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COMMENTARY

Understanding how variable thermal environments affect the molecular mechanisms underlying temperature-sensitive phenotypes: lessons from sex determination

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ABSTRACT

The thermal environment that organisms experience can affect many aspects of their phenotype. As global temperatures become more unpredictable, it is imperative that we understand the molecular mechanisms by which organisms respond to variable, and often transient, thermal environments. Beyond deciphering the mechanisms through which organisms respond to temperature, we must also appreciate the underlying variation in temperature-dependent processes, as this variation is essential for understanding the potential to adapt to changing climates. In this Commentary, we use temperature-dependent sex determination as an example to explore the mechanistic processes underlying the development of temperature-sensitive phenotypes. We synthesize the current literature on how variable thermal conditions affect these processes and address factors that may limit or allow organisms to respond to variable environments. From these examples, we posit a framework for how the field might move forward in a more systematic way to address three key questions: (1) which genes directly respond to temperature-sensitive changes in protein function and which genes are downstream, indirect responders?; (2) how long does it take different proteins and genes to respond to temperature?; and (3) are the experimental temperature manipulations relevant to the climate the organism experiences or to predicted climate change scenarios? This approach combines mechanistic questions (questions 1 and 2) with ecologically relevant conditions (question 3), allowing us to explore how organisms respond to transient thermal environments and, thus, cope with climate change.

KEY WORDS: Climate Change, Development, Gene Expression

Introduction

The long-standing interest of biologists in how organisms respond to environmental temperatures goes back to Aristotle (Peck, 1942) and has taken on increased urgency as scientists try to predict the consequences of a rapidly changing climate (Radchuk et al., 2019; Schleuning et al., 2020). Fortunately, there have been many technological advances to study organismal responses to temperature (e.g. transcriptomics), and we can take advantage of this new information to better understand how organisms might adapt to a changing climate. Although we are making important progress on this front, the plethora of information seems to be creating as many (if not more) questions than it is answering. For example, it is quite common for studies that house animals at different temperatures to identify hundreds to thousands of genes that respond to temperature manipulation (Stager et al., 2015; Czerwinski et al., 2016; Yatsu et al., 2016; Nitzan et al., 2019), which is a powerful indication of the importance of temperature effects on molecular processes across a range of organisms. Some questions that arise from these studies are: (1) which genes directly respond to temperature-sensitive changes in protein function and which genes are downstream and respond indirectly; (2) how long does it take different proteins and genes to respond to temperature?; and (3) are the experimental temperature manipulations relevant to the climate the organism experiences or to predicted climate change scenarios? Questions such as these are often complex and difficult to answer. The goal of this Commentary is to provide a framework for approaching these complex questions, with the hope that using a more systematic approach will help us make headway towards understanding current and future responses to temperature. Here, we use the process of sex determination as an example of how individual transcription factors can influence the expression of a large number of genes and use temperature-dependent sex determination (TSD) to explore how the expression of transcription factors can be regulated by temperature-dependent changes in protein function (Czerwinski et al., 2016; Radhakrishnan et al., 2018; Whiteley et al., 2021). These examples help illustrate how a single transcription factor might be regulated by temperature-dependent changes in protein function to subsequently modulate the expression of other genes to produce a physiological response to temperature. This research also provides a possible explanation for how so many genes exhibit altered expression in response to changing temperatures, without the need for those genes to be directly responsive to temperature. We also discuss temperature effects on gene expression within the context of more natural conditions where temperatures fluctuate on a regular basis, resulting in transient temperature exposure. Understanding how, or whether, organisms respond to transient temperature exposures, especially unseasonably warm or cool conditions, is critical to predicting the effects of changing climates.

Vertebrate sex determination: how one transcription factor affects gene expression on multiple levels of a temperature response cascade

When it comes to phenotypic variation, perhaps no single factor is studied more frequently than sex. Males and females often exhibit pronounced anatomical, physiological and behavioral differences. Although much of this phenotypic variation is attributed to the consequences of endocrine differences that arise from the presence of sexually dimorphic gonads, the development of gonadal tissue into either ovaries or testes is often determined by a single transcription factor (Fig. 1). In mammals, the sex-determining region of the Y chromosome gene (*Sry*) is responsible for inducing

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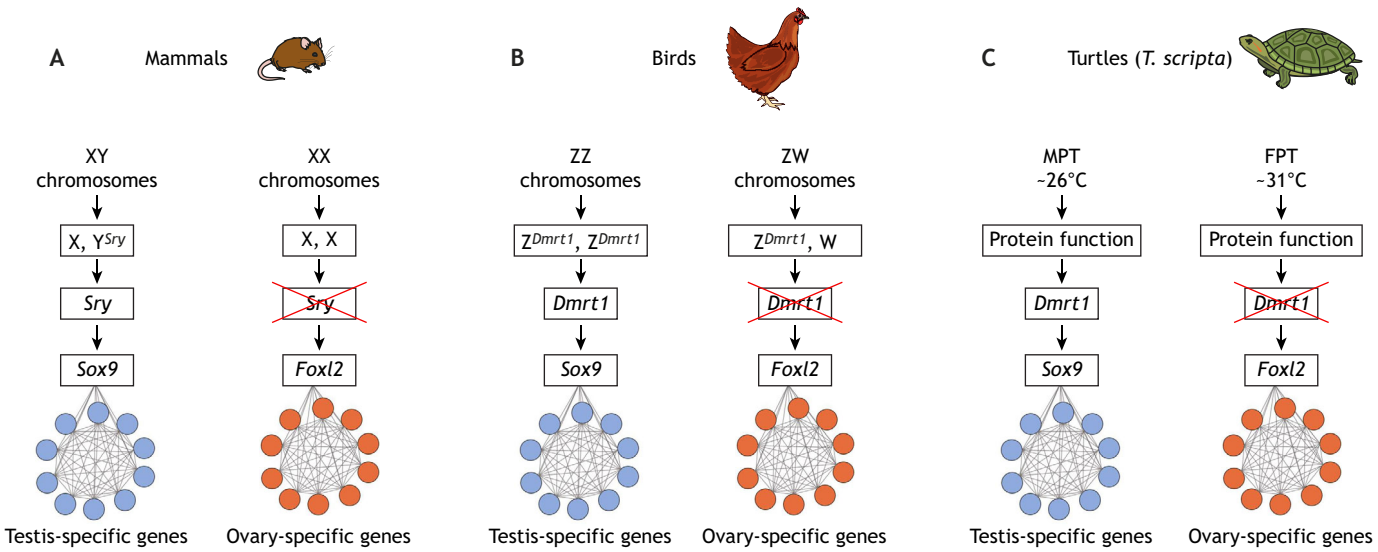

Fig. 1. Transcription factor regulation of genes involved in gonadal differentiation. (A) For mammals, inherited variation in *Sry* (presence or absence) determines gonadal fate. (B) For birds, inherited variation in *Dmrt1* (one copy or two) determines gonadal fate. (C) For turtles such as *Trachemys scripta*, temperature-sensitive regulation of *Dmrt1* (cool temperatures or warm temperatures) determines gonadal fate.

testis development (Gubbay et al., 1990; Koopman et al., 1991; Kurtz et al., 2021), whereas in birds, this function is carried out by Doublesex and Mab-3-related transcription factor 1 (*Dmrt1*) (Smith et al., 2009; Ioannidis et al., 2021). Although the role these genes play in regulating gonadal differentiation is critical, it is worth noting that these transcription factors also regulate gene expression independently of gonadal cues (Arnold, 2012). In mammals, approximately one-third of the genome exhibits sex-specific expression at the blastula stage (Bermejo-Alvarez et al., 2010), which is long before gonads develop. In birds, adult secondary sex characteristics arise independently of gonadal sex but are determined by the autonomous regulation of *Dmrt1* within cells (Ioannidis et al., 2021). *Sry* and *Dmrt1* provide good examples of how differential regulation of individual transcription factors can initiate a cascade of events that leads to the induction of a highly conserved group of genes that result in testis or ovary development (Capel, 2017; Fig. 1).

Temperature-dependent regulation of a transcription factor in TSD: a case study

This Commentary builds upon recent advances in TSD research identifying temperature-sensitive processes that regulate gene expression. Although we have known for decades that some genes are differentially expressed at temperatures that induce ovary development versus testis development in species with TSD, many of those genes are also known to be sexually dimorphic in species with genotypic sex determination (in which the chromosomal make up determines sex), suggesting that the pathways involved in ovary and testis differentiation are relatively well conserved (Capel, 2017). Recently, we have started to characterize the temperature-sensitive processes that regulate the expression of sex-determining genes in species with TSD. Processes such as calcium influx (Castelli et al., 2020; Weber et al., 2020), protein phosphorylation (Haltenhof et al., 2020; Weber et al., 2020), RNA splicing (Haltenhof et al., 2020; Bock et al., 2020a,b; Marroquín-Flores et al., 2021) and histone demethylation (Ge et al., 2018) interact to regulate the expression of transcription factors that determine sex (e.g. *Dmrt1*). These processes have been investigated in a variety of species (Yatsu et al., 2015; Radhakrishnan et al., 2018; Bock et al., 2020a,b; Castelli et al., 2020), of which the red-eared slider turtle (*Trachemys scripta*) is one of the most commonly studied and provides a relatively detailed picture of how sex determination is affected by temperature.

In many species of turtles with TSD, including *T. scripta*, eggs incubating at a constant 31°C develop as females, whereas eggs incubating at 26°C develop as males. The pivotal temperature (29°C) represents a threshold where higher temperatures promote female development and lower ones promote male development (Crews et al., 1994; Wibbels and Crews, 1995). By comparing gene expression patterns in developing gonads under the sex-specific constant temperature conditions of 31 and 26°C, researchers were able to identify genes critical to ovary and testis development, but these constant conditions are not reflective of natural nests where temperatures routinely fluctuate between those that induce ovary formation and those that induce testis formation (Les et al., 2007; Paitz et al., 2010; Carter et al., 2017; Valenzuela et al., 2019). Relatively little work has been done on how gene expression is regulated under fluctuating conditions (but see Breitenbach et al., 2020), but this line of inquiry is essential for identifying temperature-sensitive processes regulating gene expression. Such work would provide major advances in our efforts to understand this mode of sex determination by helping to explain how embryos develop ovaries or testes while experiencing cues for both.

As mentioned above, the mechanisms through which incubation temperature regulates the expression of genes involved in gonadal differentiation are complex and involve numerous processes. We also know that these processes take time to occur, and this is evidenced by studies exposing *T. scripta* eggs to varying numbers of days where temperatures reach female-producing conditions against a baseline of male-producing conditions (Carter et al., 2018; Breitenbach et al., 2020). For some embryos, being exposed to just 5 days where temperatures reach female-producing conditions (moved to 29.5±3°C from 27±3°C) is sufficient to induce ovary development, whereas others develop testes even after being exposed to 20 days at 29.5±3°C (Carter et al., 2018). These data indicate that individual embryos can vary in the time it takes for warm temperatures to induce ovary production or in their thermal responsiveness (Bowden and Paitz, 2021). Even when embryos

experience a sufficient number of warm days to induce ovary development, some genes involved in gonadal differentiation may not exhibit induction until well after the exposure to warm temperature has subsided (Breitenbach et al., 2020). The delayed response likely results from the number of steps in the cascade of events between the initial temperature-sensitive element and the expression of the sex-determining gene (Fig. 2). This might be especially true for genes that require several sequentially expressed transcription factors to be induced prior to their own induction.

The regulation of gene expression by temperature appears to start with an increase in calcium influx into gonadal cells as temperatures move from cooler to warmer conditions (26°C to 31°C; Weber et al., 2020). Transient receptor potential (TRP) channels are thought to be the 'thermo-sensors' that mediate the temperature-sensitive influx of extracellular calcium into cells by opening at warm temperatures (Held et al., 2015; Yatsu et al., 2015). Warmer temperatures lead to increased intracellular calcium in gonadal cells, which promotes phosphorylation of signal transducer and activator of transcription 3 (STAT3), which in turn represses the expression of a histone demethylase (*Kdm6b*; Weber et al., 2020). Warmer temperatures also lead to the dephosphorylation of CDC-like kinases (CLKs), rendering them inactive and unable to phosphorylate serine–arginine-rich proteins (SR proteins) that regulate mRNA splicing (Haltenhof et al., 2020). Although more work is needed to characterize how temperature regulates processes such as calcium influx and protein phosphorylation in developing gonads, these initial findings demonstrate how changing temperatures elicit rapid changes in protein function that are independent of any changes in gene expression.

The phosphorylation status of proteins such as STAT3 and SR proteins dictates their effect on gene expression. Phosphorylated STAT3 (warm temperatures) directly binds the *Kdm6b* locus to repress its expression (Weber et al., 2020). Conversely, phosphorylated SR proteins (cool temperatures) increase intron retention, which alters gene expression in a temperature-dependent manner (see below) (Haltenhof et al., 2020). Characterizing the downstream genes directly affected by proteins such as STAT3 and SR proteins has important implications for understanding how temperature regulates gene expression. Two genes that have received attention in this regard are *Kdm6b* and *Jumonji And AT-Rich Interaction Domain Containing 2* (*Jarid2*). These genes respond to temperature early in development (Czerwinski et al., 2016), exhibit temperature-sensitive intron retention (Deveson et al., 2017) and are involved in epigenetic regulation of gene expression; specifically, histone demethylation. *Jarid2* is a cofactor of the Polycomb Repressive Complex 2 that methylates histones to silence expression (Kuzmichev et al., 2002; Kasinath et al., 2021), whereas *Kdm6b* is a histone demethylase that promotes transcription (Ge et al., 2018). For *Jarid2* and *Kdm6b*, intron retention mediated by differentially phosphorylated SR proteins (Haltenhof et al., 2020) could be a critical aspect of how their expression responds to temperature cues (Marroquín-Flores et al., 2021). This creates a situation where a limited number of genes involved in epigenetic regulation may be directly responding to biochemical signals of temperature exposure, such as protein phosphorylation, to subsequently regulate the expression of numerous genes.

The specific consequences of intron retention are context dependent and in the case of TSD, are only starting to be explored. Intron retention can lead to premature stop codons and loss of protein function (Deveson et al., 2017) or it can facilitate transcript accumulation and increased expression (Rose, 2019). For *Kdm6b*, intron retention appears to be associated with increased transcription and function in *T. scripta* (Ge et al., 2018). At cool temperatures, levels of *Kdm6b* transcripts retaining an intron

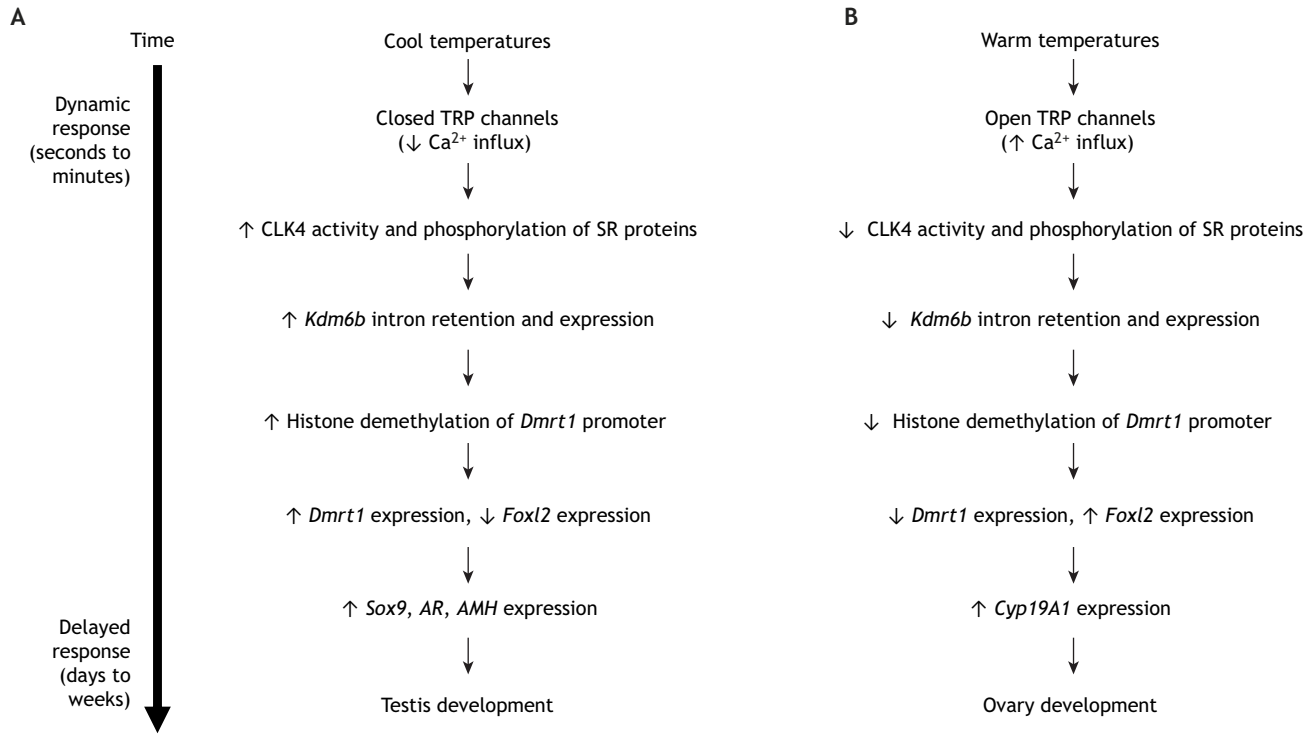

Fig. 2. Sequence of events in temperature-dependent sex determination (TSD) and the time span over which they occur. (A) Cascade of events indicating how cool temperatures result in testis development. (B) Cascade of events indicating how warm temperatures result in ovary development. Variation in most of these processes could affect the time it takes for an individual to respond to a thermal cue.

accumulate in the developing gonad, but short exposures to heat (2 warm days) result in a rapid decrease in levels of intron-retaining transcripts (Marroquín-Flores et al., 2021). *Kdm6b* is necessary at cool temperatures to induce testis development, as knocking down *Kdm6b* expression results in ovary formation (Ge et al., 2018). This is thought to occur because *Kdm6b* demethylates the promoter of *Dmrt1* (Ge et al., 2018), which is the critical regulator of testis development. Direct knockdown of *Dmrt1* expression also results in ovary formation (Ge et al., 2017). By demethylating histones at the promoter of *Dmrt1*, *Kdm6b* is considered the critical mediator that converts biochemical temperature cues (e.g. protein phosphorylation and alternative splicing) into gene expression changes via epigenetic processes such as histone demethylation (Fig. 1). In simple terms, temperature-sensitive epigenetic regulation of *Dmrt1* may serve as a molecular switch that dictates gonadal fate (Fig. 2). Although processes such as protein phosphorylation and *Kdm6b* expression may fluctuate with changing temperatures, the eventual induction of *Dmrt1* resulting from sufficient demethylation of the promoter could be indicative of a commitment to testis formation (Bock et al., 2020a,b).

Thermal and temporal thresholds and their potential role in adapting to climate change

When thinking about how organisms respond to temperature, many phenological traits, such as insect emergence, plant flowering and bird migration could involve thermal and temporal thresholds. These thresholds are likely relevant to most, if not all, dichotomous traits that respond to temperature. TSD provides a good example of how both thermal and temporal thresholds are involved in the temperature-dependent regulation of gene expression. For TSD, the pivotal temperature represents a thermal threshold, which, in turtles, is remarkably conserved across species that inhabit a wide range of climatic conditions (Bull et al., 1982; Carter et al., 2019a,b). From sea turtles nesting near the equator (Mrosovsky, 1994) to freshwater turtles nesting in Canada (Ewert and Nelson, 1991), the pivotal temperature is approximately 29°C, which suggests there may be a limited capacity for this threshold to facilitate an adaptive response to different climates. It is worth noting that some studies have found limited latitudinal variation in pivotal temperatures within a species (0.3–0.4°C lower in northern populations) (Bull et al., 1982), while other studies have found pivotal temperatures do not vary between populations inhabiting different climates (Carter et al., 2019a,b). From a mechanistic perspective, this threshold could be defined by something like the temperature at which proteins such as TRP channels undergo conformational changes, thus constraining evolution of the threshold.

Temporal thresholds have received much less attention, but they may prove to be important for allowing organisms with TSD to respond to thermal variability (Bowden and Paitz, 2021). Variation in how long embryos must be exposed to a temperature cue before committing to a gonadal fate (i.e. thermal responsiveness) could underly adaptive responses to different climatic conditions. There has been some effort to quantify the cumulative temperature exposure necessary before embryos commit to a gonadal fate (Valenzuela, 2001; Bock et al., 2020a,b), but this work has not been done in the context of individual or population variation (Bowden and Paitz, 2021). Thermal responsiveness is a trait that should receive more attention as laboratory studies move to incorporate more ecologically relevant temperature conditions, such as exposing organisms to transient thermal cues. The small amount of work that has examined variation in the time it takes turtle embryos to respond to thermal cues has yielded some valuable findings. First, in some embryos, ovary development can be induced after experiencing just 5 days where temperatures temporarily surpass the pivotal temperature (29.5±3°C), whereas other embryos develop testes even after experiencing 15 days under these conditions (Carter et al., 2018), suggesting that there is ample variation in this trait upon which natural selection can act. Second, the accumulation of the molecular signal to induce ovary formation is not simply additive (Breitenbach et al., 2020). For example, experiencing a 12 day heatwave early in development results in female-biased sex ratios, but experiencing two 6 day heatwaves separated by a gap of cool temperatures (12 total heat wave days in either case) results in male-biased sex ratios (Breitenbach et al., 2020). These results suggest that molecular signals that accumulate when temperatures increase can be lost when temperatures cool. Some processes, such as histone demethylation, can be reversed but this involves a different set of proteins (histone methylases rather than demethylases) (Michalak et al., 2019). A process like RNA splicing cannot be reversed. Thus, the rate at which molecular signals accumulate can be different from the rate at which molecular signals are lost. Variation in the rates of signal accumulation and loss may be critical to the potential to adapt to climatic variation. In this context, a slower accumulation of molecular signals in response to warm temperatures and/or a faster loss of molecular signals could result in a decreased sensitivity to warmer climates. Unfortunately, there is a paucity of information available on this topic.

Many organisms use seasonal changes in temperature as a cue to physiologically prepare for major events such as reproduction, emergence or migration. For some of these changes, organisms must experience specific temperatures (thermal thresholds) and do so for a specific duration of time (temporal thresholds). Experiencing a temperature for a few hours is unlikely to induce changes in most of these traits, so the question becomes: for how long must exposure occur before changes are induced? This question takes on increased urgency as the frequency of exposure to unseasonably warm or cool events is predicted to increase with climate change (Rahmstorf and Coumou, 2011; Stillman, 2019). Will these transient exposures to unseasonably warm or cool temperatures induce temperature-sensitive changes in physiology? Whereas many of the rapid responses to temperature may be conserved across vertebrates, downstream events are likely to vary among species or even individuals. Understanding the cascade of events involved in transducing temperature cues into physiological responses is critical to deciphering how biological processes may respond to changing temperatures.

A new framework for assessing responses to temperature

Here, we explore three areas in critical need of further investigation: (1) quantification of relevant processes, (2) appropriate timing of quantification and (3) use of relevant temperatures. By incorporating these ideas into experiments, research can move beyond simply describing how organisms respond to temperature to characterizing important variation in how organisms respond to temperature. It is this variation that is likely to serve as the substrate upon which the selective pressures of a changing climate will act. We need to move beyond thinking that the genes that are differentially expressed under different temperatures are in fact the same genes that are likely to facilitate adaptations to novel climatic conditions.

What processes should be quantified (direct versus indirect responses)?

Most temperature-induced changes in physiology and phenotype are going to involve a suite of processes, only some of which

directly respond to temperature cues, whereas others respond indirectly. To fully characterize a process, researchers will likely need to study protein function and gene expression. However, many questions will not require a complete characterization of the process. Which portions of the process to focus on will depend on the research question. When attempting to explain the mechanism underlying temperature-induced phenotypes, it is important to keep in mind that changes in gene expression are likely indirect responses to temperature, mediated by direct temperature effects on protein function (Fig. 3). In species with TSD, temperature-dependent regulation of TRP channels and subsequent calcium-regulated changes in the phosphorylation of several proteins (STAT3 and SR proteins) appear to regulate gene expression. Using a non-TSD example, temperature-induced assembly of heat shock factor 1 from a monomer to a homotrimer activates the expression of heat shock protein genes (Ahn and Thiele, 2003). These are just a two examples of how temperature-induced changes in protein function regulate the expression of temperature-induced genes (Fig. 3). Importantly, these proteins can be vital regulators of the response to temperature without their respective expression levels being influenced by temperature.

How long do different processes take to respond to temperature?

One goal of this Commentary is to discuss the effects of temperature on gene expression as it pertains to potential climate change effects. With that in mind, it is important to consider how organisms respond to a transient exposure to unexpected temperatures, such as heat waves or cold snaps. As these transient exposures could occur at any time of the year, the temperatures which the organisms are exposed to will not necessarily be the annual maximum or minimum temperatures. When these transient exposures to unexpected temperatures do occur, it is important to understand whether the exposure is sufficient to induce phenotypic change and determine why some organisms may respond, whereas others do not. This variation in responsiveness may be critical to adapting to changing climates (Bowden and Paitz, 2021).

One way to investigate variation in how responsive different individuals are to temperature is to expose them to temperatures for varying lengths of time. Using shorter, more ecologically relevant temperature exposures creates a question as to when one should quantify the response. Findings from TSD demonstrate that an 8 day exposure to warm temperatures induces ovary development in about 50% of exposed embryos (Carter et al., 2017), but many of the temperature-induced genes do not exhibit differential expression until after the exposure has ended (Breitenbach et al., 2020). This highlights that temperature-induced changes in gene expression may not occur until after a transient exposure has subsided and this has logistical implications for studying temperature effects. Although some processes will not exhibit a response during the exposure, the exposure likely initiates a cascade of events that will result in a response that occurs hours to days post-exposure.

Are relevant temperatures being used?

Defining what can be considered a relevant temperature is almost always going to be context dependent. For most organisms used in scientific research, individuals are likely to experience diurnal fluctuations under ambient temperatures. This is true of reptile embryos developing in natural nests, and there have been numerous calls to incorporate more naturalistic fluctuating temperatures into

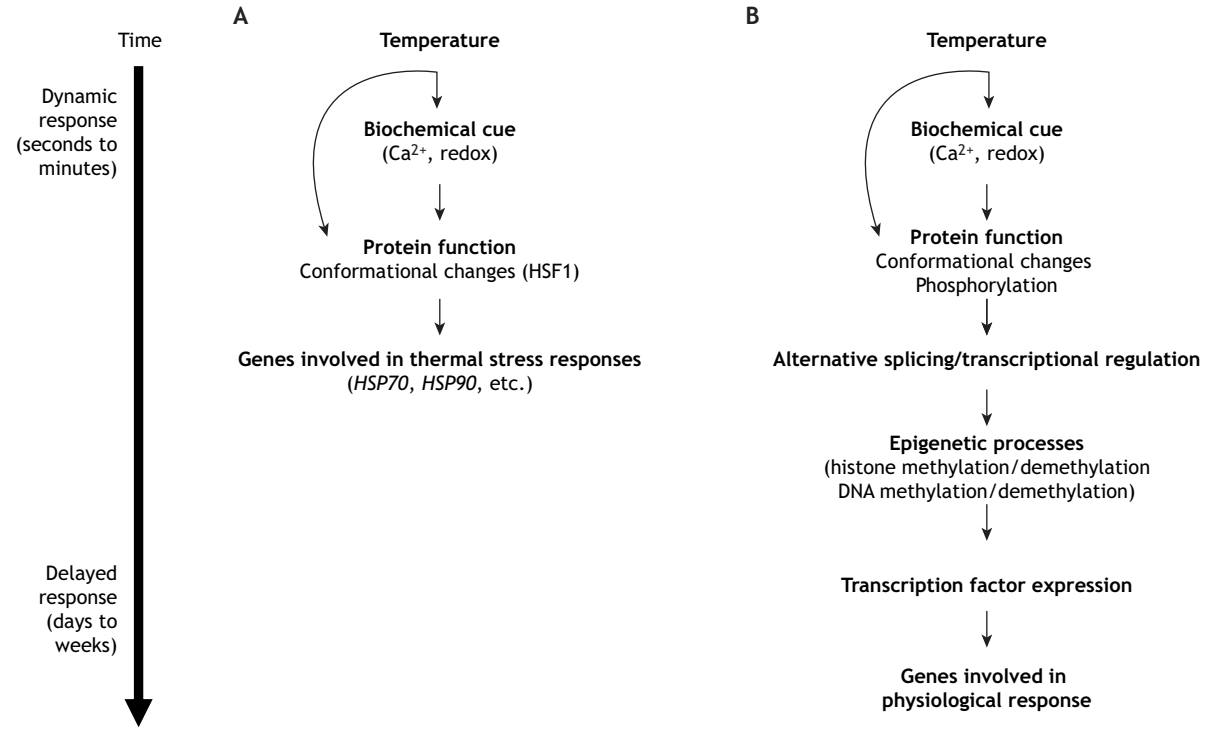

Fig. 3. Examples of how temperature-sensitive pathways can vary in the length of time it takes for a response to occur. (A) Thermal stress response. HSF1 directly responds to temperature by trimerization, thereby altering the protein's function as a result of conformational change. This altered protein function induces expression of downstream genes involved in thermal stress responses (such as *HSP70*, *HSP90*, etc.). (B) TSD. Protein function can directly respond to temperature or indirectly respond to temperature via processes such as phosphorylation. This altered protein function can directly regulate the transcription of genes via processes such as alternative splicing or indirectly regulate processes such as histone demethylation. Increased expression of critical transcription factors can regulate levels of multiple downstream genes and this cascade of events can continue to create a delayed response to temperature cues.

laboratory studies of TSD (Georges et al., 1994; Bowden et al., 2014; Bowden and Paitz, 2018; Carter et al., 2019a,b; Valenzuela et al., 2019). Consequently, more studies are now using fluctuating temperatures in the laboratory and thus have been instrumental in advancing our understanding of how TSD occurs under natural conditions (Valenzuela et al., 2019; Bock et al., 2020a,b). We argue that laboratory studies of temperature-induced phenotypes generally need to incorporate more transient temperature exposures that vary in severity and duration to characterize the variation between individuals in the temporal aspect of the response to temperature exposure. Understanding this variation will contribute to our understanding of the potential consequences of climate change. As a result, research can begin to characterize how organisms respond to unanticipated exposure to unseasonal temperatures. Although there is value in understanding how organisms exposed to temperatures 1°C warmer or cooler than their counterparts may differ in phenotype (Crews et al., 1994; Carter et al., 2017), this type of research misses the day-to-day variation in temperature that organisms are likely already experiencing as a result of climate change. A focus on transient exposure to temperatures enables us to better characterize the rapid biochemical and downstream physiological processes that take place in response to these more natural conditions. Similar to the designation of 'relevant', the definition of 'transient' is also going to be context dependent and will likely need to be empirically characterized for many traits. For example, exposing *T. scripta* embryos to 3 days of warm temperatures (29.5±3°C), from a baseline of 27.0±3°C, failed to induce the production of any female hatchlings. However, extending that exposure to 8 days resulted in 50% of the embryos developing as female hatchlings (Carter et al., 2018). For this trait, transient exposure of around a week was necessary to induce phenotypic change. As with all foundational research, this process will need to be systematic and iterative. Laboratory studies that systematically characterize thermal responsiveness to transient temperature exposure are needed. Studies that attempt to mimic the full stochasticity seen in nature are unlikely to resolve the specific temperatures and duration of exposure needed to induce phenotypic change, as mimicking the stochasticity of natural temperatures produces thermal profiles too complex to allow for specific conclusions concerning specific temperatures and exposures. Ultimately, more work on short-term/transient exposure to temperatures is needed to understand and predict the consequences of climate change.

Going forward

There are obviously many ways one can study the effects of temperature on phenotypic variation and most of these studies can advance our understanding of this topic. We argue that with regards to predicting the effects of climate change, we need to increase our understanding of how transient exposures to temperature induce phenotypic change. Transient exposures may (or may not) initiate a cascade of events that may manifest as temperature-induced changes in phenotype. For most of these changes, the cascade of events is going to involve temperature-induced changes in protein function that will result in changes in gene expression. The specific aspects of the cascade one chooses to study will dictate when a response may be observable. Changes in protein function are likely to be more dynamic and could occur almost immediately upon a change in temperature, whereas changes in gene expression may not occur until after the transient exposure to temperature has subsided. It is plausible that hundreds of proteins may exhibit changes in function in response to changes in temperature, and this could result in a similar number of genes exhibiting changes in expression as temperature changes. However, the link between changes in protein function and gene expression may be mediated by just a few transcription factors that are regulated by changing protein function and subsequently regulate downstream genes. Linking changes in protein function to the regulation of transcription can help explain how temperature induces phenotypic change.

When moving beyond studies aimed at deciphering how temperature induces phenotypic change, we can begin to investigate individual variation in how organisms respond to temperature. In this context, it will be important to examine why some individuals may be more responsive to temperature than others, as this variation could be critical to adapting to changing climates. For example, tree swallows have been shown to initiate egg laying earlier in years with earlier warmth during the spring (Shipley et al., 2020). Unfortunately, this early warmth is often temporary and the subsequent return to more seasonal, cool temperatures often results in higher rates of chick mortality. Thus, the females that laid eggs in response to this early warmth had decreased reproductive success. Investigations into the mechanisms underlying this temperature-dependent egg laying could use a variety of approaches. We would argue the most common approach would be to house different groups of birds under different temperatures to characterize variation in the temperatures at which females lay eggs (i.e. thermal threshold). In this scenario, it could be suggested that selection under future environmental conditions might favor females that require higher temperatures to initiate laying. However, a different experimental approach would be to house different groups of birds under elevated temperatures for different durations to characterize variation in the duration of exposure necessary to initiate laying (i.e. temporal threshold). In this scenario, it could be suggested that selection under future environmental conditions might favor females that require longer exposure to initiate laying. Importantly, the mechanisms (and adaptive potential) underlying thermal and temporal thresholds can be very different. To relate this back to the TSD example, the pivotal temperature (thermal threshold) is very similar in turtle species inhabiting a wide range of climates (Mrosovsky, 1994; Carter et al., 2019a,b), but the temporal threshold appears to be more variable in that some individuals initiate ovary development after only 5 days of heat exposure while others maintain testis development even after 20 days of heat exposure (Carter et al., 2018). By understanding the sequence of events that occur from the onset of the response (thermal threshold is surpassed) until the time a response occurs (an egg is laid), we can better understand how organisms might adapt to a changing climate that involves more transient exposure to unseasonal temperatures.

Given that the response to temperature involves a cascade of events that includes changes in protein function and gene expression, there are a variety of ways in which the response to temperature could change, adapt or evolve. Genetic variation that alters protein function and how a protein is affected by temperature would alter how temperature influences phenotype. The same can be said for genetic variation that influences how gene expression is indirectly regulated by temperature-induced changes in protein function. These are just a few possible examples that demonstrate how important genetic variation in genes whose expression is not temperature sensitive could be to adapting to changes in climate. It is important to recognize that genes do not have to be differentially expressed at different temperatures to mediate the response to temperature. Genes for proteins that exhibit a change in function in response to temperature are also a critical part of this process,

especially if there is genetic variation that affects how the protein responds (or does not respond) to temperature. Overall, incorporating transient exposures to temperature into laboratory-based studies and considering the complete cascade of events that occurs, from altered protein function to how that regulates gene expression, is likely necessary to improve our ability to predict how climate change may affect different organisms and how organisms may be able to adapt to climate change.

Competing interests
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