the side of the eggshell that is facing upward at that time. Most of the time, the position of the embryo under the eggshell can be identified by the formation of a lighter “chalky” white spot on both the inside and outside of the shell. This white spot must remain up at all times during development so that the embryo remains above the yolk within the egg. If the egg is rotated so that the white spot is on the bottom of the egg, the embryo will be smothered by the yolk and die. This may be less of an issue later in development when the size of the yolk has been reduced and the membranes surrounding it are more developed. However, at early stages, it is absolutely critical that the side of the egg containing the embryo remain upward at all times.

5.6 Derivation of Turtle Cell Lines
With no current ability to do genetic experiments in turtles, and the limitations of in ovo experimentation, ex vivo methods of experimenting on live turtle tissues are much needed.
5.6.1 Cell Culture Media: (RPMI media with phenol red (Gibco), 20% FBS (not heat-inactivated), 1x Anti-Anti (Gibco))

Keep cells in a cell culture incubator with 5% CO2 between 26 °C (MPT) and 31°C (FPT). Growth is more rapid at 31 °C for most cell types, however 26 °C often times allows for the maintenance of various differentiated cell types better than 31°C. Some cell lines can tolerate lower FBS concentration (10%) after the initial seeding process but may tend to expand more slowly.

5.6.2 There Are Two Techniques for Establishing Primary Cell Lines with Different Effectiveness Based on Tissue of Origin

I have used two techniques for initial seeding of primary embryonic turtle cells; (1) tissue can be minced; or (2) cells can be dissociated using enzymatic methods. These techniques vary in effectiveness depending on tissue of origin. Contamination is the primary reason for failure to establish a turtle cell line. For both techniques, it is critical to wash the dissected tissue multiple times to remove as many of the contaminating microbes as possible. To wash the tissue, place a small fragment into a drop of PBS with 1x Anti-Anti (Gibco) in a sterile petri dish. Stir the tissue around the droplet for about 15-30 seconds then transfer the tissue with either sterile forceps or a sterile mouth-pipet to a new, clean drop of PBS with 1x Anti-Anti, and repeat stirring/mixing for another 30 seconds. Wash the tissue piece in PBS with 1x Anti-Anti one more time, then transfer it into a drop of culture media. Repeat the washing procedure in culture media drops two more times for a total of 3 PBS washes and 3 culture media washes. The tissue piece, if sufficiently small (~3mm cube or less), can be incubated in this droplet overnight if covered and placed in a humidified incubator, or continued through processing immediately.

1) Mincing: To establish primary turtle cell line cultures from minced tissue, remove most of the culture media droplet around the tissue, then mince with a sterile scalpel or razor blade for about a minute, or until no large pieces remain. It helps to mince the tissue in one direction first, then rotate the dish to mince at 90 degrees to the original blade angle. After mincing, pipette some culture media onto the tissue, then transfer to a sterile 1.5ml microcentrifuge tube. Distribute the cell suspension into one or more wells of a 6-well or 12-well tissue culture-treated plate and add with just enough culture media to cover the entire bottom of the plate. It is important not to use too much media during the initial seeding of the cell line because you want the cells and tissue chunks to be touching
the bottom of the plate with no, or minimal, room to float above it. Place the plate into a cell culture incubator with 5% CO₂ for 1-2 hours to allow the cells to adhere to the plate. After this initial period of adherence, remove all the media and gently rinse the well with culture media 2-3 times. This rinsing procedure is important to reduce the likelihood of microbe contamination, as well as to remove cellular and extracellular debris that can inhibit the spreading and expansion of the initial seed cells. Cells should be seeded at high density, so that there is no more than 5-10 cell widths between uniformly spaced cells in a dish. To seed cultures from smaller pieces of tissue do the initial seeding step in a smaller sized well to increase cell density. Once cells reach 85-90% confluence, they can be expanded to a larger well for further expansion, or if originating from a 6-well plate they can usually be seeded into a 25cm² tissue culture flask at this point.

2) Enzymatic Digestion: To establish primary turtle cell line cultures by enzymatic digestion, after the washing steps, the tissue should be placed in a 1.5ml microcentrifuge tube and washed one time quickly with trypsin (Trypsin-EDTA 0.25%, Gibco), after which the trypsin (500uL is usually sufficient) should be replaced and the tube incubated at 37°C for 10 minutes. During the initial trypsinization, the tube should be mixed by inverting several times. This is sufficient to generate a single cell suspension for most embryonic tissues, however, fibrous tissues such as heart and skeletal muscle may need to be gently pipetted up and down several times over the 10-minute incubation period to maximize cell extraction from the tissue. The cell suspension should then be pipetted into one or more wells of a 6-well or 12-well plate and 3x the volume of cell culture media should be added to neutralize the enzyme. Incubate the cells for 1-2 hours to allow them to adhere, then rinse the well with cell culture media 2-3 times to remove floating cells and debris. After reaching 85-90% confluence, this culture can be expanded to either a larger well or directly to a 25cm² culture flask if originating from a well of a 6-well plate. Most tissues will produce fibroblast-like cells using these techniques with some, such as embryonic testis, producing a mix of fibroblast- and epithelial-like cells. After the culture is split a few times, it often results in a roughly homogenous culture of a single cell type, though the type of cell that dominates the line can be manipulated based on the way cells are split between passages (see below).

In general, trypsinizing cells has produced better outcomes than the mincing method for most tissues. The primary advantage to using trypsin is that a much more uniform cell suspension can
be produced and cells tend to adhere and expand more rapidly after initial seeding. There are some tissue types from which I have been unable to derive cell lines using the trypsin technique, and these seem to be tissues with highly specialized/differentiated cell types such as neurons in brain and multi-ciliated cells in mesonephros.

5.7 Specific Cell Types Can Be Isolated Based on Techniques Used in Splitting Cultures

I have yet to derive a primary cell line from embryonic turtle cells that only persists for a certain number of passages then becomes non-proliferative. Many cell lines have been carried for over 30 passages with no indication of any loss in proliferative ability. Splitting cell lines should always be done by enzymatic means and not be scraping, as scraped cultures often do not expand well afterward. To split these primary cultures a standard trypsinization protocol can be used. In short, aspirate all media from the flask with a sterile pipet, then apply a thin layer of “sacrificial” trypsin (Trypsin-EDTA 0.25%, Gibco) to the dish briefly, use just enough to cover the entire bottom of the culture vessel where the cells are adhered. Quickly remove this sacrificial layer with a sterile pipet and apply fresh trypsin. For a 25cm² plate, 1-1.5 ml of trypsin is usually enough. The trypsin solution need not be warmed to 37 °C degrees prior to use and works just as well immediately after it’s removed from storage at 4 °C. After applying the second trypsin solution, place the plate at 37 °C for 5-10 minutes, checking regularly to see if the cells have become dislodged from the surface. Occasionally rocking the culture vessel to mix the trypsin layer helps detach the cells more quickly. You can select for different types of primary cells to dominate the subsequent cell lines by shortening or lengthening the time of trypsin incubation. In some cases, the epithelial cells will dislodge from the surface of the plate more quickly than the fibroblasts, or vice versa. You can test this by taking cells at different stages of trypsin incubation and transferring to new culture wells.

For standard splitting of cultures, remove the culture vessel when most of the cells have dislodged. To facilitate this process, you can place the flask a few millimeters above the bench top and drop it a few times to jar the cells free. Neutralize the trypsin with 3x the volume of cell culture media then split according to your needs, though no thinner than 1:8 to maintain proliferation. The process may need to be repeated every 3-6 days depending on the cell type and the incubation temperature, but avoiding confluence is advised as the cells generally divide more slowly after a period of confluence.
Most cells show some degree of contact inhibition, but fibroblasts from heart and gut seem to lose this behavior at high cell density. If left at confluence for extended periods of time, fibroblasts will tend to migrate into evenly spaced clumps a few cell layers tall then stop proliferating until they are split again (Figure 17). These cultures can be convinced to expand again after passaging, but they will have an extended lag period immediately after the clumped stage.

5.8 Differentiated Cells Can Be Cultured for a Few Passages by Culturing Whole Organs

To produce cultures of various differentiated cells, other techniques can be used to maintain them for short periods of time. Cardiomyocytes don’t proliferate in culture and lines derived of heart tissue are almost always made up entirely of fibroblasts after a few passages. However, cardiomyocytes can be maintained and expanded in culture for a few passages by establishing them from whole embryonic hearts. When a heart from a turtle embryo at about stage 17 or younger is washed and placed in culture on the bottom of a multi-well plate with just enough media to cover the bottom of the well, it will adhere to the dish and remain beating for days or even weeks. After a few days in culture, numerous cells from the exterior of the heart begin to migrate away from the organ in multiple layers consisting of multiple cell types including differentiated, beating cardiomyocytes. After about a week, these heart cultures can be lightly trypsinized for a few minutes so that cells remain in clumps and are not reduced to single cell suspensions. Cell clumps can then be passaged to continue the expansion of the differentiated cells. This process has also worked for whole embryonic gonads, gut, and liver. This often allows for the derivation of multiple cell lines of different cell types from a single organ and is more likely to produce a line that is not composed of simple embryonic fibroblast-like cells. This technique also works on whole early-stage embryos between stages 10 to 12 as well. Embryos are more likely than individual organs to become contaminated over time, but the heart will remain beating and the embryo will grow and develop for many days in culture before cell extravasation along the surface of the plate causes the tissue to become indistinct and the heart stops beating.
Figure 17. Primary Cells in Culture Migrate Into Clumps After Extended Time at Confluence

A tiled image of multiple 10x images of primary turtle cells derived of embryonic gut. Cells that are in the later stages of clumping can be seen in the left of the image. A section of the plate that more recently reached cellular confluence and is in the early stages of clumping is on the right.
5.9 A New Culture Media Formulation Allows for Longer Culture of Embryonic Turtle Gonads

We have previously published a protocol for short-term culture of embryonic turtle gonads ex vivo (Mork & Capel, 2013). In general, this technique is limited in its effective duration by tissue survival and culture contamination so that experiments can only last for around 10 days. By experimenting with culture conditions and media formulations, I have improved our existing gonad culture system so that tissue can survive and develop for at least one month in culture.

With the new organ culture media formulation described here (Gonad culture media: (RPMI media with phenol red (Gibco), 10% FBS, 1x Anti-Anti (Gibco)), gonads have been successfully cultured for over a month without any noticeable negative effects on gonad morphology or development. Before beginning any ex vivo gonad culture experiment, since the FBS used in the media is best if not heat-inactivated, it is important to find a proven lot of FBS that does not contain hormones that might interfere with proper TSD-based sexual development. This can be done by using a new lot of FBS for making a batch of gonad culture media, then culturing gonads at MPT and FPT with the same media and assaying for appropriate sex determination with FPT causing down regulation of SOX9 and cortical enrichment of β-catenin, and MPT gonads retaining SOX9 expression in the supporting cells. This can be easily assayed through immunofluorescence of SOX9 and β-catenin on gonads that are cultured beginning at stage 17-18 for at least one week or longer. Using validated FBS with minimal hormone contamination, gonads can be cultured using the original protocol published from the Capel lab with only a few modifications (rCIT). Stock RPMI media should be used to produce 1.5% agar blocks. The blocks should then be soaked overnight in 1ml of complete gonad culture media at 31°C. Any block that was contaminated during production will be obvious after 24 hours of incubation. In addition, the block will be equilibrated with the FBS and Anti-Anti from the completed media prior to initiating the culture. For turtle gonads we use a narrow well in comparison to the agar pad used for mouse gonad culture. The turtle gonad is cultured without the mesonephros attached and the wells are 0.4mm deep, 0.25mm wide on the bottom sloping outward to be 0.75mm wide at the mouth. When culturing gonads on agar pads made from 800-1000µL of 1.5% agar, the dish should contain no more than 350µL of media as any more risks overtopping the pad and rinsing the gonads from the wells. Once placed in culture, gonads should not be moved or manipulated in any way to avoid damaging them or contaminating the culture unless it is critical.
for the experiment itself. The wells containing the gonads do not need to be “flushed” or drained ever
during the culture period, and I have found that doing so does not seem to improve the cultures but
does significantly increase the chance of contamination. If media is replaced at least every 3 days, the
gonads will survive and develop for a very long period of time with the limiting factor seeming to
be a maximal size at which the media cannot readily diffuse through the entire volume of the gonad.
CHAPTER VI
Conclusions and Future Directions

Our current understanding of temperature dependent sex determination is still very minimal even after the 50 year of research in the field. With recent advances in sequencing technology and new discoveries of species with coexisting TSD and GSD many new avenues have been opened for addressing long standing questions in the field. We have produced evidence that TSD occurs much earlier than was previously thought and may be more widespread within the embryo instead of simply restricted to the gonad. Given these results, more focus needs to be given to non-gonadal sex determination as well as early and/or systemic systems that might be regulating or buffering TSD before canalization occurs toward the end of the temperature sensitive period.

Our findings that gonad pairs develop concordantly ex vivo at PvT was initially difficult to interpret. The two major explanations were that a systemic factor, genetic or otherwise, was shared between each gonad in the pair, or that sex had been partially but stably determined at stage 17, the time of the initial dissection. After our later analysis of gene expression in a stage 12 embryo, as well as multiple early gonad stages, it’s clear that a significant amount of the male and female gene expression networks are in place by stage 17. In fact, all previously known regulators of male and female development are already established at this point. It is, however, still possible that in the absence of strong thermal influence, such as at PvT, genetic or other global factors act to bias an embryo toward either the male or female fate. To address this possibility, we have begun experiments in genome sequencing, assembly and multiple reassemblies to identify loci that strongly correlate with sexual phenotype after incubation at PvT. We will sample a large number of individuals from many different clutches across the entirety of the egg laying season, sequence their individual genomes and perform genome-wide association studies to find consistent correlations with male or female development. To facilitate this goal and provide greater genetic tools for future genetic analysis of TSD and T. scripta, we have already sequenced and will soon begin assembly of the T. scripta genome.

To aide in the assembly of the T. scripta genome, we are working with collaborators who have developed novel assembly methods that leverage the multiple diverse transcriptome assemblies we have already generated. The large number of transcriptome assemblies, their high quality and existing annotation will allow us to rapidly annotate and release the T. scripta genome for public
consumption in our own experimental purposes. A high quality annotated genome assembly for T.
scripta will aide in the pursuance of our other major experimental goals in the study of TSD.

Using the new genome assembly, we will be able to generate high quality exon maps for every gene.
This, coupled with our RNA-seq data, will allow us to do detailed splicing analysis to uncover the
exact quantity and nature of the splicing differences induced at MPT and FPT. We will be able
to map existing reads to the genome for multiple points across the temperature sensitive period,
calculate alternative exon usage as well as more accurately map intron retention as it differs between
incubation temperatures. Another avenue of future research that the genome assembly will aide in
is the analysis of epigenetic regulation of TSD.

In light of the identification of epigenetic regulators, such as Kdm6b and Jarid2, we require new
genetic tools to further our understanding of TSD. Again, using the new genome assembly, we can
apply techniques such as ChIP-seq to identify genomic regions under the regulation of these genes.
This, along with standard TSD techniques, such as temperature shifts, will allow us to determine the
position of epigenetics in the regulatory cascade controlling male and female development. Aside
from expanded applications of next-gen sequencing, more traditional experimental avenues have
been opened up by our timecourse analysis.

With the new expanded role of sex hormone signaling predicted from our data, assaying hormone
levels within the embryo prior to gonad formation is now a priority. If the early embryo is establishing
a male or female environment dictated by temperature, turtles may be in direct opposition to the
mammalian paradigm that gonadal sex dictates secondary sexual development. Our existing data is
limited in scope to expression differences at the transcript level. To verify the existence of differential
hormone signaling environments in the early embryo, we will measure embryonic hormones
directly and identify when and how these differences occur. It is well established that exogenous
hormones can be readily applied and absorbed into T. scripta eggs. The two proposed mechanisms
for MPT enrichment of 5 beta-reductase Akr1d1 can be tested by a combination of blocking the
enzyme at MPT or adding exogenous 5 beta-DHT at FPT. If the purpose of 5 beta-reductase is to
reduce available testosterone, blocking the enzyme at MPT may produce some degree of embryo
feminization. If instead, the production of 5 beta-DHT is the critical component, we would expect
to see masculinization at FPT. The proposed interaction between embryonic calcium regulation and hormones requires further investigation as well. One candidate that should be analyzed is the CRISP venom-like gene that is enriched in female gonads shortly after the initiation of gonadogenesis. If the function of this protein is similar to the venom-peptides with which it shares homology, it would function as a potent inhibitor in intracellular calcium release. Fortunately, there are sources of venom from species like the Mexican beaded lizard *Heloderma horridum* which contain a highly homologous protein to the *T. sciptra* CRISP venom-like peptide based on sequence homology. Simple experiments in which venom is applied to gonads in culture could provide some answers as to whether this peptide might provide a block of aromatase inhibition at MPT leading to feminization.

Without the ability to generate transgenic or knockout animals, our ability to interrogate the role of calcium in TSD is fairly limited. One possibility is the manipulation of available calcium in organ culture ex vivo. If increasing free calcium at MPT is critical for the negative regulation of Aromatase function, chelating calcium in culture may block the MPT gonad from preventing estrogen production. Conversely, raising intracellular calcium concentration in FPT gonads by pharmacological means may inhibit estrogen production and thus feminization of the gonad. We can also identify if calcium is upstream of AMH expression through these same experiments. These combined future directions will provide a major step forward in the study of TSD as well as provide immense public resources for the entire field.
WORK CITED


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Michael James Czerwinski was born on November 22nd in Jackson Michigan to his parents Kevin and Kathy Czerwinski. He spent his formative years living first in Bay City, Michigan then Essexville, Michigan through high school graduation. He came to science late in his undergraduate career after deciding to veer from a “more practical” business major into biology, something that has always been dear to his heart. After graduation he stayed on in the lab of Jennifer Schisa to complete a master’s degree studying oocyte dynamics in aged C. elegans worms. After finishing his first foray in graduate school, he took a technician and lab managing position in the lab of Deborah Gumucio at the University of Michigan department of Cell and Developmental Biology. After the amazing introduction to top tier science under Deb’s tutelage, he began having dreams of one day being a PI himself. With the very strong encouragement of Dr. Gumucio, he decided to head back to graduate school in the fall of 2009. He eventually landed in the lab of Blanche Capel at Duke University in the early summer of 2010 to start his graduate rotations 3 months early. His love for vertebrate organogenesis that began in the Gumucio lab led him to continue studying under Dr. Capel for the remainder of his time at Duke. Through a fortuitous set of circumstances, an incredible project studying temperature dependent sex determination became his unintended main focus.

Michael’s early life experience with computers and programming, along with the incredible help of the lab’s resident computer scientist Anirudh Natarajan, lead him to undertake a nearly entirely computational project. After an initial steep learning curve, the resources at Duke allowed him to brush up on his coding skills and begin pushing the boundaries of non-model system computational biology. Michael was very lucky to be given the freedom and trust to pursue his project in a way that allowed him to develop not only a solid set of data and hypothesis but also a great new skill set in computational biology.