Original Article

Sexual Development

Sex Dev 2018;12:308–319
DOI: 10.1159/000492188

Accepted: July 2, 2018
by M. Schmid
Published online: October 3, 2018

Thermal Response of Epigenetic Genes Informs Turtle Sex Determination with and without Sex Chromosomes

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Keywords
Embryonic gonad development · Epigenetic regulation · Genotypic sex determination · Reptile · Temperature-dependent sex determination · Vertebrates

Abstract
Vertebrate sexual fate can be established by environmental cues (e.g., temperature-dependent sex determination, TSD) or by genetic content (genotypic sex determination, GSD). While methylation is implicated in TSD, the influence of broader epigenetic processes in sexual development remains obscure. Here, we investigated for the first time the embryonic gonadal expression of the genome-wide epigenetic machinery in turtles, including genes and noncoding RNAs (ncRNAs) involved in DNA/histone acetylation, methylation, ubiquitination, phosphorylation, and RNAi. This machinery was active and differentially thermosensitive in TSD versus GSD (ZZ/ZW) turtles. Methylation and histone acetylation genes responded the strongest. The results suggest these working hypotheses: (i) TSD might be mediated by epigenetically controlled hormonal pathways (via acetylation, methylation, and ncRNAs), or by (ii) hormonally controlled epigenetic processes, and (iii) key epigenetic events prior to the canonical thermosensitive period may explain differences between TSD and GSD. Novel epigenetic candidate regulators other than methylation were identified, including previously unknown ncRNAs that could potentially mediate gonadogenesis. These findings illuminate the molecular ecology of reptilian sex determination and permitted hypothesis building to help guide future functional studies on the epigenetic transduction of external cues in TSD versus GSD systems.

Most vertebrates utilize genotypic sex determination (GSD) where the individual's genotype (e.g., via sex chromosomes) determines its sexual fate. Other vertebrates employ environmental sex determination (ESD) where environmental cues mediate gonadal differentiation into testes or ovaries [Tree of Sex Consortium et al., 2014], as

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in temperature-dependent sex determination (TSD) [Valenzuela and Lance, 2004]. Intermediate mechanisms are reported in increasing numbers in reptiles, including bona fide cases where temperature overrides sex chromosomes in nature [Shine et al., 2002; Radder et al., 2008; Holleley et al., 2015], whereas other purported instances of TSD and GSD co-occurrence have been disproven [Valenzuela et al., 2014; Mu et al., 2015]. This diversity has long defied scientific explanation partly because our understanding of the molecular basis of TSD and GSD is incomplete [Bachtrog et al., 2014].

Epigenetic modifications are heritable alterations to the genome that affect gene expression without changing the DNA sequence, such as by DNA methylation and histone modification [Jones, 2001; Allis and Jenuwein, 2016], or RNA interference which alter gene expression or translation [Wilson and Doudna, 2013].

Epigenetic mechanisms (methylation and piRNA [Piwi protein-associated RNA]) have been proposed to underlie sex determination in a number of species [Okamoto et al., 2011; Kuroki et al., 2013; Kiuchi et al., 2014; Venegas et al., 2016; Radhakrishnan et al., 2017a], and few genes of the epigenetic machinery have been reported to respond differentially to temperature in TSD taxa such as *Kdm6b* (also called JMJD3) in alligator [Yatsu et al., 2016] and slider turtles [Czerwinski et al., 2016], and JARID2 and JMJD3 in Jacky dragons that have a mixed GSD/TSD system [Deveson et al., 2017], both related to changes in histone methylation. Following the lead from such differential transcription evidence, it was recently demonstrated that the higher gene expression of *Kdm6b* at male-producing temperatures (MPT) in slider turtles yields higher KDM6D protein levels which act to remove the triple methylation of the histone H3K27 bound at the promoter of *Dmrt1*, thus activating this testis development gene as part of the male differentiation cascade [Ge et al., 2018]. Despite these significant advances, whether TSD is mediated by just a couple types of epigenetic modifications or by many remains unknown. As a first step to answer this question, here we tested the hypothesis that the epigenetic machinery responds broadly to incubation temperature by characterizing the expression of genes involved in epigenetic mechanisms including various histone modifications (acetylation, methylation, phosphorylation, and ubiquitination), DNA methylation, and RNA interference in the developing gonads of a TSD and a GSD turtle at various embryonic stages. We sought to illuminate the molecular ecology of sex determination in vertebrates and to identify candidate epigenetic processes for a role in sexual development to guide future research by the scientific community.

In particular, we examined *Chrysemys picta*, a TSD turtle lacking sex chromosomes [Bull and Vogt, 1981; Valenzuela et al., 2014], and *Apalone spinifera*, a GSD turtle with ZZ/ZW sex chromosomes [Bull and Vogt, 1979; Badenhorst et al., 2013], referred to hereafter as *Chrysemys* and *Apalone*. We leveraged RNA-seq transcriptomes obtained at high and low incubation temperatures that induce male-only or female-only development in *Chrysemys* [Radhakrishnan et al., 2017b], compared the expression of genes in the epigenetic machinery that are present in the *Chrysemys* reference genome [Shaffer et al., 2013; Badenhorst et al., 2015] at each of 5 embryonic stages, and contrasted these patterns to the response of *Apalone* embryos to identical incubation conditions. Our approach revealed stronger thermal regulation of genes related to methylation and acetylation plus noncoding RNAs (ncRNAs) not previously reported, with a strong link to hormonal pathways and differentially for TSD and GSD turtles. These findings point to potentially key epigenetic processes that are likely to mediate the differences in plasticity of vertebrate sexual development we observe in nature and thus warrant further research.

Materials and Methods

The dataset analyzed here is a subset not previously investigated of a RNA-seq time series obtained during an earlier study [Radhakrishnan et al., 2017b] as detailed below.

Sample Collection

Embryos were obtained from *Chrysemys* and *Apalone* turtles at developmental stages [Yntema, 1968] before and during the canonical thermosensitive period (TSP) of *Chrysemys* [Bull and Vogt, 1981]: stages 9 (before TSP), 12 (before TSP), 15 (TSP onset), 19 (mid-TSP), and 22 (late TSP) (Fig. 1) incubated at 26 and 31°C. These values are MPT and female-producing temperatures (FPT), respectively, in *Chrysemys* and fall within the permissible thermal range of *Apalone* (referred to as low-T° and high-T°). The genital ridge may be present by stage 12, because urogenital tissue is present by stage 12 in *Trachemys scripta* [Spotila et al., 1998]. These stages were chosen because temperatures experienced before the canonical TSP influence sex ratios of TSD turtles in the wild [Valenzuela, 2001] and induce differential transcription of genes in the sexual development regulatory network in TSD and GSD turtles [Czerwinski et al., 2016; Radhakrishnan et al., 2017b]. Additionally, examining stages before the canonical TSP helps uncover candidate genes that might govern the activation of this important developmental window of time. Egg incubation followed standard protocols using boxes (that were rotated within incubators daily to account for potential thermal clines) containing moistened sand [Valenzuela, 2009]. Moisture was maintained constant by restoring lost water weekly (determined by changes in the weight of the boxes). Because *Apalone*'s sex ratios are independent of the incubation temperature [Bull and Vogt, 1979], this turtle serves as a

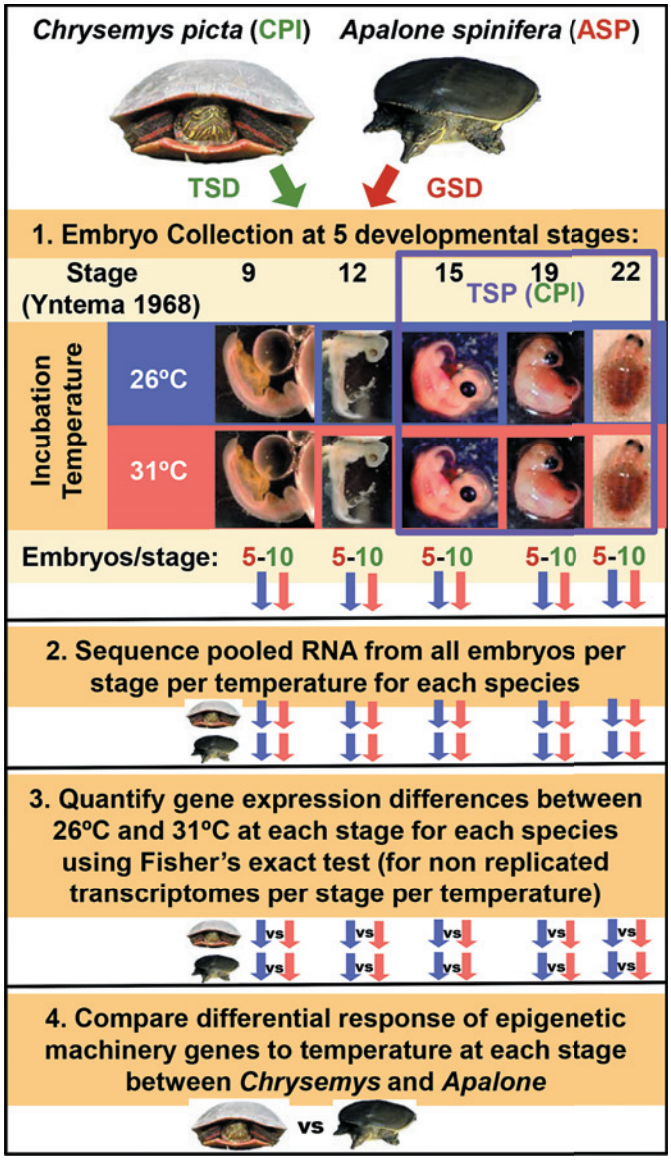

Fig. 1. Experimental design of the study of transcription of epigenetic machinery genes by temperature during embryonic development in *Chrysemys picta* and *Apalone spinifera* turtles.

negative control to disentangle the responses that are due to TSD per se from general thermal sensitivity in gene expression that may exist. From both turtles, total RNA was extracted from trunks (stage 9), adrenal-kidney complex (stage 12), adrenal-kidney-gonadal complex (stages 15), and just gonads (stages 19, 22) using RNeasy kit (Qiagen) [Radhakrishnan et al., 2017b]. Thus, gene expression from these samples reflect differences by temperature/stage/species in non-cephalic tissue beyond the fields where gonads will form (stage 9), urogenital tissue (stages 12, 15), and developing gonads (stages 19, 22). This is important because gene expression in adrenal-kidney or other nongonadal tissue can mask biologically important but subtle differences in the gonadal tissue

Table 1. Fisher's exact test based on a 2 × 2 contingency table

	26°C	31°C	Total
Gene X	n_{11}	n_{12}	$n_{11} + n_{12}$
Remaining genes	n_{21}	n_{22}	$n_{21} + n_{22}$
Totality of genes	$n_{11} + n_{21}$	$n_{21} + n_{22}$	N

alone [Pieau and Dorizzi, 2004; Ramsey and Crews, 2007; Valenzuela et al., 2013]. RNA was quantified with a NanoDrop® ND-1000 Spectrophotometer, and the quality was assessed by the presence of ribosomal bands in agarose gels. Total RNA was submitted to Duke University's GCB Genome Sequencing facility where mRNA-seq libraries (not stranded) were constructed for each species starting from 1 µg pooled total RNA samples per stage per temperature (using TruSeq RNA Sample Prep Kit v2): 10 for *Chrysemys* and 5 for *Apalone* (a single pool per temperature-by-stage), and sequenced using Illumina's HiSeq 2000 protocol, generating on average 35 million 100-bp paired-end raw reads per library.

Transcriptome Bioinformatics

Our analyses are identical to those reported previously [Radhakrishnan et al., 2017b]. Briefly, RNA-seq reads were first processed using Trimmomatic [Bolger et al., 2014] for quality filtering and removal of adapter sequences using default parameters. Species-specific de novo transcriptomes were then constructed using Trinity [Grabherr et al., 2011]. Species-specific reads were mapped back to the de novo reference transcriptomes of each species and resulted in a high mapping efficiency: 92% for *Chrysemys* and 93% for *Apalone*. To identify the expressed genes, the de novo transcriptomes were annotated using Trinotate [Haas et al., 2013] and the SwissProt protein database [Boeckmann et al., 2003]. Additionally, and because not all transcripts were found in SwissProt, these de novo assembled transcripts were mapped back to the 22,380 annotated *Chrysemys* coding sequences [Shaffer et al., 2013] using GMAP (v2012-03-23) [Wu and Watanabe, 2005] with default parameters. To quantify gene expression by species, temperature, and stage, the reads from the respective libraries were mapped back to the 22,380 species-specific coding sequences identified in the previous step. Sex effects could not be tested in *Apalone,* because molecular sexing methods for this GSD turtle [Literman et al., 2014, 2017a] were unavailable at the time these data were collected, thus precluding sex diagnosis of the embryos [Radhakrishnan et al., 2017b]. However, our approach permits detecting temperature effects on gene expression as previously reported for *Apalone* [Valenzuela et al., 2006; 2013 Valenzuela and Shikano, 2007; Valenzuela, 2008a, b, 2010] which was our goal in this study. Read counts per gene were generated using HTSeq [Anders et al., 2015]. Raw gene counts were normalized with regard to the upper quartile [Bullard et al., 2010] and by dividing the expression of each gene by the sum of the expression of 2 housekeeping genes, *transferrin receptor* (*Tfr*) and *hypoxanthine phosphoribosyl transferase 1* (*Hprt1*), which were chosen because they were constitutively expressed across all stages in both turtles. Fisher's exact tests were then performed between the expression levels at 26 and 31°C to identify differentially expressed genes by temperature at each developmental stage in each species, as this statistical method permits the analysis of un-replicated samples (Table 1) [Auer and

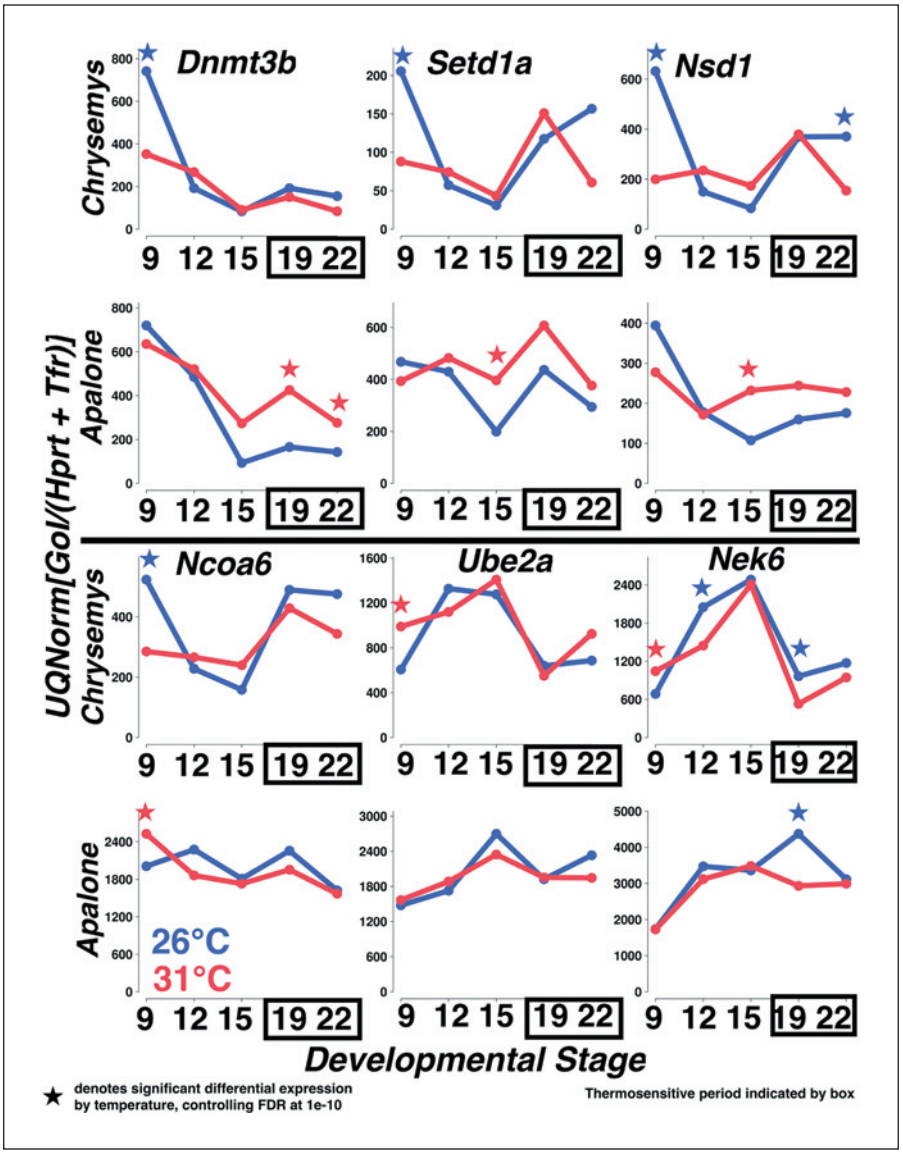

Fig. 2. Normalized expression profiles of 6 exemplary genes involved in DNA methylation (*Dnmt3b*), histone methylation (*Setd1a, Nsd1*), histone acetylation (*Ncoa6*), ubiquitination (*Ube2a*), and histone phosphorylation (*Nek6*) that exhibit differential expression at stage 9 in *Chrysemys picta* (TSD) but not (or contrastingly) in *Apalone spinifera* (GSD) turtles. Boxed stages denote the thermosensitive period of *C. picta* [Bull and Vogt, 1981]. Asterisks indicated significant differential expression at α = 1e-10.

Doerge, 2010; Bullard et al., 2010; Radhakrishnan et al., 2017b]. In Fisher's exact test n_{ki} is the observed read count for focal gene X (k = 1) or for all other genes in the transcriptome (k = 2) for treatment *i* (*i* = 1 for 26°C and *i* = 2 for 31°C); $n_{11} + n_{12}$ is the marginal total for focal gene X; $n_{21} + n_{22}$ is the marginal total for the remaining genes in the transcriptome; $n_{11} + n_{21}$ is the marginal total for the 26°C treatment; $n_{21} + n_{22}$ is the marginal total for the 31°C treatment; *N* is the grand total [Auer and Doerge, 2010]. This approach tests the null hypothesis that H_0: θ = 1, where $\theta = ((\pi 11 + \pi 22)/(\pi 12 + \pi 21))$ and where πki is the true proportion of counts in cell *k,i* (*k* = 1 for focal gene X or 2 for all other genes; *i* = 1 for 26°C and 2 for 31°C). In other words, this approach tests the null hypothesis that the proportion of counts (gene expression) of focal gene X between 26°C and 31°C is the same as for all other genes. This procedure is appropriate to test the null hypothesis that the proportion of counts (gene expression) of a focal gene *X* between 26 and 31°C is the same as for all other genes.

We noted that the lack of replication implies that differences between 26 and 31°C detected here should be interpreted with caution and should be treated as working hypotheses that warrant corroboration by further analyses. We controlled for false discoveries at a stringent cutoff of 1e–10 [Benjamini and Hochberg, 1995]. Differentially expressed genes were tested for enriched GO categories using the DAVID bioinformatics knowledgebase [Huang et al., 2007]. The transcripts were also mapped to publicly available ncRNA [Kin et al., 2007], miRNA (www.mirbase.org), and piRNA databases (http://pirnabank.ibab.ac.in) [Sai Lakshmi and Agrawal, 2008] using BLAST [Camacho et al., 2009]. For sequences diagnosed as ncRNA and for genes in the epigenetic machinery (online suppl. Tables 1–3; for all online suppl. material, see www.karger.com/doi/10.1159/000492188), mRNA abundance by species, developmental stage, and temperature was calculated to identify differentially expressed transcripts. The effect of temperature on the number of differentially expressed piRNA, ncRNA,

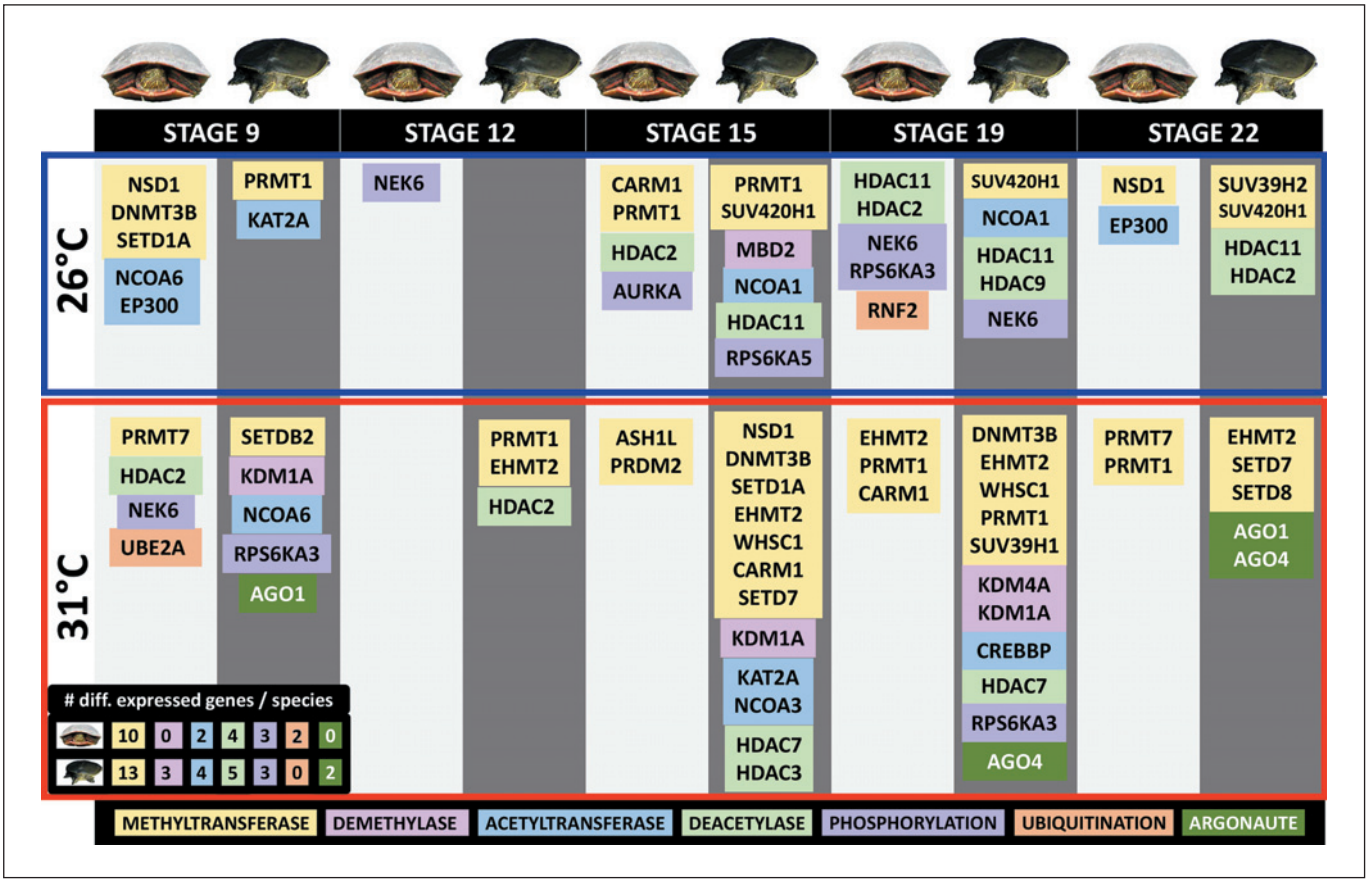

Fig. 3. Genes from the epigenetic machinery with significant differential regulation in *Chrysemys picta* (white columns) and *Apalone spinifera* (grey columns) during embryonic development. Blue outline indicates upregulation at male-producing or low temperature. Red outline indicates upregulation at female-producing (*C. picta*) or high (*A. spinifera*) temperature.

and miRNA (micro RNA) transcripts was assessed using a resampling test at $\alpha = 0.05$. Sequencing reads and gene expression data were deposited in the Short Read Archive (SRA) and Gene Expression Omnibus (GEO) under BioProject ID PRJNA371383 [Radhakrishnan et al., 2017b].

Results

Here, we interrogated a transcriptomic series of gonadal development [Radhakrishnan et al., 2017b] to characterize the response of the genome-wide epigenetic machinery to incubation temperature in the painted turtle *C. picta* (TSD) and the spiny softshell turtle *A. spinifera* (GSD) in order to identify candidate genes and epigenetic processes for a role as mediators of sexual development that might also explain the differences in the levels of plasticity between TSD and GSD turtles. In particular, we targeted 69 genes involved in histone and DNA modifications such as acetylation, methylation, phosphorylation and ubiquitination (Fig. 2, 3; online suppl. Table 1), plus multiple types of small RNAs that have a known role in epigenetic gene silencing in eukaryotes. We first scanned the *C. picta* genome assembly [Shaffer et al., 2013; Badenhorst et al., 2015] for the target genes and corroborated that they are present in the painted turtle genome.

Greater differential transcription of the epigenetic machinery genes was observed very early in development (stage 9), as well as at the onset (stage 15) and in the middle (stage 19) of the TSP, and less so in the tissue containing the bipotential gonads (stage 12) or late in development (stage 22) in both *Chrysemys* (TSD) and *Apalone* (GSD) (Fig. 3). Some contrasting patterns between TSD

and GSD were observed at early developmental stages prior to the onset of the TSP of *Chrysemys* (Fig. 2). Remarkably, *Apalone*'s response to temperature spanned more genes at stage 15 and 19 than in *Chrysemys*. Namely, 3 genes involved in histone or DNA methylation, the methyltransferases *Nsd1, Dnmt3B*, and *Setd1a,* were upregulated at MPT and one (*Prmt7*) at FPT during stage 9 in *Chrysemys*, while at stage 15 two others (*Carm1* and *Prmt1*) were upregulated at MPT and 2 at FPT (*Ash1l* and *Prdm2*). Instead, at stage 19 *Prmt1* and *Carm1* (plus *Ehmt2*) were upregulated at FPT, and *Prmt7* plus *Prmt1* did the same at stage 22, while at this late stage *Nsd1* was upregulated at MPT. No demethylases exhibited differential transcription in *Chrysemys*, whereas in *Apalone* both acetyltransferases and demethylases did (1–7 per stage). In particular, during stage 9 *Prmt1* was upregulated at low-T° and *Setdb2* at high-T°. *Ehmt2* was upregulated at high-T° during stages 12–22, *Whsc1* and *Dnmt3B* during stages 15–19, and *Setd7* at stages 15 and 22 along others at each of these time points (Fig. 3). Fewer methyltransferases were upregulated in *Apalone* at low-T° (*Prmt1* at stages 9 and 15, *Suv420h1* during stages 15–22, and *Suv39h2* at stage 22). The demethylase *Kdm1A* was upregulated in *Apalone* at high-T° during stages 9, 15, and 19 (along *Kdm4a*) and *Mbd2* at low-T° in stage 15.

Similarly, in *Chrysemys* we detected the upregulation of 3 genes involved in histone acetylation, 2 acetyltransferases (*Ncoa6, Ep300*) at MPT (plus one deacetylase, *Hdac2,* at FPT) during stage 9 of development, and one of them during stage 22 (*Ep300*). *Hdac2* was also upregulated at MPT during stage 15 and along *Hdac11* during stage 19 in *Chrysemys*. Completely different acetyltransferases were upregulated at low-T° in *Apalone* during stages 9 (*Kat2A*), 15, and 19 (*Ncoa1*) and at high-T° (*Ncoa6* at stage 9, *Kat2A* and *Ncoa3* at stage 15, and *Crebbp* at stage 19). However, some identical and some different deacetylases were upregulated in *Apalone*, namely *Hdac2* at high-T° during stage 12, *Hdac11* at low-T° during stages 15, 19 (along *Hdac9*), and 22 (along with *Hdac2*), and *Hdac7* at high-T° during stages 15 (along *Hdac3*) and 19.

Two genes that participate in histone ubiquitination and phosphorylation were upregulated at FTP (*Ube2a* and *Nek6*, respectively) during stage 9 in *Chrysemys,* and *Nek6* was also upregulated at MPT during stage 12 and 19 (along *Rps6ka3* and the ubiquitination gene *Rnf2*), while *Aurka* was upregulated at MPT during stage 15. In contrast, no gene involved in ubiquitination exhibited differential transcription in *Apalone,* whereas genes involved in phosphorylation did. Specifically, *Rps6ka3* was upregulated at high-T° during stages 9 and 19, and *Rps6ka5* and *Nek6* were upregulated at low-T° during stages 15 and 19, respectively (Fig. 3).

Although 4 argonaute protein genes were detected in both turtle transcriptomes (*Ago1, Ago2, Ago3, and Ago4*), only 2 exhibited differential transcription, exclusively in *Apalone*, and always at high-T°. Namely, *Ago1* was upregulated at stages 9 and 22, and *Ago4* at stages 19 and 22 (Fig. 3). However, the genes *Tnrc6a* and *Tnrc18* that direct argonaute protein interactors were upregulated at MPT during stage 9 in *Chrysemys* (online suppl. Table 1).

We also identified a large number of transcripts (252 in *Chrysemys* and 169 in *Apalone*) that were annotated as ncRNAs when mapped to the publicly available fRNAdb [Kin et al., 2007]. Most of these ncRNAs (184 in *Chrysemys*, 131 in *Apalone*) are long ncRNAs (lncRNAs) (>200 bp), but small RNAs were also detected (miRNAs and piRNAs). Temperature influenced the transcription of 115 ncRNAs in *Chrysemys* (95 lncRNAs) and 70 in *Apalone* (63 lncRNAs) (Fig. 4), some of which overlap between species (online suppl. Table 4). These ncRNAs were upregulated at MPT and the number of differentially transcribed ncRNAs was lowest at stage 15. Additionally, we identified 257 miRNA transcripts in the *Chrysemys* and 170 in the *Apalone* transcriptomes via BLASTing to known miRNA sequences (www.mirbase.org) [Griffiths-Jones et al., 2006], with mir-2985, mir-3064, and mir-6592 being particularly abundant. These miRNAs interact with multiple genes [Chou et al., 2016], including heat shock genes, transmembrane proteins, kinases, and ion channel transfer proteins, many of which were differentially expressed (online suppl. Table 2). Additionally, 66 piRNA transcript sequences were detected in *Chrysemys* and 61 in *Apalone*, a portion of which were differentially expressed by temperature.

Discussion

Environmental stimuli such as temperature changes can have lasting effects on the development of phenotypes (such as the sexual phenotype) by way of epigenetic modifications, which mediate responses to environmental inputs, are heritable, and influence evolution [Feil and Fraga, 2012; Matsumoto et al., 2013]. Differential transcription has been documented for many TSD and GSD turtle homologs of the genes in the regulatory network of mammalian gonadal development [Lovell-Badge et al., 2002; Eggers et al., 2014], and some evidence exists that epigenetic modifications such as DNA and histone methylation mediate some of the observed differential tran-

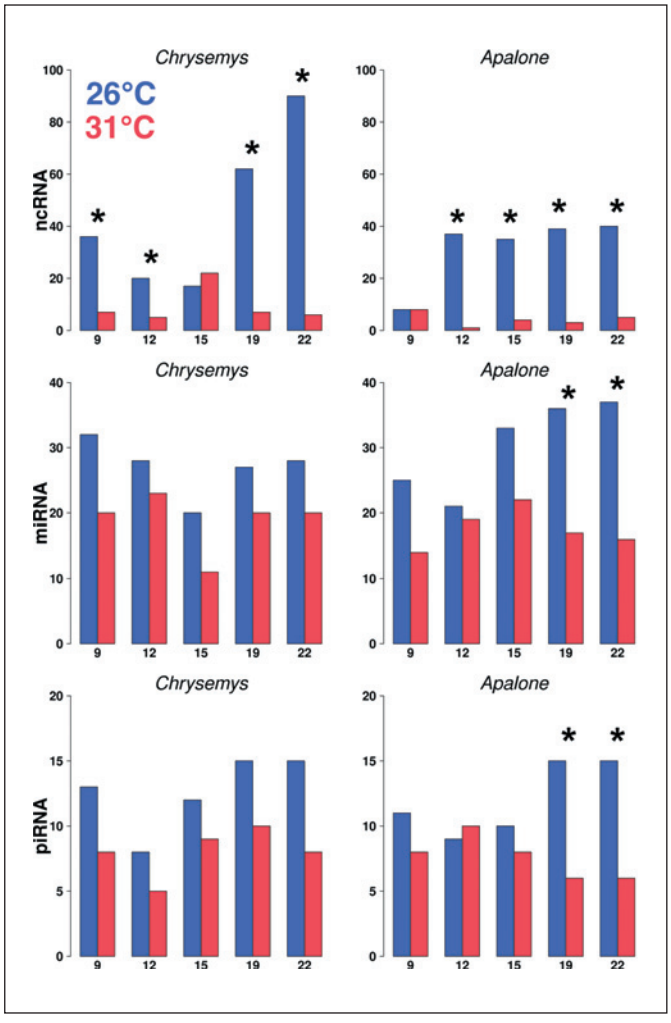

Fig. 4. ncRNA, miRNA, and piRNA transcripts differentially expressed by developmental stage and temperature in *Chrysemys picta* and *Apalone spinifera*. Asterisks denote significant differences at $p = 0.05$ determined by resampling.

scription in TSD taxa [Matsumoto et al., 2013; Radhakrishnan et al., 2017a; Ge et al., 2018]. A recent study demonstrated that differential transcription of epigenetic genes can be a powerful tool to identify candidate epigenetic processes involved in sexual development in TSD turtles that help guide subsequent functional assays [Ge et al., 2018]. Because regulation of gene expression can involve other epigenetic processes beyond methylation, including various forms of histone tail modifications and RNA interference [Jaenisch and Bird, 2003], here we explored for the first time the extent to which the transcription of this epigenetic machinery is also thermosensitive. Thus we provided an initial insight into the epigenetic control of TSD and how it may differ in GSD systems, contrasting the painted turtle *C. picta* (TSD) and the spiny softshell turtle *A. spinifera* (GSD).

We focused our attention on the temperature-specific expression of genes involved in several histone and DNA modifications, including acetylation, methylation, phosphorylation, and ubiquitination, plus lncRNAs and multiple types of small RNAs that have a known role in epigenetic gene silencing in eukaryotes. Particularly revealing are the earliest responses during development that might be key steps in sensing or transducing the temperature signal into plastic gonadal formation in TSD, such as the observed differential transcription of epigenetic machinery genes at stage 9 in *Chrysemys* (5 at the cooler MPT and 4 at the warmer FPT) (Fig. 3). Additionally, the onset (stage 15) and the middle (stage 19) of the canonical TSP in *Chrysemys* were marked by differential transcription of 6 epigenetic genes (4 at MPT and 2 at FPT) and 8 genes (5 at MPT and 3 at FPT), respectively, whereas the end of the TSP (stage 22) showed reduced responses of this subset of genes and stage 12 was especially static (Fig. 3). A similar trend was seen in *Apalone*, except that more genes were upregulated at stages 15 and 19 compared to *Chrysemys*. Why this occurs is unclear, but perhaps it is partly explained by developmental systems drift where gene regulatory networks diverge neutrally but maintain the production of conserved phenotypes [Halfon, 2017]. That is, the divergence of relict thermosensitive transcription in *Apalone* as observed here and previously [Valenzuela, 2008a, b; Radhakrishnan et al., 2017b] via genetic drift would not have been opposed by natural selection, because GSD evolution in this lineage would have rendered sexual development independent of the thermal response of these genes to environmental signals. Also in general, more genes were upregulated at the low MPT in *Chrysemys* while more were upregulated at high-T° in *Apalone*. Below, we highlight some of the most salient results with emphasis on those epigenetic machinery genes that are known to be involved in vertebrate sexual development or that interact with genes that are known to do so (Table 2). See online supplementary tables for the full list of differentially expressed genes.

Gene Enrichment

In general, chromatin organization and modification pathways were enriched during stage 9 in the differentially expressed *Chrysemys* genes but not in *Apalone*. Genes in this category include many members of the methyl- and acetyltransferase families, demethylases and deacetylases among others. The complete list is shown in online suppl.

Table 2. Genes from the epigenetic machinery discussed

Gene symbol	Description
NCOA6	Nuclear receptor coactivator 6
CREBBP	CREB binding protein
EP300	E1A binding protein p300
RARA	Retinoic acid receptor alpha
RARB	Retinoic acid receptor beta
ESR2	Estrogen receptor 2 (ER beta)
NCOA1	Nuclear receptor coactivator 1
NCOA3	Nuclear receptor coactivator 3
DNMT3B	DNA cytosine-5-methyltransferase 3 beta
SETD1A	SET domain containing 1A
NSD1	Nuclear receptor binding SET domain protein 1
PRMT7	Protein arginine methyltransferase 7
CTNNB1	Catenin cadherin-associated protein beta 1 88kDa
PRDM2	PR domain containing 2 with ZNF domain
CARM1	Coactivator-associated arginine methyltransferase 1
KDM1A	Lysine (K)-specific demethylase 1A
AURKA	Aurora kinase A
NEK6	NIMA-related kinase 6
RPS6KA3	Ribosomal protein S6 kinase 90kDa polypeptide 3
RPS6KA5	Ribosomal protein S6 kinase 90kDa polypeptide 5
UBE2A	Ubiquitin-conjugating enzyme E2A
RNF2	Ring finger protein 2
EIF5A2	Eukaryotic translation initiation factor 5A2
HSPA9	Heat shock 70kDa protein 9 (mortalin)
SRD5A1	Steroid-5-alpha-reductase alpha polypeptide 1
HNRNPM	Heterogeneous nuclear ribonucleoprotein M
HNRNPH1	Heterogeneous nuclear ribonucleoprotein H1
AGO2	Argonaute RISC catalytic component 2
AGO1	Argonaute RISC catalytic component 1
AGO4	argonaute RISC catalytic component 4
AGO3	argonaute RISC catalytic component 3
TNRC6A	Trinucleotide repeat containing 6A
TNRC18	Trinucleotide repeat containing 18

Table 3. This observation suggests the involvement of the epigenetic machinery acting early during the development of TSD reptiles well before the onset of the canonical TSP. Because trunks were examined at that early stage, chromatin modification may perhaps help orchestrate the development of the gonads and other tissues.

Methylation

Histone methylation marks can lead to transcription activation or repression depending on the context. For instance, methylations on residues H3K4, H3K36, and H3K79 are marks of active transcription, while H3K9 and H3K27 methylation is usually associated with transcription repression [Kouzarides, 2007]. Genes involved in methylation were the category that responded the most to incubation temperature in both species (Fig. 3). Among these, *Dnmt3b* induces the de novo methylation of CpG dinucleotide sequences in mammals [Okano et al., 1999; Kato et al., 2007], and it is upregulated at MPT during stage 9 in *Chrysemys* and at high-T° during stages 15 and 19 in *Apalone*, which could potentially induce temperature-specific DNA methylation patterns. Consistent with this idea, previous genome-wide studies of DNA methylation in TSD turtles detected extensive sexually dimorphic methylation between male and female hatchling gonads in *Chrysemys* [Radhakrishnan et al., 2017a] and in embryonic gonads of *Lepidochelys olivacea* [Venegas et al., 2016] incubated at MPT and FPT. We also observed stage 9 upregulation of *Setd1a* (H3K4me3) and *Nsd1* (H3K36me), both marks of gene activation [Barski et al., 2007; Morris et al., 2007] during the MPT in *Chrysemys*. The same is true for *Prmt7* at FPT, a gene that encodes a protein that binds to the estrogen receptor and is a regulator of the *Wnt* pathway that mediates ovarian development in mammals [Kim et al., 2006]. Further, *Wnt* can be regulated by the male-inducer *Sox9* via phosphorylation of the ovarian inducer beta-catenin (*Ctnnb1*) [Liu et al., 2009; Topol et al., 2009] which is upregulated in *Chrysemys* at this same stage 9 [Radhakrishnan et al., 2017b]. The early activity of *Dnmt3B, Setd1a, Nsd1*, and *Prmt7* in *Chrysemys* strongly suggests that regulation of gene expression by methylation could contribute to plastic gonadal development in TSD reptiles. *Carm1*, an interactor of beta-catenin during hormone receptor activation to induce transcription, was upregulated at MPT in *Chrysemys* at the onset of the canonical TSP (stage 15) and at high-T° in *Apalone.* At this same stage, *Prdm2* is upregulated at FPT in *Chrysemys*, and is a zinc finger protein that binds to the estrogen receptor and may regulate estrogen activity. On the other hand, some demethylases were differentially regulated in *Apalone* but not in *Chrysemys* (Fig. 3). Among them, *Kdm1A*, a coactivator of androgen-receptor-dependent transcription, was upregulated at high-T° at stages 9, 15, and 19. If the transcriptional level of these genes is a good proxy of their activity in epigenetic regulation, then combined, the observed differences in their thermal response between species lead to the hypothesis that a contrast might exist in hormone-dependent epigenetic regulation between TSD and GSD or in their hormonal regulation of epigenetic processes.

Acetylation

Histone acetylation occurs mostly on the N-terminus of histone tails, which are accessible for modification.

Acetylation occurs on lysine residues and weakens the bond between the histone and DNA and is usually associated with gene activation [Kouzarides, 2007]. *Ncoa6* encodes a nuclear receptor protein which recruits the Cbp/Ep300 complex that directly leads to lysine acetylation on histones H3, H4, H2A, and H2B [Bannister and Kouzarides, 2011]. We detected upregulation of *Ncoa6* at MPT and *Ep300* during stage 9 in *Chrysemys*, whereas it was upregulated at high-T° in *Apalone*, perhaps due to developmental systems drift after GSD evolution. *Ncoa6* is important for gonadal development, as its knockout reduces fertility in mice [Mahajan and Samuels, 2008]. Among the interactors of *Ncoa6* are the *Retinoic acid receptor* (*Rarα*) which helps induce germ cell meiosis (a marker of mice ovary determination) [Mu et al., 2013] and estrogen receptor genes *ERα* and *ERβ*. Functional studies are needed to directly test the role of *Ncoa6* in mediating the temperature signal during gonadal development in TSD turtles. In *Apalone*, the upregulation of *Ncoa1* and *Ncoa3* at stages 15 and 19 stand out because they are transcriptional coactivators of steroid and nuclear hormone receptors, and these responses were absent in *Chrysemys,* suggesting another potential change in the regulation of hormonal pathways between TSD and GSD systems that requires functional examination. Notably, hormone signaling genes are among the fastest evolving genes of the regulatory network of sexual development in turtles [Literman et al., 2017b].

Phosphorylation

Phosphorylation of residues including serine, threonine, and tyrosine on histone tails can trigger other modifications, including H3K9 acetylation and H3K4 methylation, which are switches for transcription activation [Rossetto et al., 2012]. Similar to ubiquitination, few genes involved in phosphorylation exhibited a differential response to incubation temperature in our study, but effects were detected in both species, and the genes involved (*Nek6, Rps6ka3, Rps6ka5, Aurka*) are all related to cell growth, cell cycle, proliferation, and differentiation (Fig. 3).

Ubiquitination

Lysine residues on histones that are subject to ubiquitination could be candidates for transcription activation or repression depending on the context. Only 2 genes involved in ubiquitination were differentially regulated, both exclusively in *Chrysemys*, one in early development (*Ube2a*) and one in the mid TSP (*Rnf2*). Of these, the polycomb complex protein RNF2, involved in X inactivation in mammals via the monoubiquitination of H2AK119, was upregulated at MPT in *Chrysemys* during stage 19.

ncRNAs

Noncoding RNAs are increasingly recognized to play vital roles in transcriptional regulation, translation, and DNA protection from foreign molecules among a bulk of other functions [Cech and Steitz, 2014]. We identified 252 transcripts in *Chrysemys* and 169 in *Apalone* annotated as ncRNAs. Among these, some ncRNA transcripts were also differentially expressed by temperature in both species (Fig. 4). Interestingly, the difference in number of differentially expressed ncRNA transcripts by temperature was lowest at the onset of the TSP (stage 15) in *Chrysemys.* At all other stages, including stages 9 and 12, there were more ncRNAs overexpressed at MPT than FPT, suggesting that perhaps ncRNAs could act as vital epigenetic switches in the transcriptional network of gonadal development. More research in this area is needed to test this hypothesis.

Small RNAs

miRNA sequences are short RNA fragments that are known to regulate gene expression post transcription in addition to RNA silencing [Ambros, 2004]. Importantly, miRNAs have been proposed to underlie sex differences in *Drosophila* [Fagegaltier et al., 2014]. We identified 257 miRNA transcripts in *Chrysemys* and 170 in *Apalone* transcriptomes via BLASTing to known miRNA sequences (www.mirbase.org) [Griffiths-Jones et al., 2006]. Out of these, some miRNA sequences were more abundant than others, including mir-2985, mir-3064, and mir-6592. Some of the targets of these miRNAs are particularly intriguing candidates to investigate for they are known to contribute to processes such as transcription, heat shock, and gonadogenesis (online suppl. Table 2). For instance, mir-2985 targets *Eif5a2* (a translation initiation factor) and *Hspa9,* both of which are differentially expressed in the turtles at different stages. In *Chrysemys*, *Eifa2* is upregulated at MPT during stage 19 and 22, whereas *Hspa9* is upregulated at MPT during stage 15 and at FPT during stage 22 [Radhakrishnan et al., 2017b]. In *Apalone*, *Eif5a2* is upregulated at low-T° during stage 9 and at high-T° at stage 19, while *Hspa9* is upregulated at low-T° during stage 22. Mir-3064 targets *Srd5a1,* a central player in male sexual differentiation [Andersson et al., 1989], which is expressed across all developmental stages (albeit not differentially) in both turtles [Radhakrishnan et al., 2017b], and whose molecular evolu-

tion is accelerated in vertebrates but especially in turtles [Literman et al., 2017b]. Further, mir-3064 and mir-6592 also target heterogeneous nuclear ribonucleoproteins (Hnrnps), which have been implicated in sexual differentiation in TSD turtles [Harry et al., 1990]. Importantly, we found that *Hnrnph1* and *Hnrnpm* are active and differentially expressed in turtles; *Hnrnpm* and *Hnrnph1* are both upregulated at MPT during stage 15, while *Hnrnph1* is also upregulated at FPT during stage 9 in *Chrysemys* [Radhakrishnan et al., 2017b]. Interestingly, in both *Chrysemys* and *Apalone* we identified slightly more miRNAs to be differentially expressed at 26 than 31°C, the difference generally being more pronounced in *Apalone*.

Other small RNAs, piRNAs, are sex specific in zebrafish [Zhou et al., 2010]. More recently, a single piRNA was documented as the primary sex determinant in the silkworm [Kiuchi et al., 2014]. In this study, we identified 66 piRNA transcript sequences in *Chrysemys* and 61 in *Apalone*. Multiple piRNA sequences each in *Chrysemys* and *Apalone* were differentially expressed by temperature, but their functions are currently unknown.

Argonaute Proteins

Argonaute proteins, acting in conjunction with small RNAs, are involved in posttranscriptional gene silencing [Meister, 2013]. We identified 4 argonaute genes expressed in both turtle transcriptomes (*Ago1, Ago2, Ago3,* and *Ago4*). *Ago1* and *Ago4* were upregulated in *Apalone* at high-T° during stages 19 and 22, but not in *Chrysemys*. Trinucleotide-repeat containing genes *Tnrc6a* and *Tnrc18,* which are direct interactors of *Ago* proteins [Stroynowska-Czerwinska et al., 2014], were upregulated at MPT during stage 9 in *Chrysemys*.

Conclusion

This study illuminates the molecular ecology of the process that commits developing turtles to their sexual fate by characterizing the transcriptional response of the epigenetic machinery genes to incubation temperature in species with contrasting levels of plasticity in their primary sexual development. We showed that elements involved in histone modification, DNA methylation, and posttranscriptional gene silencing are not only active but are differentially deployed by temperature between TSD and GSD turtles, and in some cases enriched even before the onset of the canonical TSP in TSD turtles. The epigenetic control of hormonal pathways via acetylation, methylation, and the participation of ncRNA emerges from our results as a strong candidate mediator of the plastic responses of TSD systems, a working hypothesis that warrants further research. Also, the distinctive enrichment of chromatin remodeler elements in TSD but not GSD turtles detected here is particularly promising. Such structural differences in genome regulation may set these 2 sex determining mechanisms apart and help explain their contrasting levels of phenotypic plasticity and canalization. Our findings indicate that future functional assays targeting these particular epigenetic pathways are promising avenues to unravel the still enigmatic diversity of vertebrate sex determination.

Acknowledgements

This work was supported in part by Sigma Xi grant G20120315162029 to S.R. and NSF grants IOS-0743284, MCB-1244355, and IOS-1555999 to N.V.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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