Post-transcriptional mechanisms respond rapidly to ecologically relevant thermal fluctuations during temperature-dependent sex determination

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Abstract

An organism’s ability to integrate transient environmental cues experienced during development into molecular and physiological responses forms the basis for adaptive shifts in phenotypic trajectories. During temperature-dependent sex determination (TSD), thermal cues during discrete periods in development coordinate molecular changes that ultimately dictate sexual fate and contribute to patterns of inter- and intra-sexual variation. How these mechanisms interface with dynamic thermal environments in nature remains largely unknown. By deploying thermal loggers in wild nests of the American alligator (Alligator mississippiensis) over two consecutive breeding seasons, we observed that 80% of nests exhibit both male- and female-promoting thermal cues during the thermosensitive period, and of these nests, all exhibited both male- and female-promoting temperatures within the span of a single day. These observations raise a critical question— How are opposing environmental cues integrated into sexually dimorphic transcriptional programs across short temporal scales? To address this question, alligator embryos were exposed to fluctuating temperatures based on nest thermal profiles and sampled over the course of a daily thermal fluctuation. We examined the expression dynamics of upstream genes in the temperature-sensing pathway and find that post-transcriptional alternative splicing and transcript abundance of epigenetic modifier genes JARID2 and KDM6B respond rapidly to thermal fluctuations while transcriptional changes of downstream effector genes, SOX9 and DMRT1, occur on a delayed timescale. Our findings reveal how the basic mechanisms of TSD operate in an ecologically relevant context. We present a hypothetical hierarchical model based on our findings as well as previous studies, in which temperature-sensitive alternative splicing incrementally influences the epigenetic landscape to affect the transcriptional activity of key sex-determining genes.
Introduction

Embryos often experience dynamic environmental conditions during development which can exert lasting influences on organismal phenotypes. This is especially true for species with temperature-dependent sex determination (TSD) in which the sex of offspring is determined by temperature cues experienced during discrete periods in development. Occurring in a diverse range of taxa, including all crocodilians, many turtles, and some fish (Bull, 1980; Lang & Andrews, 1994; Ospina-Alvarez & Piferrer, 2008), TSD provides a unique window through which to examine how transient thermal cues coordinate molecular changes that ultimately establish patterns of inter- and intrasexual variation. Major advances have recently been made in understanding the proximate developmental mechanisms underlying TSD (Czerwinski et al., 2016; Deveson et al., 2017; Ge et al., 2018, 2017; Yatsu et al., 2016), yet little is known regarding how these mechanisms interface with the dynamic thermal environments experienced by embryos in nature.

For many species with TSD, nest temperatures fluctuate considerably on daily, monthly, and seasonal timescales (Carter, Bowden, & Paitz, 2017; Carter, Sadd, Tuberville, Paitz, & Bowden, 2018; Escobedo-Galván, López-Luna, & Cupul-Magaña, 2016; Warner & Shine, 2011). While the consequences of these temperature fluctuations for offspring phenotype are incompletely understood, in some species including the red eared slider turtle, *Trachemys scripta*, temperature fluctuations appear to play critical roles in influencing population sex ratios. Mean temperatures of *T. scripta* nests in the field rarely exceed those necessary to produce females under constant laboratory conditions, yet mark-recapture studies indicate females are frequently recruited into these populations. This apparent discordance is reconciled by the observation that female development can be triggered in *T. scripta* by as few as 5 days of
exposure to female-promoting temperatures (Carter et al., 2018). How do embryos integrate brief
periods of female-promoting temperature cues experienced in the field during “heatwaves” into
lasting physiological responses that drive sexual fate commitment? And, how do these
mechanisms function in other TSD species? These questions remain unanswered and hold
important implications for our understanding of TSD as it occurs in ecologically relevant
contexts.

Epigenetic mechanisms, including DNA methylation and histone modifications, appear to
play upstream roles in integrating transient temperature cues into persistent transcriptional
responses during TSD (Ge et al., 2018; Matsumoto, Buemio, Chu, Vafae, & Crews, 2013;
Navarro-Martín et al., 2011; Parrott, Kohno, Cloy-McCoy, & Guillette, 2014). Two genes with
roles in chromatin regulation, JARID2 and KDM6B (or JMJD3), are among the earliest genes
displaying temperature-sensitive differential expression during TSD in both T. scripta and
Alligator mississippiensis (Czerwinski et al., 2016; Yatsu et al., 2016). JARID2 is a component
of the master Polycomb repressive complex 2 (PRC2) which plays a key role in localizing the
PRC2 complex to its target loci for subsequent transcriptional silencing via histone 3 lysine 27
trimethylation (H3K27me3) (da Rocha et al., 2014; Kaneko et al., 2014; Landeira & Fisher,
2011; Peng et al., 2009; Sanulli et al., 2015). KDM6B encodes a histone demethylase which
removes the repressive H3K27me3 mark to activate transcription of target loci (Agger et al.,
2007; Lan et al., 2007). Intriguingly, interruption of KDM6B activity via RNA interference in T.
scripta results in the inhibition of male-development at a male-promoting temperature and is
associated with increased H3K27me3 at the promoter of DMRT1, a transcription factor involved
in male development (Ge et al., 2018). Together, these findings suggest that epigenetic
modifications and the conserved genes that regulate them play causal roles in integrating
transient temperature cues experienced during development into sexually dimorphic transcriptional programs during TSD.

Interestingly, the role of these upstream epigenetic regulators in mediating TSD is likely more nuanced than previously realized. While much of the focus of TSD research has centered on investigating transcriptional responses to temperature, mounting evidence suggests post-transcriptional mechanisms, particularly alternative splicing, may contribute an added layer of regulatory complexity (Agrawal, Wessely, Anand, Singh, & Aggarwal, 2009; Anand et al., 2008; Deveson et al., 2017; Harry, Williams, & Briscoe, 1990; Rhen, Fagerlie, Schroeder, Crossley, & Lang, 2015). Alternative splicing, the process by which pre-mRNA generates variable mature mRNA transcripts via differential exon usage and intron retention, plays a widespread role in contributing to transcriptional diversity across metazoan taxa (Tapial et al., 2017) and serves as the decisive sex-determining signal in some species (Salz, 2011). Notably, multiple species possessing different forms of TSD including A. mississippiensis, T. scripta, and Pogona vitticeps, exhibit temperature dependent alternative splicing of the epigenetic modifier genes, JARID2 and KDM6B (Deveson et al., 2017). In particular, a unique intron containing premature stop codons is differentially retained depending on incubation temperature in mature transcripts of each of these genes (Deveson et al., 2017). How environmental variability experienced in the field interfaces with these molecular pathways is currently unknown.

Here, we quantified the level of thermal variation experienced by alligator embryos in wild nests and implemented experimental thermal fluctuations based on empirically derived nest thermal profiles to examine how transcription and post-transcriptional alternative splicing of genes involved in sex determination and sexual differentiation change over the course of a daily thermal fluctuation during TSD. We found that alternative splicing of the epigenetic modifier
*JARID2*, in both the gonad and brain, responded rapidly to thermal fluctuations. Further, transcript abundance of both *JARID2* and *KDM6B* also fluctuated with temperature in the gonad, albeit with an apparent delay of approximately 7 hours. In contrast to these epigenetic regulators, genes with downstream roles in sex determination and differentiation, *SOX9* and *DMRT1*, did not exhibit clear responses to thermal fluctuations. Together our findings suggest fluctuating thermal cues experienced in nature are integrated via a temporal hierarchy of molecular responses during TSD.

**Methods**

*Field-derived nest thermal profiles*

Twenty alligator nests were monitored at the Tom Yawkey Wildlife Center (YWC; Georgetown, SC; (Bock et al., 2020)). At each nest (Figure 1A), one Onset (UTBI-001) HOBO temperature logger preprogrammed to record temperature at 5-minute intervals was deployed in the center of the nest cavity and one temperature logger was attached to vegetation in close proximity to the nest out of direct sunlight to record air temperatures of the nest microclimate. Nests were left undisturbed until after hatchlings emerged (second week of September), after which temperature loggers were retrieved. Raw data from the temperature loggers were processed to only include measurements for the dates encompassing the thermosensitive period (TSP), Ferguson stage 15 to 24 (Ferguson, 1985; Lang & Andrews, 1994; McCoy, Parrott, Rainwater, Wilkinson, & Guillette, 2015). Dates of the TSP were estimated based on hatch date, average nest temperature during the first 49 days of the incubation period, and the established relationship between temperature and developmental rate in *A. mississippiensis* (Kohno & Guillette, 2013).

*Laboratory incubation experiment*
Field collections were performed under permits obtained from the Florida Fish and Wildlife Conservation Commission and the U.S. Fish and Wildlife Service. In 2018, three clutches of alligator eggs were collected shortly after oviposition at Lake Woodruff National Wildlife Refuge (De Leon Springs, FL). Following transport to the Savannah River Ecology Laboratory (Jackson, SC), a representative embryo was examined to determine the developmental stage of each clutch (Ferguson, 1985). Eggs were kept in damp sphagnum moss and incubated at 32°C, a temperature promoting the development of both males and females, until the opening of the thermosensitive period at Ferguson stage 15. Thermal sensitivity of sex determination does not begin until stage 15 and embryos were kept at a single constant temperature prior to this stage to minimize variation resulting from other thermosensitive embryonic traits. Eggs were then shifted to one of three temperature treatments—a constant 29°C (female-promoting temperature), constant 33.5°C (male-promoting temperature), or fluctuating thermal regime based on field-derived nest thermal profiles (average 31.25°C, minimum 29°C, maximum 33.5°C) (González et al., 2019). We derived the fluctuating thermal regime from the eight nest temperature profiles measured during 2015 by determining the average deviation from the mean nest temperature for every 5 minutes of the day during the TSP, then increasing that deviation by a factor of seven (Figure 1B). This resulted in a thermal regime that fluctuated on a daily basis between the male-promoting (33.5°C) and female-promoting (29°C) temperatures and exhibited the same periodicity as a wild nest. While daily thermal variation of the experimental fluctuation was greater than average (average daily nest temperature shift = 0.84°C; experimental daily temperature shift = 4.5°C), it was not outside the scope of measured daily fluctuations (e.g., nests were observed to experience shifts of ≥ 7°C within a day).
At stage 22, the middle of the thermosensitive period, alligator embryos were sampled at four time points spanning a daily thermal fluctuation in the fluctuating temperature treatment (Figure 2). The sampling time points corresponded to the minimum temperature (29°C, female-promoting temperature; T2; n = 10), intermediate increasing temperature (31.5°C, T3; n = 10), maximum temperature (33.5°C, male-promoting temperature, T4; n = 9), and intermediate decreasing temperature (31.5°C, T1; n = 8) of the fluctuating temperature treatment. Embryos exposed to a constant female-promoting temperature (29°C; n = 11(T1), 10(T2), 7(T3), 8(T4)) and constant male-promoting temperature (33.5°C; n = 9(T1), 8(T2), 7(T3), 11(T4)) were sampled at the same time points to assess daily variability in gene expression unrelated to temperature and to yield a baseline to which the fluctuating temperature treatment group could be compared.

**Nucleic acid isolation, cDNA synthesis, and qPCR**

Gonadal and brain RNA were extracted using the SV total RNA isolation system (Promega; Madison, WI) and resulting concentrations were quantified via spectrophotometry (NanoDrop One; Thermo Fisher Scientific; Waltham, MA). Synthesis of cDNA was carried out using the iScript cDNA synthesis kit (Bio-Rad; Hercules, CA) according to the manufacturer protocol and using 903.4 ng and 999 ng of input RNA from the gonad and brain respectively. Gene expression was assessed via quantitative real-time PCR and reactions were performed in triplicate using a SYBR green reaction mix. Intron-retaining transcripts of *JARID2* and *KDM6B* were selectively targeted for quantification via specially designed primer sets. In particular, we targeted transcripts retaining the same introns reported to be differentially retained in previous studies (11th intron in *JARID2* and 19th intron in *KDM6B*; (Deveson et al., 2017)). Information for all associated primers is provided in Figure 3. All primers were designed to be intron-
spanning. Those primers targeting intron-retaining isoforms were designed to span the preceding intron. For each gene and isoform, all expression values were derived from triplicate replicates (for biological and standard samples) using a standard curve comprised of target-containing plasmid standards of known concentration (copies/µl) that was run on each qPCR plate. Expression values for gonad and brain samples were normalized to that of beta-actin (ACTB) and ribosomal protein L8 (RPL8) respectively, which served as internal controls and were also derived from standard curves. There were no significant effects of temperature, sampling timepoint, or their interaction on the absolute expression levels of either of these housekeeping genes (SI Figure 2).

**Statistical analyses**

Statistical analyses were conducted using R statistical software version 3.6.1. Distributions of relative expression values for each gene were tested for normality and homoscedasticity using a Shapiro-Wilk test and Levene’s test, respectively. If necessary, a transformation was applied to relative expression values to achieve normality. A log-transformation was applied to values for JARID2 (all transcripts, intron-retention ratio), KDM6B (all transcripts, intron-retention ratio), and SOX9. A square-root transformation was applied to values for DMRT1. Transformations were not sufficient to alleviate non-normality for the JARID2 intron-retention ratio in both the gonad and brain. We examined the influence of temperature treatment, sampling timepoint, and their interaction on relative expression of each gene and isoform using linear mixed effects models with clutch identity included as a random effect. These models were fit using the ‘lme4’ package in R. We obtained p-values from the models using likelihood ratio tests. Due to the persistent non-normality of the JARID2 intron-retention ratio following transformation, we also assessed the effect of temperature and sampling
timepoint in the gonad and brain by conducting non-parametric Kruskal-Wallis tests. In both cases, results of the Kruskal-Wallis test conformed to those of the linear mixed effects model. To further examine potential relationships among the responses of genes in the gonad to thermal regime, we assessed pairwise Pearson correlations between each of the isoforms of JARID2 and KDM6B, as well as DMRT1 and SOX9.

Results

Nest thermal profiles

Thermal profiles of 20 alligator nest cavities were monitored between 2015 and 2016 at the Tom Yawkey Wildlife Center (Georgetown, SC, USA). The overall mean temperature during the TSP for all nests was 32.02°C, a temperature predicted to produce 69.8% males (Lang & Andrews, 1994). The coolest nest monitored occurred in 2015 and had a mean nest temperature of 29.00°C, a temperature predicted to produce 100% females. The warmest nest monitored occurred in 2016 and had a mean nest temperature of 33.85°C, a temperature predicted to produce 51.9% males (Lang & Andrews, 1994). We observed considerable within-nest variability in temperature (Figure 1C), with 80% (16 of 20) of nests exhibiting both male- and female-promoting temperatures within the TSP. All of these nests also exhibited both male- and female-promoting temperatures within the span of a single day during at least one day during the TSP. On average, nest temperatures varied by 0.84°C within the span of a day. The maximum daily temperature range for an individual nest was 7.61°C and the minimum daily temperature range was 0.08°C. While individual nests varied considerably in their mean temperatures during the TSP, there were distinct commonalities in the shape of their daily thermal cycles (SI Figure 1). In particular, nests tended to exhibit their coolest temperatures between 06:00 and 18:00 hours followed by a temperature maximum occurring between 18:00 and 24:00 hours. Nests also
tended to warm faster than they cooled (SI Figure 1). Each of these thermal characteristics was retained in our experimental thermal fluctuation wherein embryos experienced male- and female-promoting temperatures over the course of a daily thermal cycle.

*Epigenetic modifiers*

In the gonad, there was a significant interactive effect of temperature treatment and sampling timepoint on the relative expression ratio of the *JARID2* intron-retaining transcripts to all transcripts (IR: ND; \(X^2(6) = 49.86, p < 0.001\); Figure 4A). A Kruskal-Wallis test of the combined effect of temperature and timepoint on the *JARID2* intron-retention ratio supported this result (\(H(11) = 81.28, p < 0.001\)). While embryos exposed to the constant female-promoting temperature (FPT) exhibited consistently higher intron-retention in *JARID2* compared to embryos exposed to the constant male-promoting temperature (MPT), embryos exposed to the fluctuating temperature treatment (FLUX) exhibited variable levels of intron-retention. In particular, intron-retention of *JARID2* peaked in FLUX embryos at the timepoint in which these embryos were experiencing a female-promoting temperature (T2), consistent with a rapid response of intron-retention to temperature (Figure 4A). We also detected a significant interactive effect of temperature treatment and sampling timepoint on overall transcript abundance of *JARID2* (Non-discriminating; \(X^2(6) = 20.64, p = 0.002\); Figure 4B). Similar to the pattern of intron-retention in *JARID2*, transcript abundance of *JARID2* was more variable in FLUX embryos. However, in contrast to intron-retention, transcript abundance of *JARID2* peaked at the timepoint after they experienced a female-promoting temperature (T3; ~7 hours after T2; Figure 4B).

In the brain, intron-retention in *JARID2* exhibited a nearly identical response to temperature as it did in the gonad suggesting a tissue-independent mechanism. There was a
significant interactive effect of temperature treatment and sampling timepoint on *JARID2* intron-retention in the brain (IR: ND; $X^2(6) = 34.105, p < 0.001$; Figure 5A), with intron-retention peaking in FLUX embryos at the FPT timepoint, T2. This result was also supported by a Kruskal-Wallis test of the combined effect of temperature and timepoint on *JARID2* intron-retention ($H(11) = 83.70, p < 0.001$). The response of *JARID2* transcript abundance to temperature in the brain was less clear, but there was a significant interactive effect of temperature treatment and sampling timepoint (Non-discriminating; $X^2(6) = 28.954, p < 0.001$; Figure 5B).

Levels of intron-retention in *KDM6B* were lower than those of *JARID2* in both the gonad and brain. There was a significant effect of temperature treatment ($X^2(2) = 8.6665, p = 0.013$) but not sampling timepoint or their interaction on the ratio of *KDM6B* intron-retaining transcripts to all transcripts in the gonad (Figure 5C). There was also a significant effect of temperature treatment ($X^2(2) = 84.346, p < 0.001$) but not sampling timepoint or their interaction on *KDM6B* transcript abundance in the gonad (Figure 5D). While gonadal *KDM6B* transcript abundance appeared highest in the FLUX embryos at the timepoint after embryos received a female-promoting temperature cue (T3), this variation was not statistically significant. In contrast to *JARID2*, there was no significant effect of the temperature-by-timepoint interaction on *KDM6B* transcript abundance (Figure 5D). In the brain, intron-retention levels in *KDM6B* were low and there were no effects of temperature, sampling timepoint, or their interaction on the ratio of intron-retaining to all *KDM6B* transcripts. We did, however, detect a significant interactive effect of temperature and timepoint on *KDM6B* transcript abundance in the brain ($X^2(6) = 14.478, p = 0.025$) (Figure 5D).

*Downstream effector genes of sex determination*
There was a significant interactive effect of temperature and timepoint on gonadal SOX9 relative expression ($X^2(6) = 19.2, p = 0.004$). Consistent with its role in promoting testicular differentiation, SOX9 relative expression was higher in embryos incubated at MPT compared to those incubated at FPT (Figure 4E). FLUX embryos exhibited variation in their relative expression of SOX9 with highest relative expression observed at timepoint T3 (one timestep after experiencing a female-promoting temperature or three timesteps after experiencing a male-promoting temperature; Figure 4E). Given the current experimental design, it is not yet possible to distinguish whether this reflects a rapid response of SOX9 relative expression to increasing temperatures following exposure to FPT or a delayed response to MPT.

We observed a similar pattern of relative expression of DMRT1 compared to that of SOX9, though we did not detect a significant interactive effect of temperature and timepoint on DMRT1 relative expression. There was, however, a significant additive effect of temperature and timepoint ($X^2(3) = 9.3691, p = 0.025$) on DMRT1 relative expression in the gonad. Similar to SOX9, relative expression of DMRT1 in FLUX embryos was highest at timepoint T3 (one timestep after experiencing a female-promoting temperature or three timesteps after experiencing a male-promoting temperature; Figure 4F), though this did not result in a significant temperature-by-timepoint interactive effect.

The strongest gene expression correlations we observed in the gonad were between the intron-retaining and non-discriminating isoforms for JARID2 and KDM6B, the non-discriminating isoform for JARID2 and the non-discriminating isoform for KDM6B, and the intron-retaining isoform for JARID2 and non-discriminating isoform for KDM6B (SI Figure 3). While we observed a correlation between DMRT1 and SOX9 expression, correlations between
expression of upstream epigenetic regulators, *KDM6B* and *SOX9*, and downstream effector
genes, *DMRT1* and *SOX9*, were comparatively weaker (SI Figure 3).

**Discussion**

During temperature-dependent sex determination, alligator embryos experience rapid
fluctuations between female- and male-promoting temperatures in wild nests. Findings from this
study suggest embryos integrate these opposing environmental signals via a temporal hierarchy
of responses. Alternative splicing of the epigenetic modifier gene *JARID2* responds quickly to
thermal fluctuations during sex determination in both the gonad and brain. Transcript abundance
of both *JARID2* and *KDM6B* similarly fluctuates with temperature, though with an apparent time
delay relative to the splicing response. In contrast to these upstream epigenetic regulators,
expression patterns of downstream effector genes of sex determination, *DMRT1* and *SOX9*, are
highly variable and do not exhibit as clear a relationship to temperature under fluctuating
conditions. These results are consistent with a hypothetical model wherein alternative splicing
and transcript abundance of upstream chromatin modifiers fluctuate with temperature resulting in
incremental epigenetic changes that influence the transcriptional activity of key sex determining
genes (Figure 6). However, additional experiments testing other key components of this
conceptual model, such as how temperature-dependent alternative splicing affects the function of
*KDM6B* and *JARID2*, are clearly still needed.

The relatively rapid responses of *JARID2* and *KDM6B* to thermal fluctuations suggest
these factors are likely proximate targets of a cellular thermo-sensory mechanism(s) and thus
occupy upstream positions in the sex-determining transcriptional cascade. Despite extensive
research efforts, the thermo-sensory mechanism governing TSD has remained elusive. Several
candidates have been hypothesized to initially translate incubation temperature into a biological
response during TSD including transient receptor potential (TRP) channels (Lin et al., 2018; Yatsu et al., 2015), heat shock proteins (HSPs) (Bentley, Haas, Tedeschi, & Berry, 2017; S. Kohno et al., 2010), and cold-inducible binding protein (CIRBP) (Chojnowski & Braun, 2012; Lin et al., 2018; Rhen & Schroeder, 2010; Schroeder, Metzger, Miller, & Rhen, 2016) (Figure 6). All of these factors are generally well-conserved and coordinate cellular responses to temperature across diverse systems (Clapham, 2003; Kohno et al., 2010; Zhong & Huang, 2017) though evidence for their role in TSD is currently equivocal. CIRBP represents an intriguing candidate as it belongs to a family of RNA recognition motif binding proteins with roles in regulating splicing, translation, and mRNA stability (De Leeuw et al., 2007; Dreyfuss, Kim, & Kataoka, 2002; Xia et al., 2012). Based on data from Chelydra serpentina, temperature-induced transcriptional responses of CIRBP would appear to lag behind the shifts in alternative splicing and transcript abundance of JARID2 and KDM6B reported here (Schroeder et al., 2016). This, however, does not rule out the possibility of rapid post-transcriptional regulation of CIRBP by temperature (Gotic et al., 2016; Haltenhof et al., 2020). A recent report demonstrated a role for CDC-like kinases (CLKs) in regulating temperature-sensitive alternative splicing of both CIRBP and JARID2 (Haltenhof et al., 2020), lending support to the possibility that multiple thermo-sensory mechanisms may act in a coordinated manner during TSD. Further investigations into temperature-induced changes in intracellular signaling cascades (Rottingen & Iversen, 2000; Stamm, 2008), protein conformation (Haltenhof et al., 2020), and/or conformation of RNA molecules themselves (Chowdhury, Maris, Allain, & Narberhaus, 2006; Johansson et al., 2002; Loh et al., 2013) during TSD are likely to shed light on the molecular interactions that transduce temperature into a sex-determining signal.
Exposure to female-promoting temperatures in alligator embryos resulted in both increased intron-retention and increased overall transcript abundance of JARID2. Counterintuitively, the intronic sequence retained in mature JARID2 transcripts contains premature stop codons and intron-retaining transcripts are predicted to produce truncated products with altered or ameliorated function if they avoid nonsense-mediated decay and are translated (Deveson et al., 2017). This result is consistent with previous findings in which intron-retention in both JARID2 and KDM6B was more frequent at the temperature promoting expression of these genes in A. mississippiensis and T. scripta (Deveson et al., 2017; Georges & Holleley, 2018). Various explanations have been posited to reconcile these seemingly incongruent observations. For example, intron-retention in JARID2 and KDM6B may influence which genes these factors target for transcriptional regulation or alter how these factors interact with other chromatin modifier complexes, though these ideas have yet to be tested experimentally (Georges & Holleley, 2018; Marasca, Bodega, & Orlando, 2018; Wong, Au, Ritchie, & Rasko, 2016). Our finding that intron-retention and transcript abundance respond to fluctuating temperatures on different timescales may provide additional insight into the consequence of this temperature-sensitive alternative splicing in ecologically relevant contexts. It is possible that rapid intron-retention in JARID2 stabilizes the female-promoting signal during transient decreases in nest temperature during alligator TSD by promoting the storage of intron-retaining transcripts in the nucleus which are later spliced and transcribed to functional proteins after the female-promoting temperature cue. An analogous phenomenon is observed in a fern species, Marsilea vestita, and sea anemone, Nematostella vectensis (Boothby, Zipper, Van der Weele, & Wolniak, 2013; Moran et al., 2008; Wong et al., 2016) in which intron-retaining transcripts are stored in the nucleus and serve as “sentinel RNAs” that are later spliced.
facilitating rapid stage-specific protein translation. Testing whether a similar process plays a role in TSD would necessitate further experiments to determine if intron-retaining transcripts are eventually spliced and translated to functional products (e.g. experiments employing pulse-chase methods). Alternatively, intron-retention serves widespread roles in gene regulation and repression of protein translation (Braunschweig et al., 2014; Wong et al., 2016, 2013), and may serve to buffer against premature sexual fate commitment resulting from temperature-induced transcription of JARID2 and KDM6B, thereby extending the period of temperature-sensitivity during TSD. Previous work suggests intron-retention can take on diverse functions across taxa, especially depending on the genomic context of the retained introns (Wong et al., 2016), and thus further experiments to resolve the function of intron-retention in chromatin-modifier genes are likely to yield important insights into TSD.

Many genes with conserved roles in sex determination and differentiation display robust sexually dimorphic expression patterns in TSD species after the thermosensitive period (Czerwinski et al., 2016; Yatsu et al., 2016). Yet in this study, we detect highly variable expression patterns of two of these genes, SOX9 and DMRT1, in the middle of the thermosensitive period, especially in embryos exposed to a thermal fluctuation. This finding raises the question – how do sexually dimorphic transcriptional patterns arise during TSD and how does this relate to the regulatory actions of JARID2 and KDM6B over time? Ge and colleagues demonstrated that in T. scripta under constant male-promoting temperatures, KDM6B promotes commitment to the testicular fate via direct removal of the repressive H3K27me3 mark at the promoter of DMRT1 (Ge et al., 2018). However, under fluctuating conditions in alligator embryos, JARID2 and KDM6B transcript abundance fluctuates with temperature while downstream effector genes exhibit apparent delays in their responses. This raises the possibility
that under fluctuating thermal conditions, chromatin landscapes resulting from the actions of JARID2 and KDM6B may be dynamically remodeled over time to eventually repress or activate target downstream effector genes. However, it remains unknown whether incremental epigenetic changes occur during TSD under fluctuating conditions, and if so, what ultimately regulates the threshold for sexual fate commitment (Figure 6). Further, the full battery of genes targeted by JARID2 and KDM6B is yet to be resolved in most TSD species, including A. mississippiensis.

While the present study revealed intriguing patterns in the responses of upstream epigenetic regulators and downstream effector genes of sex determination to a thermal fluctuation between male- and female-promoting temperatures, there are limitations to the current experimental design that leave several important questions unanswered. In particular, while transcript abundance of JARID2 and KDM6B reached a maximum at the timestep after receiving a female-promoting temperature cue (~7-hour delay), this observation is, in part, a result of our sampling scheme and may not reflect the true timescale of this response. In order to better resolve the temporal scale on which alternative splicing, transcription, and translation respond to fluctuating thermal cues during TSD, future experiments must sample embryos across a higher resolution time-series and across multiple days. Further, the thermal regime implemented here represents a simplification of the thermal fluctuations occurring in wild nests. Nests frequently exhibit thermal fluctuations of smaller magnitudes and daily fluctuations often vary over the course of the thermosensitive period. It remains unknown how characteristics such as the magnitude and repeatability of fluctuations impact molecular responses to these thermal cues and, ultimately, how these complex thermal profiles shape sex ratio outcomes in species with TSD.
Many questions regarding the epigenetic mechanisms of TSD await further exploration and studies like the one presented here underscore the importance of implementing thermal regimes that accurately reflect the dynamic environment in which TSD evolved and examining these processes across multiple temporal scales in future experimental investigations of this phenomenon.

**Competing interests**

The authors declare no competing interests.

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**Figure Legends**

**Figure 1.** (A) Image of a representative alligator nest. (B) Design of experimental daily thermal fluctuation using the temperature deviation from the daily mean at 5-minute increments in empirically derived nest thermal profiles. Solid black line depicts the average deviation from the mean daily nest temperature across all days in the thermosensitive period for eight nests monitored in 2015. Dark grey shading depicts the middle 50th percentile of deviations from the daily mean temperature and light grey shading depicts the middle 90th percentile of deviations from the daily mean temperature. Red and blue lines depict the 95th and 5th percentile of temperature deviations respectively. Black dotted line depicts the deviation from the daily mean temperature of the experimental thermal fluctuation. Although the amplitude of the experimental trace is greater than the average thermal fluctuation observed in nature, the experimental trace is not outside of ecological relevance as natural nests are observed to fluctuate by 7°C within a single day. (C) Nest thermal profiles from nests monitored during 2015 on which the experimental thermal fluctuation was based. Horizontal dotted lines represent constant temperatures promoting female (blue, maroon) and male (red) development.
**Figure 2.** Experimental daily thermal regimes – constant male-promoting temperature (MPT), fluctuating temperature (FLUX), and constant female-promoting temperature (FPT). Vertical dotted lines depict timepoints at which stage 22 embryos were sampled.

**Figure 3.** Gene isoforms with primer locations. (A) *JARID2* gene model for *Alligator mississippiensis*. (B) *KDM6B* gene model for *A. mississippiensis*. (i) Entire transcript with locations of primers selecting for all transcripts (not discriminating based on intron-retention status). (ii) Locations of primers selecting for intron-spliced transcripts. (iii) Locations of primers selecting for intron-retaining transcripts (11th intron in *JARID2* and 19th intron in *KDM6B*). Primer information is included in associated tables on right.

**Figure 4.** Gonadal gene expression of upstream epigenetic modifiers and downstream effectors of sex determination in response to different thermal regimes. (A) *JARID2* relative expression ratio of intron-retaining transcripts to all (non-discriminating) transcripts (IR:ND) for each treatment across sampling time points. (B) *JARID2* relative expression of all transcripts. (C) *KDM6B* relative expression ratio of intron-retaining transcripts to all transcripts (IR:ND). (D) *KDM6B* relative expression of all transcripts. (E) *SOX9* relative expression. (F) *DMRT1* relative expression. All gene expression values normalized to the housekeeping gene *ACTB*. Central dots depict mean, vertical lines depict ± 1SD.

**Figure 5.** Brain gene expression of upstream epigenetic modifiers in response to different thermal regimes. (A) *JARID2* relative expression ratio of intron-retaining transcripts to all
transcripts (IR:ND) for each treatment across sampling time points. (B) *JARID2* relative expression of all transcripts. (C) *KDM6B* relative expression ratio of intron-retaining transcripts to all transcripts (IR:ND). (D) *KDM6B* relative expression of all transcripts. All gene expression values normalized to the housekeeping gene *RPL8*. Central dots depict mean, vertical lines depict ± 1SD.

**Figure 6.** Hypothesized model linking functional and temporal hierarchies of molecular responses to fluctuating thermal cues during temperature-dependent sex determination. Temperature is initially transduced into a biological signal via one or more thermo-sensory mechanisms. Possible mechanisms include transient receptor potential (TRP) channels which alter intracellular calcium levels, heat shock proteins (HSPs), cold-inducible binding protein (CIRBP), RNA thermosensors, or CDC-like kinases (CLK4) which phosphorylate serine arginine (SR)-rich proteins with roles in alternative splicing. Upstream epigenetic regulators, *JARID2* and *KDM6B*, are proximate targets of this cellular thermo-sensory mechanism and their transcription and post-transcriptional regulation respond rapidly to thermal fluctuations as a result (1). In particular, transcript abundance of *JARID2* and *KDM6B* increases in the bipotential gonad following exposure to transient female-promoting thermal signals. Intron-retention in *JARID2* also responds rapidly to temperature potentially leading to the generation of a truncated protein with altered function or sequestration of ‘sentinel RNAs’ in the nucleus that are later spliced and translated. Once translated, JARID2 and KDM6B influence H3K27me3 marks at the promoters of genes associated with the male and female pathway resulting in incremental changes to the chromatin landscape (2) in response to temperature. Over time, the repressive H3K27me3 mark accumulates at the promoters of genes involved in the male-pathway via the
actions of JARID2 in complex with PRC2, while genes involved in the female-pathway progressively lose H3K27me3 at their promoters via actions of KDM6B. As a result, expression of genes involved in the female-pathway increases gradually (3) until reaching a threshold (*) for sexual fate commitment. (References: 1Lin et al. 2018; 2Yatsu et al. 2015; 3Bentley et al. 2017; 4Kohno et al. 2010; 5Rhen & Schroeder 2010; 6Schroeder et al. 2016; 7Chowdhury et al. 2006; 8Johansson et al. 2002; 9Haltenhof et al. 2020).

**Supplemental Information**

**SI Figure 1.** Daily thermal fluctuations across all days in the thermosensitive period for eight nests monitored in 2015. Solid black line depicts mean daily thermal fluctuation. Grey shaded area depicts middle 50th percentile of daily thermal fluctuations. Blue and maroon dotted lines depict constant temperatures promoting fully female development and red dotted line depicts constant temperature promoting fully male development.

**SI Figure 2.** Absolute expression of housekeeping genes in the gonad and brain. (A) There was no significant effect of temperature, sampling timepoint, or their interaction on the absolute expression of \( ACTB \) in the gonad. (B) There was no significant effect of temperature, sampling timepoint, or their interaction on the absolute expression of \( RPL8 \) in the brain.

**SI Figure 3.** Scatterplot matrix depicting pair-wise correlations between all genes and isoforms in the gonad. Panels above the diagonal depict scatterplot of relative expression values. Points are colored by temperature treatment (blue=FPT, black=FLUX, red=MPT). Panels below the diagonal depict pair-wise correlation coefficients and font size is proportional to the magnitude.
of the correlation.
Figure 1. (A) Image of a representative alligator nest. (B) Design of experimental daily thermal fluctuation using the temperature deviation from the daily mean at 5-minute increments in empirically derived nest thermal profiles. Solid black line depicts the average deviation from the mean daily nest temperature across all days in the thermosensitive period for eight nests monitored in 2015. Dark grey shading depicts the middle 50th percentile of deviations from the daily mean temperature and light grey shading depicts the middle 90th percentile of deviations from the daily mean temperature. Red and blue lines depict the 95th and 5th percentile of temperature deviations respectively. Black dotted line depicts the deviation from the daily mean temperature of the experimental thermal fluctuation. Although the amplitude of the experimental trace is greater than the average thermal fluctuation observed in nature, the experimental trace is not outside of ecological relevance as natural nests are observed to fluctuate by 7°C within a single day. (C) Nest thermal profiles from nests monitored during 2015 on which the experimental thermal fluctuation was based. Horizontal dotted lines represent constant temperatures promoting female (blue, maroon) and male (red) development.
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(References: 1Lin et al. 2018; 2Yatsu et al. 2015; 3Bentley et al. 2017; 4Kohno et al. 2010; 5Rhen & Schroeder 2010; 6Schroeder et al. 2016; 7Chowdhury et al. 2006; 8Johansson et al. 2002; 9Haltenhof et al. 2020).
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