

Genetic Polymorphisms in Aromatase (*cyp19a1*) Are Not Associated with Gonadal Phenotypes in Red-Eared Slider Turtle Hatchlings Developed at a Pivotal Temperature

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Keywords

Pivotal temperature · Temperature-dependent sex determination · *Trachemys scripta*

Abstract

Embryonic gonad sex in many reptilian species is determined by the incubation temperature of the egg, a differentiation process known as temperature-dependent sex determination (TSD). Incubation at the pivotal temperature (PvT) results in approximately an equal number of offspring of both sexes. We investigated the potential contribution of genetic variations that drives the gonadal differentiation into testes or ovaries under this temperature in the red-eared slider turtle (*Trachemys scripta*). Four male and 4 female hatchlings of eggs that had been incubated at the PvT were examined for polymorphisms at an approximately 23-kb region of the aromatase (*cyp19a1*) gene. By aligning the red-eared slider aromatase gene to a reference genome of the western painted turtle (*Chrysemys picta bellii*), we discovered 22 exonic and 1,268 intronic polymorphisms. Of these, 12 (55%) exonic polymorphisms were unique to the individuals of the red-eared slider; 10 were synonymous and 2 were nonsynonymous changes. We found no pattern in these genetic variants as well as intronic variants that are consistent-

ly different between male and female hatchlings at the PvT. Overall, our study suggests that polymorphisms within the aromatase gene – at least by themselves – do not constrain the gonad sex differentiation in embryos developed at the PvT.

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Temperature-dependent sex determination (TSD) occurs in a variety of reptiles [Shoemaker and Crews, 2009; Gamble, 2010; Merchant Larios and Díaz-Hernández, 2013; Bachtrog et al., 2014; Holleley et al., 2016]. This phenomenon was first described by Charnier [1966] from her extended work with the rainbow lizard (*Agama agama*) in Senegal, and these findings were replicated with the European pond turtle (*Emys orbicularis*) by Pieau [1971]. However, as is the nature of modern science, their work did not penetrate the domain of English language journals until more than a decade later in the work of Bull and Vogt [1979] and Yntema and Mrosovsky [1982]. These pioneering works initiated an extremely active area of research, both in the laboratory [Crews et al., 1989; Ewert et al., 1994; Lang and Andrews, 1994; Mrosovsky, 1994] and in the field [Vogt and Bull, 1982] that continues today.

TSD has 2 remarkable features, but only one has been fully addressed to date. The first is the basic phenomenon itself, namely, how the sex of hatchlings varies according to the temperature they were incubated at as eggs. Much has been already revealed of this process, such as the different patterns of response between species [Bull and Vogt, 1981; Conover and Fleisher, 1986; Bull, 1987], the demarcation of the window of temperature sensitivity during incubation [Vogt and Bull, 1982; Crews et al., 1989; Ewert et al., 1994; Lang and Andrews, 1994; Mrosovsky, 1994], the ability of exogenous estrogen and estrogenic components to reverse the effects of a male-inducing temperature [Crews, 1996; Pieau and Dorizzi, 2004; Matsumoto et al., 2014], the effects of varying incubation temperature [Standora and Spotila, 1985; Pieau et al., 1999], and, more recently, the associated molecular mechanisms that underlie the TSD process [reviewed by Shoemaker and Crews, 2009; Rhen and Schroeder, 2010; Matsumoto and Crews, 2012]. In the transition from single-sex temperature, there is a narrow window that produces varying sex ratios, increasing as it approaches the opposite sex temperature [Crews and Bergeron, 1994]. This is the other remarkable but heretofore unexplored feature of TSD. What is it that drives the gonadal differentiation into testes or ovaries at temperatures that produce mixed sex ratios? How this actually occurs has yet to be investigated and is the starting point of our study. We contend that the study of the pivotal temperature (PvT), which produces mixed sex ratios even within the same clutch, will ultimately provide the answers to the question of how the physical stimulus of incubation temperature and other unknown factors are translated into a molecular switch that produces a male versus a female phenotype.

Because the temperature effect on the gonad type is considered to be equipotent at the PvT, many studies with TSD species have been conducted at this temperature in an attempt to reveal potential heritable components driving the embryonic gonad sex to become male or female. For example, among-family (i.e., clutch) genetic variation for the sex ratio in embryos developed at the PvT was found in map turtle (*Graptemys ouachitensis*), painted turtle (*Chrysemys picta*), common snapping turtle (*Chelydra serpentina*), and American alligator (*Alligator mississippiensis*) [Bull et al., 1982a; Janzen, 1992; Rhen and Lang, 1998]. More recent studies suggest the molecular explanation for the potential existence of heritable components, segregating variation, and genotype by temperature interactions in a TSD system [Rhen et al., 2011; Yamamoto et al., 2014].

In the red-eared slider turtle (*Trachemys scripta*), gonad differentiation is not completely stochastic between 2 gonad explants from a single embryo cultured separately at the PvT; rather the development of the 2 gonads is coordinated, suggesting the existence of shared components between them [Mork et al., 2014]. Schroeder et al. [2016] demonstrated that a single nucleotide polymorphism (SNP) in the cold-inducible RNA-binding protein (*CIRBP*) gene was significantly associated with the gonad sex in the common snapping turtle. These studies suggest that the TSD system may be influenced or even directed by genetic components, which could be masked under the incubation environment at extreme temperatures.

Several genes have been proposed to be key modulators of the temperature response: *CIRBP* [Schroeder et al., 2016], *TRPV4* [Yatsu et al., 2015], and the best studied gene by multiple laboratories, aromatase (*cyp19a1*) [D'cotta et al., 2001; Lance, 2009; Navarro-Martin et al., 2011; Matsumoto et al., 2013]. Aromatase is an enzyme that irreversibly converts androgens into estrogens in the steroidogenesis pathway. In nonmammalian vertebrates, the activation of aromatase in embryonic gonads is a hallmark of ovarian differentiation [Smith et al., 1997; Gabriel et al., 2001; Devlin and Nagahama, 2002; Ramsey et al., 2007]. In species with TSD, such as the red-eared slider turtle and American alligator, the expression of aromatase mRNA in embryonic gonads is activated at the female-producing temperature (FPT), while it is almost undetectable at the male-producing temperature (MPT) [Gabriel et al., 2001; Murdock and Wibbels, 2003; Ramsey et al., 2007].

Several polymorphic regions have been identified in the aromatase gene that could be responsible for both serum estrogen and testosterone levels [Sowers et al., 2006; Garringer et al., 2013] as well as various diseases such as polycystic ovary syndrome and cancers in humans [Tao et al., 2007; Jin et al., 2009; Zhang et al., 2012; Zins et al., 2014]. This genetic marker could be important in understanding how some individuals are susceptible to a specific environment while others are not. Despite the recent studies with TSD species, however, we still do not know how the embryonic gonad sex is determined under the PvT, how the genotype can affect the TSD system, or the potential interaction of the genetic background with various environmental cues including incubation temperatures.

In this study, we examined hatching sex ratios in embryos developed at the PvT and determined if any polymorphic variants of the aromatase gene may be differentially represented in male and female hatchlings. PvT pro-

vides a unique environment to assess whether a certain genotype or polymorphism(s) makes the embryo more susceptible to becoming a male or female during gonad differentiation. This narrow window that exists between the temperature ranges that cause either a male- or female-biased sex ratio spans only about 1°C and is reported to be at approximately 29.2°C in red-eared slider turtles [Crews and Bergeron, 1994; Crews et al., 1994]. The coding and noncoding regions of the aromatase gene along with a 1,391-bp upstream region relative to the transcription start site (TSS) were sequenced in 4 males and 4 females incubated and hatched at the PvT. Other embryos ($n = 4$) that failed to reverse their gonad sex when shifted from MPT to FPT, or vice versa, at the beginning of the temperature sensitive period (TSP), were also sequenced for any polymorphisms. We are referring any polymorphic variants at a single nucleotide site discovered in this study as a polymorphic variant instead of a SNP. Although we are confident that polymorphic variations that we found in this study are real variants rather than PCR or sequencing errors due to a high quality of reads (see Results), a larger sample (population) size would be required to refer to these polymorphic variants as SNPs in the traditional sense.

We used a closely related species, the western painted turtle (*C. picta bellii*) genome [Bradley Shaffer et al., 2013], as a reference sequence, since the sequence of the red-eared slider is not available in public resources. By aligning the genomic sequences of red-eared slider aromatase, we discovered 22 exonic and 1,268 intronic polymorphisms. Approximately 55 and 76% of these in the exons and introns, respectively, were unique to the individuals of red-eared slider turtles (i.e., intraspecies variations), and the rest were common among this species compared to the western painted turtle reference genome (i.e., interspecies variations). We found no pattern in the polymorphic variants throughout the gene that were consistently different between males and females hatched at the PvT. Overall, our study suggests that the polymorphic variation within the aromatase gene, at least by itself, does not determine the gonad sex in embryos developed at the PvT.

Materials and Methods

Animals

Freshly laid red-eared slider turtle eggs (mixed clutches) were collected within 24 h of laying and shipped from Tangi turtle farm (Ponchatoula, LA, USA) during the months of May–July in 2014 and in 2015. Eggs were maintained at room temperature for the

first 10 days upon arrival and assessed for viability of the embryos by candling. Those containing viable embryos were placed in plastic trays with moistened vermiculite. Embryonic sex at a constant PvT is very sensitive to slight temperature fluctuations; therefore manually producing an exact 50:50 hatching sex ratio in a laboratory setting is extremely difficult [Bull et al., 1982b; Yntema and Mrosovsky, 1982; Lance, 2009]. Further, the development of red-eared slider turtle embryos can be influenced by other environmental factors, such as the climate and precipitation of the year of egg collection, time of the egg laying season, and/or inherent differences in the incubators. Therefore, we used 3 separate cohorts obtained from different years and incubators to test the effect of the PvT on the sex ratio at hatching. Eggs obtained in the summer of 2014 were assigned into 2 different incubators, both set at the PvT (29.2°C) to examine an incubator effect on the hatching ratio (Cohort I and III). The incubator which produced a sex ratio closest to 50:50 was used to incubate the eggs obtained in the following summer 2015 at the PvT (Cohort II). Eggs obtained in the summer of 2015 were also assigned to 2 additional temperature regimens, MPT (26°C) and FPT (31°C), to compare the rate of development with embryos developed at the PvT. Incubation temperature was monitored by digital thermometers and recorded every 2–3 days. The daily temperature fluctuation was monitored using HOBO data loggers (Onset Computer Corp.). The trays containing eggs were rotated between different shelves of the incubator 3 times a week to ensure even exposure to the temperature within the incubator. Embryos were periodically staged using morphological features according to the Greenbaum's staging series [Greenbaum, 2011].

Tissue Collection and Genomic DNA Extraction

At hatching, each embryo's gonad sex was determined by phenotypic features of the gonads and the presence or absence of the sex cords under the microscope. No intersex gonads (e.g., ovotestes) or mismatches between gonad and sex cord phenotypes (e.g., ovaries without oviducts or testes with oviducts [Matsumoto et al., 2014]) were observed in the current study. The adrenal-mesonephros tissue from randomly chosen female ($n = 4$) and male ($n = 4$) hatchlings from Cohort III, which produced a sex ratio closest to 50:50 among the 3 PvT cohort groups and therefore was considered reflecting the accurate PvT environment, was dissected, snap-frozen immediately in liquid nitrogen, and stored at –80°C until genomic DNA extraction was performed. The adrenal-mesonephros tissues from individuals ($n = 4$) that failed to reverse their gonad sex when shifted from MPT to FPT, or vice versa in a previous study, were also included for genomic DNA extraction. Genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol. The concentration and purity of DNA was measured with a NanoDrop 2000 (Thermo Fisher Scientific). The integrity of genomic DNA was confirmed on a 1.2% agarose gel. Purified genomic DNA was stored at 4°C until use for subsequent experiments.

Prediction of Aromatase Gene and Long PCR

Since the genome sequence of the red-eared slider turtle is not available to the public, we computationally extracted the genomic region of the aromatase gene from the western painted turtle (*C. picta bellii*) genome (Dec.2011, v3.0.1/chrPic1) using the UCSC genome browser (<http://genome.ucsc.edu>) [Kent et al., 2002]. The published aromatase cDNA sequence of the red-eared slider turtle

(GenBank accession no. AF178949) was aligned with the extracted genome sequence of the western painted turtle to predict the location of exons, and this information was used for primer construction for subsequent PCR reactions. Approximately 400 ng of genomic DNA was amplified using a nested PCR method with i) one set of outer PCR primers spanning from position 2,235 bp upstream of the TSS to the position 38 bp downstream of the transcription stop codon, and ii) 3 sets of inner primers which overlap each other to cover from the 1,391-bp position 5' region of the TSS to the 23-bp position 3' region of the stop codon (amplicon locations are shown schematically in online suppl. Fig. 1, for all online suppl. material, see www.karger.com/doi/10.1159/000471940). Primer and amplicon sizes are shown in online suppl. Table 1. All PCR amplifications were carried out using TAKARA primeSTAR GXL DNA Polymerase (Clontech) on an ABI2720 Thermal cycler (Thermo Fisher Scientific). All primers were ordered from Integrated DNA Technologies. Conditions for the first outer PCR were as follows: 25 cycles of 10 s at 98°C, 10 min at 68°C. A volume of 1 µL of outer PCR product was subsequently used for the inner PCR with the following conditions: 35 cycles of 10 s at 98°C, 10 min at 68°C for the Arom1 and Arom3 primer sets vs. 32 cycles of 10 s at 98°C, 15 s at 62°C, 10 min at 68° for the Arom2 primer set. The specificity of the PCR reactions was visualized on a 1.2% agarose gel. Samples with an extra band(s) due to nonspecific amplification were purified using QIAquick gel extraction kit (Qiagen), and those with specific amplification (a single band) were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter) and diluted in 25 µL Tris-HCL. All 3 inner PCR amplicons from each embryo were quantified using a NanoDrop 2000 (Thermo Fisher Scientific), and equal amounts were pooled for library construction.

Illumina Miseq Next-Generation Sequencing

Fragmentation and library construction were performed using Nextera XT (Illumina), and the final library quantification was run on the 2100 Bioanalyzer (Agilent technologies) by the Genomic Sequencing and Analysis Facility (GSAF) at the University of Texas at Austin. The barcoded libraries were sequenced using a 250-paired-end read protocol on the Illumina Miseq sequencer with v2 kits (Illumina) at GSAF at the University of Texas at Austin.

Sequence Data Analysis and SNP Identification

As an initial analysis, raw sequence data were evaluated for sequence quality using the FAST QC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Adapters were trimmed and low quality reads (Q <20) were filtered using cutadapt tool [Martin, 2011]. Filtered reads were then aligned to the genomic region of the painted turtle aromatase gene (v3.0.1/chrPic1, JH584779:38000–63300) as mentioned above using bwa-mem [Li and Durbin, 2010] with default settings for paired-end reads. Unmapped or unspecific reads were filtered based on mapping quality. Mapped reads were visualized using Integrative Genomic Viewer [Robinson et al., 2011; Thorvaldsdottir et al., 2013]. Subsequently, we used samtools mpileup to call variants and the wANNOVAR web server to annotate variants detected from the resulting sequence data. Heat maps of allele frequencies (only of variants with more than 1,000× sequence depth) were created with R (The R project for Statistical Computing). Variant calls and annotations were collaborated with

the Center for Computational Biology and Bioinformatics at the University of Texas at Austin. The raw data files have been deposited in the NCBI sequence read archive under accession number SRP098760.

Results

Rate of Development in Embryos Incubated at PvT

First, we compared the rate of embryonic development under various incubation temperatures (Fig. 1). The rate of development within a cohort is typically homogeneous under the same temperature regimen except when development is hindered by unknown developmental defects. We excluded embryos from the current study if the development was more than 1 stage behind the rest of the cohort. In this species, the development at the cooler MPT takes longer than at the warmer FPT due to a difference in metabolic rates [McCue, 2004; Shoemaker-Daly et al., 2010]. The typical duration of TSP is between stage 15 and 19 at FPT and between stage 15 and 20 at MPT [for review see Shoemaker and Crews, 2009]. In this study, we refer to the developmental period between stage 15 and 20 as TSP for the embryos developed at the PvT. The incubation of embryos at the PvT (Cohort group II in Fig. 2) resulted in a developmental rate slightly slower than those at FPT incubation but faster than those at MPT incubation. The average day to reach embryonic stage 16, the early TSP, was 26 days at the PvT, while they were 37 and 24 days at MPT and FPT, respectively. Embryos incubated at the PvT reached a hatching average at 69 days, while embryos incubated at MPT and FPT reached a hatching average at 82 and 63 days, respectively (Fig. 1). No significant difference in developmental rate between male and female hatchlings was observed within the PvT cohort group.

Hatchlings' Sex Ratio at PvT

Next, we compared the average incubation temperatures and resulting sex ratios in 3 different cohorts incubated at the PvT. As mentioned previously, manually producing a 50:50 male-to-female sex ratio is extremely difficult in a laboratory setting. The daily fluctuation of incubation temperature is approximately 0.2–0.4°C, and this variability in temperature range is sufficient to lead a cohort of embryos to hatch with a skewed sex ratio. Therefore, we prepared 3 cohorts from different years and/or different incubators. As a result, we obtained a variety of sex ratio of 96, 82, and 63% females at 29.4, 29.2, and 29.1°C during TSP, respectively (Fig. 2; online suppl. Ta-

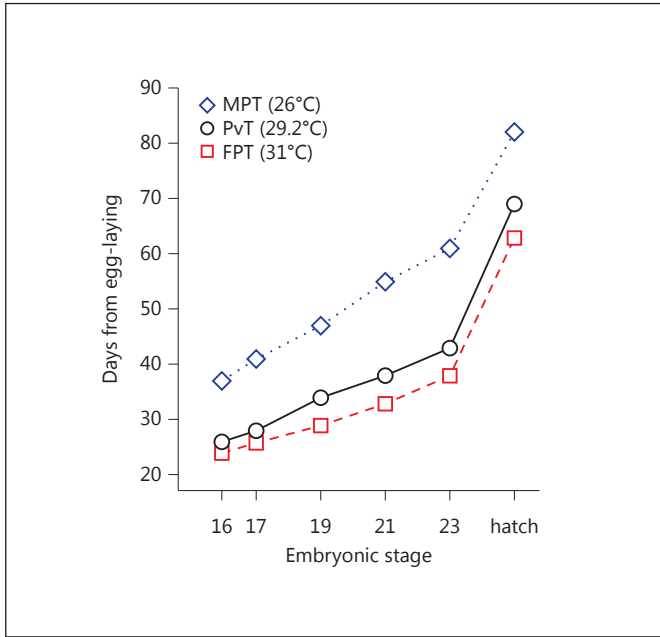


Fig. 1. Rate of development of red-eared slider turtle embryos at different incubation temperatures. All eggs were laid on the same day during the summer of 2015 and split into 3 different incubation temperature regimes. Embryos ($n = 10\text{--}12$ per each data point) were dissected and staged by morphological features according to the Greenbaum's staging series [Greenbaum, 2011]. Actual average incubation temperature (average of temperature fluctuation range) throughout development was as follows: MPT = 25.74°C ($\pm 0.20^\circ\text{C}$), PvT = 29.16°C ($\pm 0.09^\circ\text{C}$), FPT = 30.97°C ($\pm 0.10^\circ\text{C}$). Incubation at MPT and FPT produced 100% male and female hatchlings, respectively. Incubation at PvT produced 18% males and 82% females (= Cohort group II in Fig. 2). MPT, male-producing temperature; PvT, pivotal temperature; FPT, female-producing temperature.

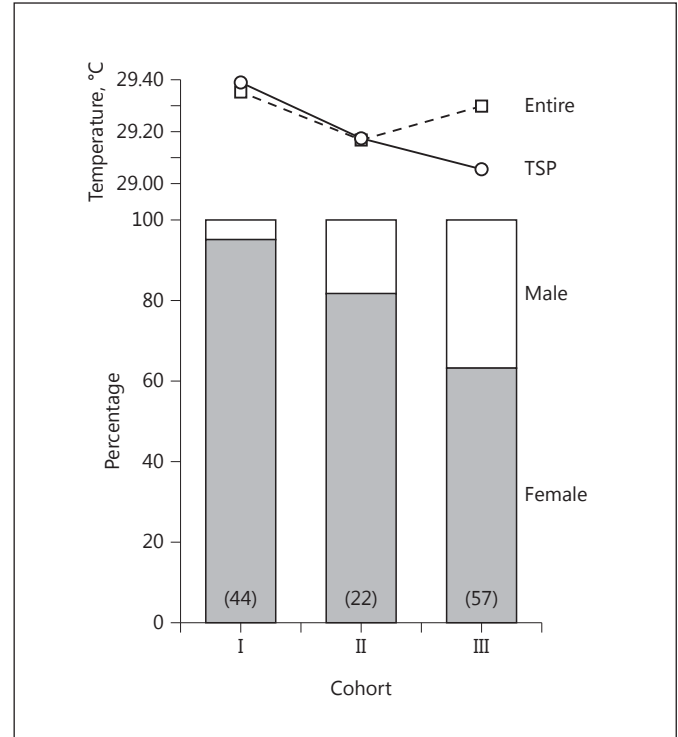


Fig. 2. Hatching sex ratio of embryos incubated at the pivotal temperature in the red-eared slider turtle. Shown are 3 cohorts of eggs (I–III) that were incubated at the pivotal temperature (29.2°C). The sex of each embryo was determined by morphological features of the gonads at hatching. The corresponding average recorded temperature during the temperature-sensitive period (TSP) and throughout the entire developmental process (entire) is indicated on the top of the graph. The sample size for each group is shown in parentheses. Actual average temperatures and percentage of hatching sex are shown in online suppl. Table 2.

ble 2). The sex ratio is proportional to the average incubation temperature during TSP but not to the average temperature throughout the entire development (“TSP” vs. “entire” in Fig. 2).

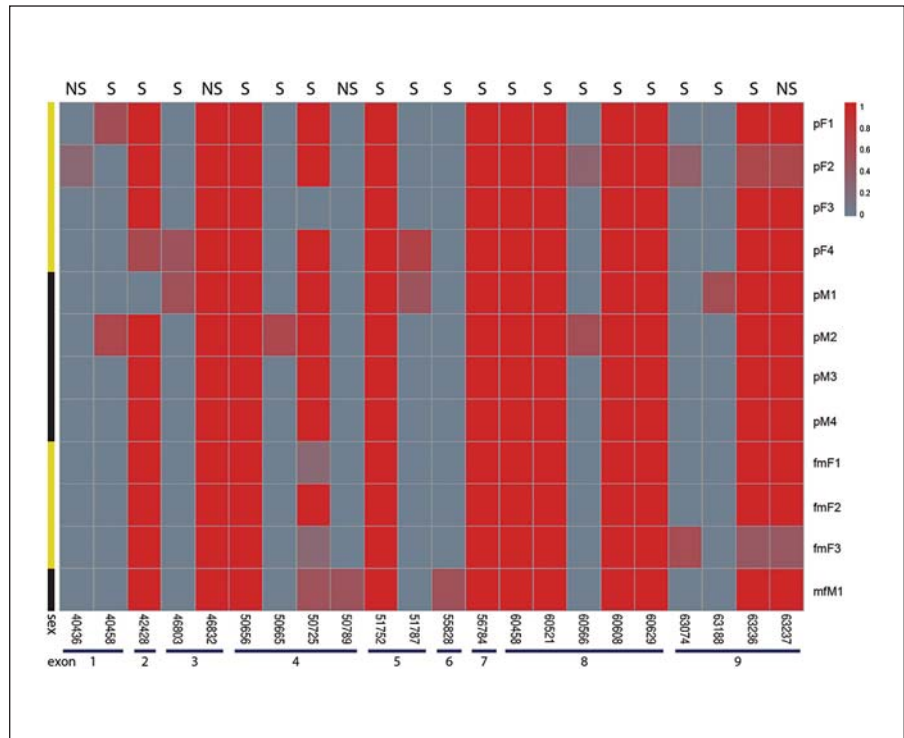
Prediction of the Gene Structure of Aromatase

The location of exons was predicted by aligning the aromatase cDNA sequence of the red-eared slider turtle (GenBank accession No. AF178949) to the extracted genome sequence of the western painted turtle. We discovered 9 exons spanning an approximately 23-kb genomic region (online suppl. Fig. 1). Sequences from these 2 different turtle species share 99.2% of nucleotide (1,500/1,512 bp) and 99.6% of protein (502/504 aa) sequence identity (online suppl. Fig. 2). No CpG island was found using EMBOSS CpG plot.

Next-Generation Sequencing Analysis and SNP Discovery

On the resulting sequencing data, on average, 95.1% of reads (range 93.7–95.7%) among the 12 samples passed the quality filter of cutadapt and were used for subsequent analyses. Overall, of the 2 million initial reads (1 million from each end in paired-end reads) for each sample, an average of 1,581,979 reads was uniquely mapped to the reference gene (74% of quality filtered reads). The average coverage of the targeted regions was approximately $13,000\times$ (range 6,000–22,000 \times) among the 12 samples, of which on average 96.5% (range 96.0–99.7%) had coverage of over $100\times$ and 97.0% (range 96.5–97.5%) was covered at least once (online suppl. Fig. 3). A total of 1,290 polymorphisms, 22 exonic and 1,268 intronic, were discovered within a 23-kb genomic region of the aromatase gene

Fig. 3. Heat map showing observed allele frequencies of exonic polymorphisms in the aromatase gene of the red-eared slider turtle in relation to the western painted turtle genome. Twenty-two polymorphisms were discovered within 9 exons of the aromatase gene of red-eared slider turtles. NS, nonsynonymous change; S, synonymous change. The bottom numbers represent the corresponding locations of the western painted turtle genome database (v3.0.1/chrPic1, JH584779:38000–63300) and exons. The yellow and black bar on the left indicates hatching sex (yellow, female; black, male). pF1–pF4, individuals developed at the pivotal temperature and hatched as female (ovary) gonad phenotypes; pM1–pM4, individuals developed at the pivotal temperature and hatched as male (testis) gonad phenotypes; fmF1–fmF3, individuals shifted from female to male producing temperature at embryonic stage 16 and hatched as female (ovary) gonad phenotype; mfM1, individual shifted from male to female producing temperature at embryonic stage 16 and hatched as male (testis) gonad phenotype.



(online suppl. Table 3) when they were aligned to the western painted turtle genome database (v3.0.1/chrPic1, JH584779:38000–63300). Of those, a total of 78 polymorphisms within upstream, exon 1, and intron 1 regions were validated with Sanger sequencing in selected individuals ($n = 8$). Approximately 70% of calls are perfectly matched between Illumina Miseq and Sanger sequence results, 22% are partially matched (miscalls between homozygous vs. heterozygous), and 8% are mismatched (online suppl. Table 4). The estimated density of interspecies polymorphisms in the aromatase gene showed that exonic regions had a lower density than the average as expected (online suppl. Fig. 4).

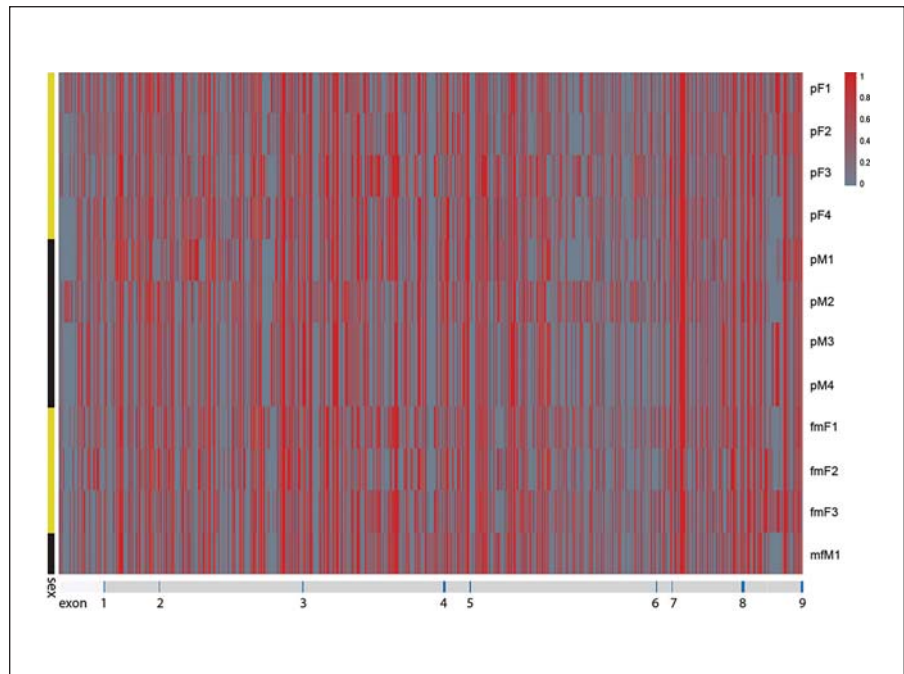
Twelve (55%) of the exonic polymorphisms discovered by Illumina Miseq sequencing were unique to the individuals of the red-eared slider turtle (intraspecies variations), while 10 (45%) were common among individuals (interspecies variations to the western painted turtle). Of the 12 exonic intraspecies variations, 2 were nonsynonymous and 10 were synonymous, and of the 10 exonic interspecies variations, 2 were nonsynonymous and 8 were synonymous changes (Fig. 3; online suppl. Table 5). We found no pattern in the exonic polymorphic variants that consistently differentiated males or females hatched at the PvT or among the shifted embryos (Fig. 3). In the intergenic/up-

stream regions and introns, 962 (76%) of polymorphisms were unique to the individual red-eared slider turtles (intraspecies variations), while 306 (24%) were common among individuals (interspecies variations). These intergenic/intronic variants were also not specific to the hatching gonad phenotype (Fig. 4; online suppl. Fig. 5).

Discussion

In both TSD and genotypic sex determination (GSD), the bipotential embryonic gonad differentiates into 1 of 2 gonad phenotypes (i.e., testes or ovaries). The fundamental difference lies in the trigger, with an external cue (temperature) serving in the former and heritable genetic factors acting in the latter. The evolutionary history of transitions between GSD and TSD is not completely understood. However, phylogenetic analyses in reptiles indicate that it likely occurred many times [Janzen and Phillips, 2006; Warner and Shine, 2008; Quinn et al., 2011]. These 2 modes of sex determination are not always mutually exclusive within a species, and the boundaries are rather ambiguous in some cases. In the Australian dragon (*Pogona vitticeps*) and the skink (*Bassiana duperryi*), the gonad sex is predominantly determined by the genotypic system in-

Fig. 4. Heat map showing observed allele frequencies of both exonic and intronic polymorphisms in the aromatase gene of the red-eared slider turtle in relation to the western painted turtle genome. Within intronic regions, 1,268 polymorphisms were discovered in the red-eared slider aromatase gene. The bottom bar indicates the positions of exons (blue) and introns (gray). The yellow and black bar on the left indicates hatching sex (yellow, female; black, male). pF1–pF4, individuals developed at the pivotal temperature and hatched as female (ovary) gonad phenotypes; pM1–pM4, individuals developed at the pivotal temperature and hatched as male (testis) gonad phenotypes; fmF1–fmF3, individuals shifted from female to male producing temperature at the embryonic stage 16 and hatched as female (ovary) gonad phenotype; mFM1, individual shifted from male to female producing temperature at the embryonic stage 16 and hatched as male (testis) gonad phenotype.



volving heteromorphic sex chromosomes. Yet, when subjected to extreme temperatures during embryonic development, these genetic cues can be overridden [Shine et al., 2002; Quinn et al., 2007; Radder et al., 2008; Holleley et al., 2015]. These studies suggest then that the opposite is also possible: i.e., the gonad sex in species commonly known as TSD species may well be influenced by its genetic blueprint, which is being masked by the fluctuation of temperature that occurs in nature or by the constant exposure to extreme temperatures in an unnatural environment such as a laboratory setting. Among TSD species, the cohort of embryos developed at the threshold or PvT produce approximately an equal number of male and female hatchlings [Yntema and Mrosovsky, 1982; Mrosovsky and Pieau, 1991]. The current study is based on the hypothesis that the individual genotype or genetic background may play a predominant role under this temperature.

Our results showed that the rate of embryonic development at the PvT (29.2°C) was more similar to that observed at FPT (31°C) than at MPT (26°C; Fig. 1). One possible explanation is that the resulting sex ratio of this PvT cohort (Cohort II in Fig. 2) was skewed and female biased (females = 82%; Fig. 2), and therefore, the rate of development was also similar to that which produces females. When we look at the developmental rate of the other 2 cohorts, Cohort I (females = 95.5%; Fig. 2) and Cohort III (females = 63%; Fig. 2), the rate of develop-

ment was still similar to the one we observed in Cohort II (online suppl. Fig. 6). This suggests that the developmental rate at the PvT is slightly faster than what is expected for this temperature thought to produce an approximate 50:50 sex ratio, and that it is not necessarily proportional to the resulting sex ratio observed.

Our current study also highlights the difficulty of producing an exact 50:50 sex ratio at the PvT in an unnatural laboratory setting. In the red-eared slider turtle, an incubation temperature of 29.2°C is reported as the PvT, where approximately 35–50% females were successfully produced in previous reports [Crews and Bergeron, 1994; Crews et al., 1994; Wibbels and Crews, 1995]. Meanwhile, 29.4°C is the temperature that produced a female-biased hatchling population (approximately 80% females) [Crews and Bergeron, 1994; Ramsey et al., 2007]. In the current study, the temperature difference of 0.34°C changed the hatching sex ratio from 95% (Cohort I) to 63% (Cohort III) females (Fig. 2). It suggests that the PvT for these cohorts actually lies somewhere around 29.0–29.1°C, slightly lower than that previously reported. As we discussed in a previous study [Matsumoto et al., 2014], embryonic development can be influenced by many environmental variables, such as climate and local weather of the given breeding season or even the source of red-eared slider eggs (i.e., population). It is not surprising if these variables also affected the observed outcome of this particular

study, which led to the female-biased sex ratio even at the reported literature PvT of 29.2°C. Our study also shows that the hatching sex ratio is proportional to the incubation temperature of TSP, but not to the temperature throughout development (Fig. 2). Our observation is consistent with previous findings that the gonadal phenotype is only influenced by the ambient temperatures during TSP and not influenced outside of this developmental period [Bull and Vogt, 1981; Conover and Fleisher, 1986; Bull, 1987].

One of the most interesting phenomenon at the PvT is that intersex (i.e., ovotestes) is extremely rare at this temperature in many TSD species, including the red-eared slider turtle [Yntema and Mrosovsky, 1982; Mrosovsky and Pieau, 1991; Crews and Bergeron, 1994; Crews et al., 1994]. Intersex at the PvT is more commonly found in other freshwater turtle species (*E. orbicularis*) which later on transform to testes [Raynaud and Pieau, 1985; Pieau et al., 1998]. In the current study, no intersex gonads (e.g., ovotestes) or mismatches between gonad and sex cord phenotypes (e.g., ovaries without oviducts or testes with oviducts) were observed, suggesting that the molecular pathway of testicular or ovarian differentiation is organized at cellular and molecular levels to produce a binary outcome of the bipotential gonads.

Aromatase, whose expression can regulate the local estrogen production, is one of the key factors that balances the 2 developmental trajectories during sex determination of embryonic gonads in nonmammalian vertebrates. Overexpression of aromatase in genetically male chicken can direct the embryonic gonads to differentiate into viable ovaries [Lambeth et al., 2013], suggesting the expression of aromatase could be a pivotal branch point of these 2 developmental pathways. Our current study indicates that polymorphic variations in the red-eared slider aromatase gene are not associated with the gonad sex at the PvT. We found 12 exonic and 962 intergenic/intronic polymorphisms that are unique to individuals; however, none of these variations in aromatase had a clear pattern associated with the phenotypic gonad sex at hatching (Fig. 2, 3). Several possibilities can be considered for this observation. One of the biggest developmental debates in discussing sex determination in species with no apparent heteromorphic chromosomes, but clearly having genetic cues, is whether embryonic sex is determined by a single gene, a few loci or a combination of a large number of genes. Several species of fish, such as European sea bass (*Dicentrarchus labrax*) and yellowtail (*Seriola quinqueradiata*), lack a major sex-determining gene or set of genetic markers, but rather seem to have a polygenic sex-determining system [Koyama et al., 2015; Palaiokostas et al.,

2015]. In some cases, however, a single SNP in a certain gene can be associated with phenotypic sex. For example, a nonsynonymous SNP in the anti-müllerian hormone receptor gene in Fugu (*Takifugu rubripes*) is important in determining the gonad sex of the individual [Kamiya et al., 2012]. Furthermore, a study with the common snapping turtle (*C. serpentina*) indicates that a synonymous SNP in the *CIRBP* gene is significantly associated with the gonad sex at hatching [Schroeder et al., 2016]. In both examples, the polymorphic change occurs within a coding region, suggesting the significance of exonic SNPs in phenotypic variation. We found 2 nonsynonymous changes that are unique to individuals (locations 40436 and 50789 in Fig. 3). At each location, only 1 out of the 12 investigated individuals had this polymorphic site, indicating that the pattern seems to be unrelated to phenotypic sex.

We previously found that 2 CpG sites adjacent to the putative TATA box, which were located approximately 80 and 10 bp upstream of the TSS, were important for temperature-specific patterns of DNA methylation [Matsumoto et al., 2013, 2016]. In the current study, we did not observe any polymorphisms within these 2 CpG sites among the individuals. We also previously discovered a polymorphic CpG site in the 5' UTR located 38 bp downstream of the TSS [Matsumoto et al., 2016]. We confirmed that this CpG site (v3.0.1/chrPic1, JH584779:40293–40294) was polymorphic among the individuals, however, the polymorphism was not related to the pattern of gonad sex at hatching (online suppl. Table 3). Genome-wide comparison between males and females developed at the PvT would probably give a different view of genetic variation among individuals and potential polygenic mechanisms underlying TSD.

Another possibility for this negative data is the lack of sample size in this study. We examined a total of 7 female hatchlings (4 developed at the PvT and 3 developed under a shifted environment from FPT to MPT) and 5 male hatchlings (4 developed at the PvT and 1 developed under a shifted environment from MPT to FPT). Because of the fact that a slight temperature difference can shift the sex ratio (Fig. 2), the PvT group may not have been an ideal condition that could be classified as the null temperature environment. Four additional embryos, which failed to reverse the gonad sex when shifted from MPT to FPT or vice versa at the early TSP, were also examined for genetic polymorphisms in the aromatase gene. These individual would more likely have defining genetic signatures, since their phenotypic sex develops against the surrounding temperature. We found 2 heterozygous polymorphisms that are unique to a male developed at FPT (locations 50789 and

55828 of mfm1 in Fig. 3). One of the polymorphisms at location 50789 is a nonsynonymous change and could be a good candidate for a further review into phenotypic sex determination. However, the failure of sex reversal in shifted embryos is rare (1–3%), and we did not have enough samples to investigate this possibility in the current study.

Nevertheless, our study was aimed to deepen our understanding of embryonic development at the PvT and how the embryonic gonad sex of TSD species is determined under this temperature condition, which in itself is a remarkable but often ignored area in the field of environmental sex determination. As the interaction of temperature and genetic components in the process of sex determination in TSD systems is still sparse, our study attempts to present an initial dive into the exploration of this rich field of study.

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Acknowledgments

We acknowledge S. Bhatnagar and A. Agarwal for technical assistance and K. Kushiro for valuable suggestions on the manuscript.

Statement of Ethics

All embryos were maintained and handled in accordance with IACUC protocol #AUP-2014-00364.

Disclosure Statement

The authors have no conflicts of interest to declare.

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