

REVIEW

Thermal variability during ectotherm egg incubation: A synthesis and framework

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Abstract

Natural populations of ectothermic oviparous vertebrates typically experience thermal variability in their incubation environment. Yet an overwhelming number of laboratory studies incubate animals under constant thermal conditions that cannot capture natural thermal variability. Here, we systematically searched for studies that incubated eggs of ectothermic vertebrates, including both fishes and herpetofauna, under thermally variable regimes. We ultimately developed a compendium of 66 studies that used thermally variable conditions for egg incubation. In this review, we qualitatively discuss key findings from literature in the compendium, including the phenotypic effects resulting from different patterns of thermally variable incubation, as well as the ontogenetic persistence of these effects. We also describe a physiological framework for contextualizing some of these effects, based on thermal performance theory. Lastly, we highlight key gaps in our understanding of thermally variable incubation and offer suggestions for future studies.

KEYWORDS

developmental plasticity, diel fluctuations, seasonal variability, temperature fluctuations, thermal variability

1 | INTRODUCTION

Variability in temperature is a key feature of natural environments. For ectotherms, this variability typically results in a wide range of experienced body temperatures due to diurnal, seasonal, and stochastic thermal fluctuations. Ectotherms have consequently adapted to perform biological functions over ranges of inconstant temperatures, with the nature of performance changing between populations, and among and within life stages (Bowler & Terblanche, 2008; Du, Warner, Langkilde, Robbins, & Shine, 2010; Sunday, Bates, & Dulvy, 2011).

The embryo life stage is particularly sensitive to temperature, owing to the relatively small body size of embryos (Gillooly, Brown, West, Savage, & Charnov, 2001). Differences in thermal incubation regimes influence myriad biological functions both during development and cascading into later ontogeny. Long-term effects of the incubation environment include changes to locomotor performance (reviewed in Booth, 2006), growth (Booth, Burgess, McCosker, &

Lanyon, 2004), survival (Parker & Andrews, 2007; Warner & Shine, 2008), behavior (reviewed in Deeming, 2004), reproductive traits (Jonsson, Jonsson, & Finstad, 2014), and, for organisms with temperature-dependent sex determination, the outcome of sex (Bull & Vogt, 1979; Valenzuela & Lance, 2004).

Given the importance of the embryonic thermal environment to producing variation that impacts ecological and evolutionary dynamics in ectotherms (Moczek et al., 2011; Sultan, 2007), it is unsurprising that considerable attention has been devoted to thermal incubation experiments. Widespread discoveries of temperature-dependent sex determination (TSD) in reptiles in the 1970s began a snowballing fascination with the vertebrate embryonic thermal environment (Bull, 2004), ultimately encouraging further research on other elements of embryonic development and physiology. A recent review by Warner, Du, and Georges (2018) found that nearly 75% of 803 studies on reptile incubation focused on the effects of temperature, and the number of publications on the effects of embryonic thermal environment has been steadily rising since 1969.

Yet, under the basic assumption that the eggs of ectothermic vertebrates typically experience natural daily and seasonal variation in temperature, the vast majority of incubation studies continue to employ constant temperature incubation conditions (Bowden, Carter, & Paitz, 2014; Noble, Stenhouse, & Schwanz, 2018). Although constant temperature incubation experiments have inarguably led to important insights regarding the influence of developmental temperatures on many phenotypic measures, it is difficult to interpret these findings with respect to thermal fluctuations. One reason for this confusion is that expected phenotypic effects, based on the means of thermal variance, often poorly align with observed effects under thermal variation (Bowden et al., 2014; Bull, 1985; Dowd, King, & Denny, 2015; Gutzke & Bull, 1986; Schulte, Healy, & Fanguie, 2011; Schwarzkopf & Brooks, 1985). As a result, our understanding of the ecological relevance of fluctuating temperatures is limited (Pearson & Warner, 2016), necessitating that we broaden our knowledge of how thermal variation affects organisms, and integrate these findings with the larger body of work on constant temperature incubation.

In the present review, we provide the first comprehensive compendium of incubation experiments that employ thermally variable regimes to incubate the eggs of all ectothermic vertebrates, including both herpetofauna and fishes. Notably, previous work on the topic has focused largely on reptiles (Noble, Stenhouse, et al., 2018), so we aimed to include additional insights from both fishes and amphibians, taxa that are frequently excluded from the broader discussion of egg incubation. Within the compendium, we also provide summaries of important findings from each study, report the temperature regimes used, and whether ecologically or physiologically relevant data informed the study. Ultimately, our goal for this compendium of studies is to act as an informative reference for authors studying thermal variability, and to facilitate taxonomic crosstalk between reptile, amphibian, and fish researchers.

We also sought to critically review several key concepts relating to the effects of thermal variation during incubation on embryos, hatchlings, and later life stages of ectothermic vertebrates, through a qualitative discussion of a selection of studies found in the compendium. First, we present a basic thermal performance framework for interpreting the phenotypic results of incubation under thermal variation, integrating what is known from constant temperature work. Next, we discuss findings from thermal variability studies not directly comparable with constant temperature work. Importantly, we also discuss the long-lasting effects of the thermal environment during incubation through developmental plasticity. We finish by discussing other biological or environmental sources of phenotypic variation, and present avenues for future research.

2 | SUMMARY OF LITERATURE COMPENDIUM

We conducted a systematic literature search, using the ISI *Web of Science* Core Collection database for vertebrate incubation studies in

which variable temperature regimes were used in the artificial incubation of eggs. We applied combinations of synonymous terms for “temperature” and “fluctuation,” and narrowed our search to the embryo life stage. The resulting advanced search query was: TS = (incubation AND [fluctuating OR variable OR seasonal] AND temperature* AND [embryo OR egg*]). The 561 results were then organized by “Relevance,” and included articles indexed until February 28, 2019. We examined abstracts for each study and followed these exclusion criteria:

- (i) We excluded studies for which the study organism was not an ectothermic vertebrate, and for which egg incubation was not artificial (i.e., in natural nests).
- (ii) We excluded studies that were unable to incubate eggs for nearly the entire incubation period, specifically those that were unable to begin artificial incubation of eggs 72 hr or more after oviposition.
- (iii) We excluded studies of viviparous squamates (e.g., those that incubated the mother rather than eggs), and squamates that undergo approximately one-half or more of development in-utero. Specifically, we imposed a cutoff of embryo oviposition at an average Dufaure and Hubert (1961) stage of 35; the majority of squamate species oviposit embryos when they have undergone one-third of development (Dufaure & Hubert, 1961) stages 25–33 (Blackburn, 1995; Shine, 1983).
- (iv) We excluded any articles that were not written in English.
- (v) We excluded studies in which the focal measurements were of sex ratios, as incubation studies focusing on temperature-dependent sex determination are already the most heavily reviewed in the egg incubation field (While et al., 2018).

If these conditions were met, or if it was ambiguous whether these conditions were met, we further analyzed the full text of the article, removing studies that did not meet all conditions.

We cross-referenced our results with literature found in a recent review, as well as results from the Reptile Development Database (Bowden et al., 2014; Noble, Stenhouse, Riley, et al., 2018), to capture additional studies that did not appear in our search; as a result, we added eight studies to our pool from the Reptile Development Database (Noble, Stenhouse, Riley, et al., 2018).

From these studies, we recorded species, the temperatures used in incubation regimes, as well as, the nature of thermal variability. We documented whether ecologically relevant nest temperatures for the population were recorded or cited and whether physiologically relevant temperatures (i.e., the thermal minimum, optimum, or maximum for development rate) were mentioned. We also reported a brief summary of the effects of thermal variability for each study.

Using the search parameters and cross-referencing materials above, we collated 66 studies (58 from the *Web of Science* search and 8 from Noble, Stenhouse, Riley, et al., 2018; the compendium can be found in Table S1). Most studies focused on squamates, followed by fishes, testudines, and anurans, with a single paper on

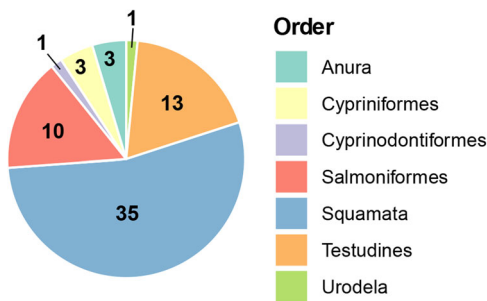


FIGURE 1 Relative (shaded region) and absolute (label) frequency taxon appearance in 66 studies incubating ectothermic vertebrate eggs under thermally variable regimes. Reptiles are best represented in these studies, followed by fishes. Amphibians are the least represented group [Color figure can be viewed at wileyonlinelibrary.com]

urodeles (Figure 1), and spanned 48 species. Several types of fluctuating regimes were employed, and we classified them into six patterns (Figure 2). The most common was diel fluctuations in temperature, characterized by sinusoidal thermal fluctuations around a stationary mean (Figure 2a). Among other thermal regimes used were seasonal shifts in temperature, characterized by a changing thermal mean throughout incubation, in the absence of diel fluctuations (Figure 2b); ambient regimes, characterized by stochastic diel changes in temperature (Figure 2c); combinations of variable regimes (e.g., Figure 2d); and heat shocks, which involved brief exposures to high temperatures (Figure 2e). We termed the last category of variability “idiosyncratic,” characterized by large changes from one constant temperature to another at different points during incubation (Figure 2f). The magnitude of thermal variability ranged from 3°C to 20°C.

We also noted whether studies investigated or cited data relevant to the thermal physiology of the study organism (specifically: upper or lower temperatures for successful development, developmental thermal performance curves, critical thermal minima, maxima, and/or optima, and temperatures that result in high or low mortality and/or embryonic growth [from previously published or pilot experiments], at the species level) and the thermal ecology of the study population (natural nest temperatures from the focal environment, at the population level). Only approximately one in three studies (23/66) reported or cited data regarding thermal physiological parameters for their study species. In contrast, most studies (43/66) reported or cited natural nest temperatures for their study population.

It is important to note that technical reports, government reports, and conference proceedings not published in books (“gray” literature) were not found in our search, and may yet represent valuable sources of information on thermal variability and egg incubation; thus, we acknowledge that our search design precluded these works from inclusion in Table S1.

3 | THE FALLACY OF THE AVERAGE: NONLINEARITY OF THERMAL PERFORMANCE CAN RESULT IN DIFFERENCES BETWEEN CONSTANT AND VARIABLE TEMPERATURE INCUBATION

There is a long history of approaches to estimating organismal performance under thermal variability, which have been used to interpret the phenotypes produced under inconstant temperatures. Centuries ago, de Reaumur (1735) described a relationship between plant development and temperature in which the sum of daily

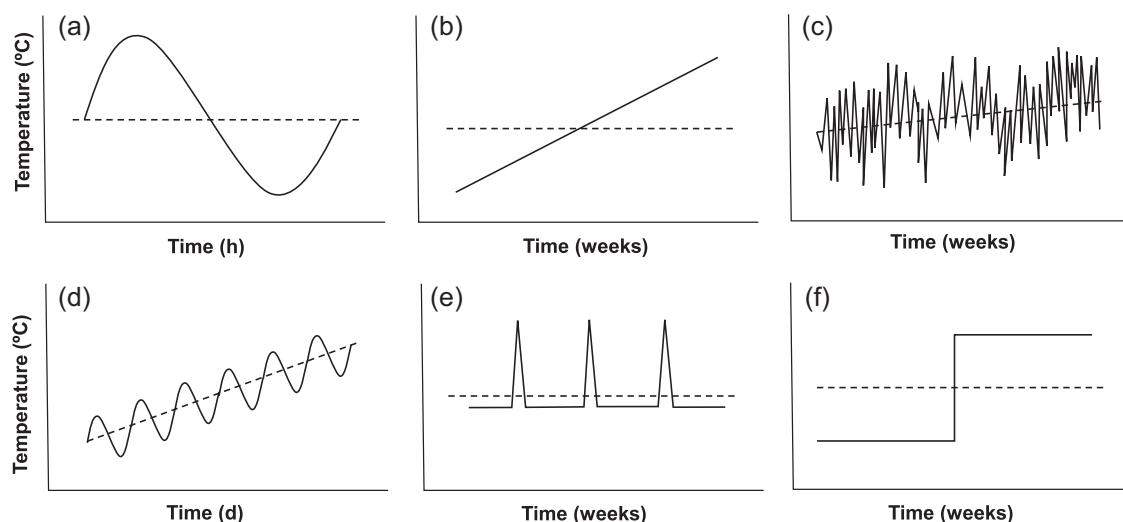


FIGURE 2 Different regimes used in studies that incubate ectothermic vertebrate eggs under thermal variability. (a) Sinusoidal fluctuations on a diel basis. (b) Seasonally changing mean, accomplished by a slow change in temperature over a long period of time. (c) Ambient temperatures, with stochastic changes in daily temperatures and seasonally shifting means. (d) Any combination of (a–c). (e) A constant mean with heat shocks applied several times per week. (f) Idiosyncratic, characterized by shifts from temperature to temperature at different stages of development

average air temperatures during development was always equal at maturity for a given plant species. This “thermometric constant” represented a way of describing the amount of heat energy required for a plant to reach maturity, and necessarily assumes a linear relationship between growth and temperature. Through time, heat summation approaches were enhanced, with modifications that take into consideration biologically informed upper and lower thermal limits for growth (Arnold, 1959; Boussingault, 1837; Sachs, 1862) and the inclusion of daily minimum and maximum temperatures (Lindsey & Newman, 1956).

Contemporarily, heat units are often measured in growing degree-days (GDD), which are calculated by subtracting a base value (T_b), representing a temperature at which the process of interest cannot occur, from a daily average temperature (T_{avg}). Although traditional GDD approaches are still widely used in agriculture and food industries, there are long-standing criticisms of their underlying assumptions (Wang, 1960). Notably, rates of biological processes respond nonlinearly to temperature, especially at thermal extremes (Sharpe & DeMichele, 1977). Under GDD (and other linear approaches), temperatures are used as a direct proxy for growth and, as a result, growth rates at thermal extremes can be overpredicted or underpredicted. Ruel and Ayres (1999) described this phenomenon as a consequence of Jensen's inequality, a mathematical principle stating that the average value of a portion of a curvilinear function (e.g., the average thermal performance under thermal variability) will differ from the value expected from the average temperature, depending on the portion's curvature (Box 1).

Box: Jensen's inequality, or the “fallacy of the average”: why incubation at variable temperatures sometimes produces different responses than incubation at the average

In this hypothetical example, a biological rate (development) has been estimated for a population of organisms across different constant temperature regimes, and modeled using a gaussian function (for other common functions used in modeling thermal performance curves [TPCs], see Angilletta, 2006) with the following equation:

$$D = 2e^{\left(-0.5\left(\frac{|T-30|}{6}\right)^2\right)}$$

where D is development rate (stages/day) and T is the temperature experienced by the organism ($^{\circ}\text{C}$). The resulting function modeling thermal performance of development in response to temperature is shown in Box Figures 1–3.

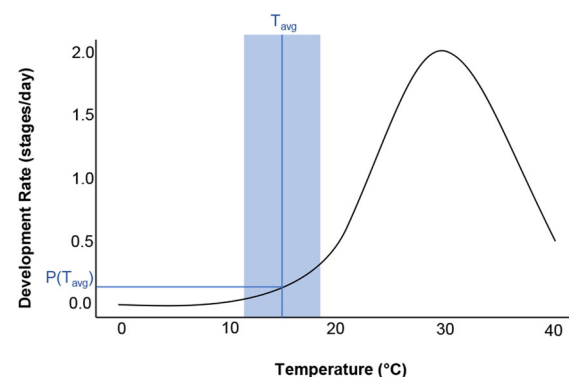
Example 1. If continuous thermal variability (blue box) from 12 $^{\circ}\text{C}$ to 18 $^{\circ}\text{C}$ around an average of 15 $^{\circ}\text{C}$ (T_{avg}) is experienced by an organism over a period of 24 hr, Jensen's inequality suggests that the realized daily performance will be the *average of the function* across the temperatures

experienced ($P_{avg}(T)$), rather than a *function of the average temperature* experienced ($P(T_{avg})$). If we calculate $P(T_{avg})$, we find an estimate of 0.087 stages for this day. However, if we take the average of the development rate function from 12 $^{\circ}\text{C}$ to 18 $^{\circ}\text{C}$, by taking the average of the integral beneath the curve, we find an estimate of 0.107 stages for this day. In this example, taking the function of the average temperature would result in a lower estimation of performance than taking the average of the function for all T experienced, which explains why, in this case, constant incubation at 15 $^{\circ}\text{C}$ (T_{avg}) should result in lower performance than thermally variable incubation from 12 $^{\circ}\text{C}$ to 18 $^{\circ}\text{C}$.

Example 2. In contrast, if we were to incubate the organism under the same magnitude of variability (Box Figure 2; red region), but in the concave portion of the curve ($T_{avg} = 30^{\circ}\text{C}$, range = 27–33 $^{\circ}\text{C}$, Box Figure 2), we estimate $P(T_{avg})$ as 2.00 stages for this day, but $P_{avg}(T)$ as 1.92 stages. Therefore, we expect higher realized performance from incubation at a constant 30 $^{\circ}\text{C}$.

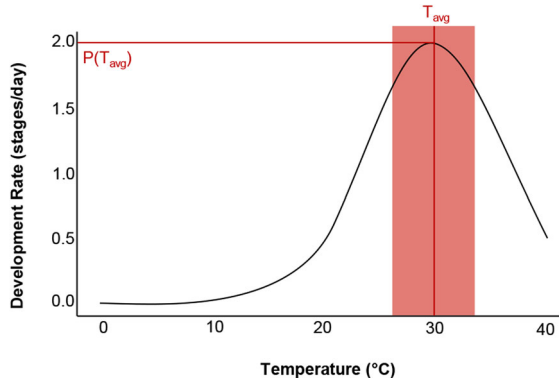
Example 3. In areas of the curve, which are approximately linear (Box Figure 3, gray region), we expect $P_{avg}(T)$ to approach $P(T_{avg})$. For example, if we incubate the organism at 21–27 $^{\circ}\text{C}$ with $T_{avg} = 24^{\circ}\text{C}$, both $P(T_{avg})$ and $P_{avg}(T)$ are 1.21 stages. We would therefore expect realized performance to be similar in constant 24 $^{\circ}\text{C}$ and variable 21–27 $^{\circ}\text{C}$ regimes because of the approximate linearity of this region of the TPC.

These generalizations are appropriate for regions of the TPC that are strictly concave, convex, or linear. However, if thermal variability occurs across a broad range encompassing more than one of these patterns, the relationship between $P_{avg}(T)$ and $P(T_{avg})$ becomes more complex. To account for Jensen's inequality in these scenarios, $P_{avg}(T)$ should be directly calculated by integrating the performance function across the temperatures experienced.

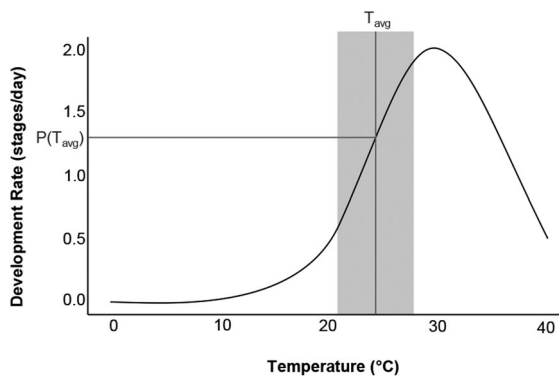


Box Figure 1. A hypothetical thermal performance curve modeled using a gaussian function, with a thermal optimum at 30 $^{\circ}\text{C}$ and a corresponding performance optimum at 2.0 stages/day. The shaded blue region from 12 $^{\circ}\text{C}$ to 18 $^{\circ}\text{C}$ represents variable incubation temperatures moving continuously from 12 $^{\circ}\text{C}$ to 18 $^{\circ}\text{C}$ over 24 hr, in a convex region

of the thermal performance curve. T_{avg} represents the average temperature of 15°C, and $P(T_{avg})$ represents the performance expected at a constant T_{avg} [Color figure can be viewed at wileyonlinelibrary.com]



Box Figure 2. In this figure, we use the same thermal performance curve (TPC) as above. The shaded red region from 27°C to 33°C represents variable incubation temperatures moving continuously from 27°C to 33°C over 24 hr, in a concave region of the TPC. T_{avg} represents the average temperature of 30°C, and $P(T_{avg})$ represents the performance expected at a constant T_{avg} [Color figure can be viewed at wileyonlinelibrary.com]



Box Figure 3. In this figure, we use the same thermal performance curve (TPC) as above. The shaded gray region from 21°C to 27°C represents variable incubation temperatures moving continuously from 21°C to 27°C over 24 hr, in an approximately linear region of the TPC. T_{avg} represents the average temperature of 24°C, and $P(T_{avg})$ represents the performance expected at a constant T_{avg} .

An alternative and a common approach to accounting for the non-linearity of biological responses (e.g., metabolism, locomotion, and growth) leverages continuous thermal reaction norms, or TPCs (Huey & Stevenson, 1979), which illustrate that the rate of biological processes increases nonlinearly with temperature to a particular optimum (T_{opt}), after which they descend sharply (Figure 3). These curves are bounded by critical thermal minima (CT_{min}) and maxima (CT_{max}),

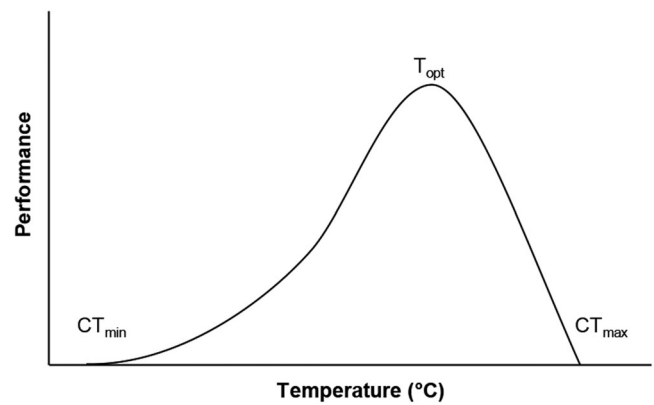


FIGURE 3 A thermal performance curve (TPC), showing that performance typically increases nonlinearly from a critical thermal minimum (CT_{min}) at which the biological process of interest cannot occur, to a thermal optimum (T_{opt}) at which performance is maximal, and then descends sharply back to a critical thermal maximum (CT_{max}) at which the biological process can no longer occur. TPCs are usually constructed by keeping organisms at a range of constant temperatures, measuring the traits of interest at each temperature, and then modeling the response curve to temperature

the minimum and maximum temperatures at which the rate of performance is zero. TPCs are typically developed by measuring performance of animals within one population at a series of constant temperatures and modeling a continuous reaction norm to fit these data. Cumulative performance under thermally variable conditions can then be estimated by integrating the TPC function, using experienced temperatures (Casagrande, Logan, & Wallner, 1987; Denny, 2019; Georges, Beggs, Young, & Doody, 2005; Niehaus, Angilletta, Sears, Franklin, & Wilson, 2012; Rollinson et al., 2018; Taylor & Shields, 1990; Worner, 1992). We note here that a recent nonlinear GDD approach has been developed, which closely resembles a TPC approach (Zhou & Wang, 2018). In contrast to traditional GDD approaches, a higher degree of accuracy is expected from using nonlinear approaches because error resulting from Jensen's inequality is minimized.

Using integration of a TPC to predict performance results in three general expectations, and assists in understanding why fluctuating temperatures during incubation often do not produce the same phenotypic effects we see at constant temperatures sharing the same mean. We walk through examples of these expectations in Box 1. First, if temperatures fall within a convex region of the TPC (e.g., near CT_{min}), the realized average performance of an organism under a fluctuating temperature regime ($P_{avg}(T)$), is expected to be greater than the performance of the same organism under a constant temperature sharing the same thermal average ($P(T_{avg})$). In other words, we expect lower performance under fluctuating temperatures than at the constant mean of fluctuation (Box Figure 1). Second, if the temperatures fall within a concave region of the TPC (e.g., near the optimum), realized average performance is expected to be lower than performance at a constant temperature sharing the same thermal average (Box Figure 2). Last, when variable temperatures fall within

an approximately linear range of the TPC, realized average performance and performance at the constant average temperature should be similar (Box Figure 3). Within these regions, as the magnitude of thermal fluctuations increases, we also expect the difference between $P_{avg}(T)$ and $P(T_{avg})$ to increase. Of course, the exact relationship between $P_{avg}(T)$ and $P(T_{avg})$ will depend on the actual thermal experience of the organism(s) in question, and can be estimated by integrating the TPC function across the temperatures experienced.

4 | INTERPRETING THERMAL VARIABILITY USING TPCs

Studies incubating ectothermic vertebrate eggs under different thermal regimes have reported complex findings with regard to the relative effects of thermally variable incubation and constant temperature incubation. Overall, authors have found that the phenotypic results of incubation are largely dissimilar between regimes; that is, variability itself does not represent a single treatment. The “fallacy of the average” provides a reasonable explanation for why these results differ in magnitude and direction of phenotypic effects, based on what might be expected from Jensen's inequality. Specifically, the fallacy of the average is useful for interpreting differences between results expected from a constant mean temperature, and the effects resulting from thermal variation around a mean (e.g., Figure 2a).

For example, Les, Paitz, and Bowden (2009) incubated painted turtle (*Chrysemys picta*) eggs at constant temperatures near the lower and upper limits for successful development (23°C and 31°C; in the area of CT_{min} and CT_{max} , respectively; Figure 4), and regimes fluctuating 3°C around those means. Based on the theory, we expect that fluctuations around the convex area of CT_{min} should accelerate

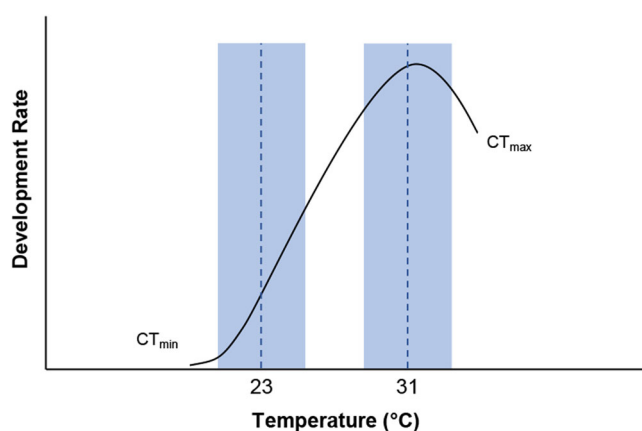


FIGURE 4 A schematic representing fluctuating temperature treatments (blue boxes) in a study on eggs of *Chrysemys picta*. CT_{min} and CT_{max} , the temperatures at which development cannot proceed, bound the curve. Temperature fluctuations around 23°C (convex region of the curve) resulted in faster development rates than those expected from a constant 23°C. The opposite was true around 31°C (concave region of the curve). Adapted from Les et al. (2009) [Color figure can be viewed at wileyonlinelibrary.com]

developmental traits, and those around the concave area of CT_{max} should decelerate them, relative to constant average temperatures. Indeed, in Les et al.'s (2009) study, *C. picta* embryos incubated near the lower limit experienced enhanced survival and development rates relative to constant temperatures, whereas those incubated near the upper limit developed slower and had higher mortality, as predicted. Similarly, Warner and Shine (2011) found that diel thermal fluctuations around a warm temperature mean near the thermal optimum decreased development rate in lizard (*Amphibolurus muricatus*) embryos, but development rate was enhanced when fluctuations occurred around a cool temperature mean, aligning with predictions. In a study incubating a lizard (*Sceloporus undulatus*) at a constant 28°C, as well as $28 \pm 5^\circ\text{C}$, no difference in development rate was found (Andrews, Mathies, & Warner, 2000). Given that this range of temperatures falls between CT_{min} and CT_{max} for *S. undulatus* (Sexton & Marion, 1974), it is likely that the temperatures experienced fall in an approximately linear portion of the TPC for development rate, which explains the lack of difference in incubation period between the two treatments.

Studies modifying the magnitude of variability around a constant thermal mean also demonstrate predictions stemming from the fallacy of the average. When eggs of Bynoe's geckos (*Heteronotia binoei*) are incubated at a constant mean (32°C), as well as treatments with fluctuations around that mean ($32 \pm 3^\circ\text{C}$, 5°C , and 9°C), increasing fluctuations result in slower developmental rates, and the largest fluctuation results in significant mortality (Andrewartha, Mitchell, & Frappell, 2010). Given that the thermal optimum for development lies at 30°C in this species (Kearney & Shine, 2004), we assume that the temperatures experienced largely fall in the concave portion of the TPC for development rate in this species, and increasing thermal variability around a mean of 32°C is expected to produce increasing reductions in performance than at a constant 32°C. Similarly, in Chinese pond turtles (*Chinemys reevesii*) and Chinese softshell turtles (*Pelodiscus sinensis*), development rate decreases as the magnitude of fluctuations around T_{opt} increases (Du, Shen, & Wang, 2009; Li, Zhou, Wu, Wu, & Ji, 2013), supporting the predictions of theory.

These experiments demonstrating the fallacy of the average show that variability should not be considered a monolithic treatment; the phenotypic response to variability appears to depend on the range of temperatures experienced, relative to the thermal sensitivity of the trait and organism in question. An existing difficulty in understanding the results of thermal variability experiments lies in the fact that thermal reaction norms for developmental and developmentally influenced traits are typically poorly characterized (Noble, Stenhouse, et al., 2018; While et al., 2018), resulting in a lack of physiological context when interpreting results. Future work would benefit, at the least, from qualitative knowledge of whether incubation temperatures are “hot” or “cold” relative to the thermal sensitivity of the trait and study organism (Les et al., 2009), or by incorporating empirical frameworks describing thermal reaction norms derived at constant temperatures for the traits in question (Georges et al., 2005; Massey, Holt, Brooks, & Rollinson, 2018).

It is important to note, however, that TPCs have several limitations (Kingsolver & Woods, 2016; Schulte et al., 2011). A key but rarely met assumption of TPCs is that thermal histories, both leading up to and during incubation treatments, do not affect resulting phenotypes through acclimation. Although we can eliminate the effect of the embryo's previous thermal history in the present review, as the studies discussed herein incubated embryos for the entirety of incubation, there is a possibility that transgenerational plasticity (via parental effects) or acclimation to temperature during incubation contributed to additional variation in phenotype. Further, given that TPCs are typically constructed under constant conditions, they may not accurately reflect instantaneous performance, especially at stressful temperatures. For example, Niehaus et al. (2012) incubated striped marsh frogs (*Limnodynastes peronii*) under two levels of diel thermal variation and found that TPCs underpredicted development rate when thermal variability was high. This finding suggests that development occurs more quickly at stressful temperatures when exposure periods are brief (i.e., under daily sinusoidal variation), but development rates are slowed under chronic exposure to high temperatures (i.e., the conditions under which TPCs are constructed). Nevertheless, given that TPCs have been able to explain the majority of variation in development rate in the wild (Rollinson et al., 2018), and modifications accounting for time-dependent effects are available (Kingsolver & Woods, 2016), TPCs can represent a useful avenue for explaining or predicting the effects of thermally variable incubation.

5 | WHAT HAVE WE LEARNED FROM OTHER THERMALLY VARIABLE REGIMES?

5.1 | Seasonal variation in temperature

In addition to experiencing diel thermal variation, embryos in seasonal environments can experience increasing or decreasing mean temperatures as incubation progresses (illustrated in Figure 2b). Seasonal thermal variation is particularly relevant to embryos that undergo long incubation periods: northern Australian saltwater crocodiles (*Crocodylus porosus*), for example, have a mean incubation period of 101 days, and mean nest temperatures decrease seasonally throughout development (Magnusson, 1979; Webb & Cooper-Preston, 1989). In combination with environmental sources of heat, both large-bodied testudines and crocodilian embryos can also generate metabolic heat throughout incubation, adding considerable heat to nests during the latter half of development (Broderick, Godley, & Hays, 2001; Carr & Hirth, 1961; Ewert & Nelson, 2003; Massey, Congdon, Davy, & Rollinson, 2019).

Seasonally variable incubation regimes appear to influence embryonic development differently than do stable temperatures, when thermal means are identical. For example, Shine (2004) simulated three realistic seasonal incubation scenarios for *Bassiana duperreyi* lizards, all with the same thermal mean: stable, increasing throughout incubation, and decreasing throughout incubation. These three treatments generated significant differences in developmental

dynamics and hatchling phenotypes, with faster development occurring in both seasonal regimes, and high deformity levels resulting in poor locomotor performance under seasonally decreasing temperatures (Shine, 2004). These results suggest that seasonal changes in temperature, in spite of mean temperatures, are sufficient to generate significant phenotypic variation, and may be of particular relevance to species that have phenologically or geographically dispersed nesting (Shine, 2004).

As many organisms with long incubation periods experience gradual seasonal thermal changes, it is also possible that embryonic thermal sensitivities can be locally adapted to seasonality, that is, thermal sensitivity changes as development progresses. Indeed, in Arctic char (*Salvelinus alpinus*), populations that spawn in relatively warm autumn temperatures and incubate throughout winter appear to have much higher hatching success and fewer deformities when warm temperatures are used during early incubation, and subsequently fall (Jeuthe, Brännäs, & Nilsson, 2016). In fact, the reversal of natural seasonal temperatures, that is, moving from cool to warm incubation temperatures, results in significantly higher mortality and deformity rate in embryos (Jeuthe et al., 2016). These results highlight the importance of understanding how natural thermal variability in study populations relate to the temporal dynamics of development. For long-incubating species in seasonal environments, the question of whether embryonic thermal sensitivities change as development progresses has yet to be explicitly explored.

5.2 | Heat shock

Heat shock refers to short exposures to sublethal, high temperatures, often resulting in increased thermal tolerance upon subsequent exposures (Bowler, 2005). If an organism experiences temperatures near the upper range of its thermal tolerance, denatured and abnormal proteins are produced, triggering the production of heat shock proteins (hsps). Hsps, among other cellular changes, ameliorate negative effects of heat on cellular proteins (Goldberg, 2003; Hightower, 1980; Parsell & Lindquist, 1993). Ultimately, brief exposures to hot temperatures in adult organisms have been shown to produce an effect known as "heat hardening" (Alexandrov, 1964) in which there is a transient increase in thermal tolerance.

For embryos, however, it appears that very brief exposures to high temperatures (i.e., heat shocks, example illustrated in Figure 2e) throughout development have minute effects on embryonic and posthatching phenotypes, at least for many of the traits investigated so far. For instance, Lim, Manzon, Somers, Boreham, and Wilson (2017) incubated lake whitefish (*Coregonus clupeaformis*) under a near-constant 2°C regime, with 1 hr temperature spikes to 5°C twice-weekly, and found that development proceeded at the same rate as in constant 2°C treatments. A slight difference in body length emerged at the prehatch stage, wherein embryos from the heat-shock regime were slightly (5%) longer. Another study on whitefish found similar results, where weekly 1 hr temperature spikes of varying magnitude (from 2°C to 5°C and from 2°C to 7°C) had no

effect on incubation period, survival, or size at hatch relative to a constant 2°C regime (Lee et al., 2016). In future experiments, it may prove interesting to increase the frequency of heat shocks as body size of whitefish embryos in these experiments was affected by twice-weekly heat shocks, but not by once-weekly heat shocks of the same magnitude, suggesting, as others have (Kingsolver & Woods, 2016; Niehaus et al., 2012), that performance responds differently as the duration of exposures changes.

The severity of the heat shock relative to the organism's thermal sensitivity may also play a role in determining whether phenotypic effects are significant. Overall (1994) incubated canyon lizards (*Sceloporus merriami*) at both moderate (31°C) and hot (34°C) constant temperatures with brief daily exposures to 37°C. Heat shocks accelerated development rate and increased hatchling body size relative to constant temperatures, but there was low embryo survival in the hot (34°C) heat-shocked treatment. In canyon lizards, constant incubation at 37°C is lethal to embryos, but short exposures to severe temperatures appear to enhance phenotype (larger body size, earlier hatch date), increasing mortality only when mean incubation temperatures are already stressful (Overall, 1994).

There are still many questions that remain unanswered regarding temperature shocks during development. First, to our knowledge, no experiments have tested the effects of cold shocks on oviparous ectothermic vertebrate embryos. Given that cold temperatures and hot temperatures illicit different gene expression responses in adult ectotherms (Podrabsky & Somero, 2004), cold shocks may result in different phenotypic responses than those observed as a result of heat shocks. Next, it is important to note that these studies generally tested whole-organism level responses to heat shocks (e.g., body size, locomotor performance). Given that acute heat shocks to embryos have been shown to illicit significant transcriptomic changes in expression of developmental and hsp genes (Bentley, Haas, Tedeschi, & Berry, 2017; Tedeschi et al., 2015), and that isolated incidents of thermal stress can transiently affect embryonic heart rates (Hall & Warner, 2019), it is likely that temperature shocks during development can significantly—and perhaps, persistently—affect other traits that have not yet been tested.

5.3 | Idiosyncratic thermal regimes

We dubbed regimes involving temperature changes at various timepoints during development “idiosyncratic” (Figure 2f). Often, these experiments aim to isolate temperature effects that occur at discrete developmental stages; for example, to determine temperature-sensitive periods for sex determination under TSD (Webb, Beal, Manolis, & Dempsey, 1987), but they can also be applied to developmental studies investigating time-sensitive effects of temperature during development.

Thus far, critical windows of physiological sensitivity to temperature have been identified by temperature-switching experiments. By changing *C. clupeaformis* embryos between cool, moderate, and warm constant thermal regimes at key milestones during development, Eme et al. (2015) found that organogenesis represented a

particularly sensitive period through which strong plasticity acts on heart rate and oxygen metabolism. Ultimately, the significant physiological changes occurring during organogenesis persisted into hatchlings. Identification of critical windows, such as organogenesis, may further elucidate mechanisms by which developmental plasticity (see Section 6 below) occurs.

Other studies that changed temperatures at various timepoints throughout development have specifically investigated embryonic acclimation capacity. Booth (1998) found no evidence for metabolic acclimation in Brisbane river turtles (*Emydura signata*), showing through a temperature-switch experiment that embryonic metabolism during the latter half of development was not dependent on embryos' previous thermal experience in early development. Likewise, Angilletta, Lee, and Silva (2006) reported that, although embryos exhibit metabolic temperature sensitivity throughout development, *S. undulatus* embryos did not undergo metabolic acclimation after being temperature-switched during development. Interestingly, these results directly conflict with the identification of organogenesis as a plastic or acclimatory window in *C. clupeaformis* (Eme et al., 2015), perhaps suggesting that taxonomic differences in embryonic metabolic acclimation capacity exist.

6 | IRREVERSIBLE DEVELOPMENTAL PLASTICITY: HOW PERSISTENT ARE THE EFFECTS OF THERMAL FLUCTUATIONS?

Previously, we discussed the effects of thermal variation primarily as they affect embryos and hatchlings. However, the developmental environment can continue to shape the phenotype of organisms beyond the incubation period, via developmental plasticity. Developmental plasticity is often considered a permanent change to phenotype as a result of the developmental environment (“irreversible nongenetic adaptation”; Kinne, 1962), with a few exceptions (e.g., Ligon, Backues, Fillmore, & Thompson, 2014; McKeown, Thompson, & Cline, 2017; Polo-Cavia & Gomez-Mestre, 2017), and plastic effects of the developmental environment arise from changes in gene expression that occur during and after development (reviewed in Beldade, Mateus, & Keller, 2011). At constant temperatures, developmental plasticity to temperature is well-described for numerous traits, but what happens when eggs are incubated at fluctuating temperatures?

In an elegant experiment in zebrafish (*Danio rerio*), Schaefer and Ryan (2006) disentangled the relative contributions of reversible and developmental plasticity to diel fluctuating temperature regimes to thermal tolerance. After rearing fish from eggs for 100 days across a range of constant and fluctuating conditions, the authors acclimated fish to constant temperatures for 12–15 days. They then tested the thermal tolerance of fish, and determined that the critical thermal maximum, or temperature at which opercular spasms occur, was significantly higher in fish incubated and reared at fluctuating temperatures when compared with constant temperatures, regardless of acclimation temperature (reversible plasticity). The ages of fish at the

time of testing (112–115 d) represent a significant portion of the lifespan of zebrafish, suggesting that their persistent physiological plastic response to fluctuating temperatures can affect long-term fitness. Likewise, long-term morphological differences arising from incubation regime have been detected in other fishes. Nathanailides, Lopez-Albors, and Stickland (1995) incubated Atlantic salmon (*Salmo salar*) at a constant temperature of 11°C and at natural, seasonal temperatures that gradually rose throughout incubation (5–10°C). Although fish from the constant regime hatched sooner and had larger muscle fibers, by 3 weeks postfeeding, fish from the ambient thermal regime exhibited significantly faster growth rates and larger muscle fiber area. Again, these effects have significant ecological importance: the posthatching growth rates of salmonids are strongly linked to fitness (Hutchings, 1993).

Several experiments in reptiles have also examined long-term responses of traits to variable developmental temperatures, finding mixed results with respect to the persistence of developmental plasticity. For example, Pearson and Warner (2016) incubated the lizard *Anolis sagrei* at fluctuating temperatures with different thermal means and found that differences in running performance between treatment groups at hatching disappeared at 3 weeks. Conversely, in the lizard *B. duperreyi*, running performance was consistently higher in individuals incubated in a warm fluctuating regime for 20 weeks posthatching relative to those incubated in cold fluctuating regimes, although morphological differences apparent at hatching disappeared within 6 weeks (Elphick & Shine, 1998). In a study on Western fence lizards (*Sceloporus occidentalis*), some morphological characters (body size and hindlimb length) persist after incubation, while others (forelimb and tail lengths) do not (Buckley, Irschick, & Adolph, 2010); it is possible that body size and hindlimb lengths, which are directly linked to sprint speed in this species (Mayr, 1956), exhibit a higher degree of developmental canalization than traits for which links to fitness are unclear (Buckley et al., 2010), although more trait-focused studies are needed to resolve this puzzling inconsistency.

The studies mentioned previously in this section described long-term effects measured in captive animals, but how do measures change when animals are reared in natural habitats? Dayananda, Gray, Pike, and Webb (2016); Dayananda, Penfold, and Webb (2017) conducted experiments on velvet geckos (*Oedura lesueurii*) in which eggs were incubated at fluctuating temperature regimes reflecting current (cold) and future (warm) conditions. After releasing hatchlings into the field, they determined that cold-incubated hatchlings had significantly higher survival and growth rates in situ than did warm-incubated hatchlings 10 months after release. Furthermore, the authors found differences in posthatching growth rates of geckos between two release sites, suggesting that posthatching environmental conditions interact with incubation conditions to produce significant effects on phenotype. Similarly, Andrews et al. (2000) found that cold fluctuating incubation temperatures resulted in higher survival 7–9 months posthatch in lizards (*Sceloporus undulatus*), when compared with high fluctuating incubation temperatures. Interestingly, in this experiment, posthatching growth rates in the field ultimately were not influenced by incubation regime; it is

possible that the degree of developmental plasticity in response to temperatures was low for growth rates, or that developmentally plastic growth rate differences could not be fully realized under natural conditions (e.g., due to low food availability). Although long-term studies under natural conditions are scarce, they raise interesting questions about whether phenotypic differences caused by incubation regime can be masked by natural conditions, and about which traits are ultimately relevant to fitness in the wild.

Long-term changes in gene and protein expression in response to developmentally variable temperatures have not been widely explored. Nonetheless, they may represent an interesting avenue for future research. Recent literature has revealed that, during acclimation to fluctuating thermal regimes, adult fish exhibit large-scale changes in messenger RNA (mRNA) levels for genes that regulate cell growth and proliferation, molecular chaperones, and cellular membrane integrity (Podrabsky & Somero, 2004). Furthermore, different genes appear to be activated at fluctuating versus constant temperature conditions (Podrabsky & Somero, 2004). With regard to proteins, increases in the concentration of hsp70 molecular chaperones in response to fluctuating temperatures have been linked to improved thermal tolerance in adult fishes (Coulter, Höök, Mahapatra, Guffey, & Sepúlveda, 2015; Nakano & Iwama, 2002), and seasonal changes in HSP levels occur in frogs (Feidantsis, Anestis, Vasara, Kyriakopoulou-Sklavounou, & Michaelidis, 2012). Given the persistent effects of thermal variability during incubation on ectothermic vertebrate thermal tolerance, morphology, growth rates, and locomotor performance mentioned herein, it is not difficult to imagine that developmentally plastic molecular changes would also be detectable. Future work should leverage established molecular techniques such as mRNA and heat shock protein assays to illuminate the proximate mechanisms of developmental plasticity in response to thermal variability.

7 | OTHER SOURCES OF PHENOTYPIC VARIATION

Although temperature overwhelmingly governs the development of oviparous ectothermic vertebrates, there are other major factors that can influence incubation and phenotype of larvae and hatchlings. First, theory predicts that population-level differences between the thermal sensitivity of traits should emerge due to adaptation to local thermal regimes (Huey & Stevenson, 1979), and indeed this prediction is founded within the studies of thermal variability herein. For example, Buckley et al. (2010) incubated *S. occidentalis* eggs from four populations under variable thermal regimes and found significant population-level effects on several traits relevant to fitness (e.g., body size and hindlimb length). Further differences that arise because of differences between clutches of eggs have also been heavily discussed. Numerous studies in reptiles (e.g., Andrews et al., 2000; Díaz, Iraeta, Verdú-Rico, Siliceo, & Salvador, 2012; Du, Shou, & Shen, 2005; Shine & Harlow, 1996; Shine, Elphick, & Harlow, 1997) have estimated significant clutch

effects on the phenotypic outcomes that were measured, although the extent to which these differences arise from genetic differences between families, maternal effects, or combinations thereof have yet to be explored in detail.

Recent studies that illustrate significant effects of transgenerational plasticity, or the ability of environmental influences during a parent's lifetime to affect offspring as well, also present interesting avenues through which to explore the effects of thermal variability. In fishes, parental acclimation to high temperatures enhances growth (Salinas & Munch, 2012) and size (Shama, Strobel, Mark, & Wegner, 2014) of offspring. Transgenerational effects are particularly strong at high temperatures, suggesting they may ameliorate negative physiological consequences of climate change (Shama et al., 2014). Given predicted rises in both mean and variability of future temperatures (Meehl & Tebaldi, 2004) and the potential of thermal variability to significantly enhance thermal tolerance (Schaefer & Ryan, 2006), studies that examine transgenerational plasticity in a variable temperature context could yield much-needed insights about the resilience of ectothermic vertebrates to climate change.

8 | CONCLUSIONS AND FUTURE DIRECTIONS

It has long been acknowledged that incubation at constant temperatures poorly reflects what organisms encounter in nature (Georges et al., 2005; Oufiero & Angilletta, 2010; Pearson & Warner, 2016). Despite this empirical reality, constant temperatures continue to dominate incubation studies (Noble, Stenhouse, et al., 2018). Variable thermal regimes experienced during incubation have resulted in unanticipated and complex effects, producing phenotypic effects that differ from what would be expected from constant thermal means (e.g., Les et al., 2009; Warner & Shine, 2011), and permanently enhancing thermal tolerance (Schaefer & Ryan, 2006), morphology (Buckley et al., 2010), and even growth rates, after release into the wild (Dayanada et al., 2016, 2017).

Existing studies that employ variable temperature incubation suggest fruitful avenues for future research. First, the field would benefit significantly from incorporating physiological frameworks that assist in explaining the phenotypes produced under variable temperature regimes (e.g., the use of TPCs). Incorporation of physiological knowledge opens up possibilities that integrate our vast knowledge of incubation under constant regimes with thermal variability, and is especially important because the majority of existing studies do not use physiologically relevant data—that is, data that are empirically anchored in what the organism is likely to experience under its own, often population-specific, natural conditions—to inform the experimental design or analyses. To facilitate this integration, authors could aim to quantify thermal reaction norms for their traits of interest, especially because broadly characterized reaction norms are uncommon in the literature (Noble, Stenhouse, et al., 2018).

Most of the attention in studies of thermal variation has been, thus far, devoted to reptiles, and currently there is a relative paucity of studies on amphibians and noncommercial fish species. This fact is puzzling, considering that many amphibians and fishes are short-lived organisms with fast life-histories, and are thus predicted to experience strong developmental plasticity to temperature (Shine et al., 1997). Researchers could consider directing future studies of fluctuating temperature incubation toward taxa that might be ideal for informing a generally applicable predictive framework for understanding organismal responses to climate change-driven thermal fluctuations. In particular, tropical stenotherms with narrow margins of thermal functionality may be more heavily impacted by climate-driven increases in thermal variation, and thus are prime candidates for further investigation (Dowd et al., 2015).

Importantly, more studies of proximate mechanisms that lead to phenotypic changes under variable temperatures are needed. Constant temperature studies have already utilized genetic tools such as transcriptomics to explain thermal acclimation (Scott & Johnston, 2012), and studies in adult organisms have investigated gene expression changes in response to variable thermal regimes in adult organisms (Podrabsky & Somero, 2004). However, there is currently a considerable gap of knowledge about the mechanisms that impart phenotype under thermal variability.

Taken together, these recommendations suggest the promise of exciting future work on thermal variability. The breadth of studies incubating ectothermic vertebrates under thermally variable conditions is much more robust than previously acknowledged, and these studies have significantly advanced our understanding of natural thermal regimes and their relationship to organismal development and ontogeny.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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