SYMPOSUM

Constancy in an Inconstant World: Moving Beyond Constant Temperatures in the Study of Reptilian Incubation

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Synopsis Variable environmental conditions can alter the phenotype of offspring, particularly in ectothermic species such as reptiles. Despite this, the majority of studies on development in reptiles have been carried out under constant conditions in the laboratory, raising the question of just how applicable those investigations are to natural conditions? Here, we first review what we have learned from these constant-temperature studies. Second, we examine the importance of temperature fluctuations for development in reptiles and highlight the outcomes of studies conducted under fluctuating conditions. Next, we report our findings from a new study that examines how the frequency of fluctuations in temperature experienced during development affects phenotype. Finally, we suggest some areas in need of additional research so that we can better understand the complex interactions of temperature and physiology, particularly in species with temperature-dependent sex determination. For questions aimed at understanding the complex effects of the environment on phenotype, we must move toward studies that better capture environmental variation. By taking such an approach, it may be possible to predict more accurately how these thermally sensitive organisms will respond to environmental perturbations, including climatic change.

Introduction

Recent work has highlighted the potential for changing environmental temperatures, driven by climatic change, to impact developmental trajectories and produce altered phenotypes in offspring in taxa that are known to be sensitive to temperature. Indeed, the effects of temperature on embryonic development have been demonstrated in a variety of invertebrate and vertebrate taxa (Walther et al. 2002; Bannerman and Roitberg 2014). In general, the effects of temperature on development are more pronounced in ectothermic species in which environmental conditions can affect a broad range of phenotypic characters. One group of particular interest with respect to thermal effects on development is the reptiles (Schwanz and Janzen 2008; Warner and Shine 2008a; Paitz et al. 2010a; Neuwald and Valenzuela 2011; Schwanz 2013; Telemeaco et al. 2013). Reptiles are an excellent group for examining the effects of environmental conditions on development because they have a diversity of life-history strategies including parity mode (Tinkle and Gibbons 1977; Blackburn 1993) and mode of sex determination (Bull 1980); in oviparous species, the eggs experience a diversity of environmental conditions (Janzen 1994). Although it is clear that the thermal environment can have important and lasting effects on the developing phenotype, few studies have attempted to capture thermal complexity in the laboratory.

What have we learned from incubation at constant temperature?

Despite the complexity of the natural environment, the majority of work on the effects of temperature on reptiles has been conducted under constant temperatures and constant hydric conditions in the laboratory. Constant-temperature studies have revealed...
that many phenotypic characters such as body size (reviewed by Deeming 2004), locomotor performance (Janzen 1993; Qualls and Andrews 1999; Du and Ji 2003; Warner and Shine 2011), immune response (Freedberg et al. 2008; Les et al. 2009), behavior (Freedberg et al. 2004; Delmas et al. 2007), and sex (Bull and Vogt 1979; Ewert and Nelson 1991) are routinely modified by incubation temperature. However, in many cases the scope and direction of the response varies with the specific incubation conditions and the species under study (reviewed by Deeming 2004; Booth 2006).

Quite possibly, the most important contribution of constant-temperature incubation studies was the characterization of temperature-dependent sex determination (TSD). The first description of TSD in a reptile was by Charnier (1966) for the rainbow lizard (*Agama agama*). Since then, TSD has been reported in all crocodilians and the tuatara, many turtles, and some species of lizards (Ewert and Nelson 1991; Ewert et al. 1994). In order to discover whether a species of interest has TSD, eggs are incubated at a range of constant temperatures and sex ratios are determined, with the expectation that species with TSD will have sex ratios that deviate from parity across the temperature range. Using this method, two major patterns of TSD have been described; TSD Pattern I with one sex formed at low temperatures and the other at high temperatures, and TSD Pattern II with one sex formed at both high and low temperatures and the other or both sexes formed at intermediate values (Fig. 1). Species exhibiting Pattern I have one pivotal temperature (i.e., the temperature that produces a population-wide 50:50 sex ratio) that coincides with one transitional range of temperature (TRT) or the range of temperatures over which mixed sex ratios are observed. Species exhibiting Pattern II have two pivotal temperatures which may coincide with two TRTs (Mrosovsky and Pieau 1991; Pieau 1996); however, when the intermediate temperature range fails to produce exclusively one sex, then there may not be multiple pivotal temperatures or TRTs. Additionally, constant-temperature studies demonstrated that the sex of the embryo is determined by the thermal conditions experienced during the middle third of development, or the temperature-sensitive period (TSP) (Yntema and Mrosovsky 1982; Desvages et al. 1993). Subsequent studies of incubation at constant temperature revealed that sex determination is also sensitive to the influence of steroids in reptiles with TSD (reviewed by Crews 1996).

![Fig. 1](https://academic.oup.com/icb/article-abstract/54/5/830/279778)

Natural nest conditions and fluctuations of temperature

While incubation at constant temperature can provide useful information on the thermal sensitivity of traits and on the mode of sex determination, it may have limited applicability for discerning how offspring’s phenotype, including sex, may be affected...
by temperature under natural conditions of incubation. In order to better understand how natural conditions affect phenotypic variation, there are two potential avenues to pursue: (1) conduct studies in the field where animals would experience a suite of environmental variables including fluctuations in temperature or (2) mimic temperature fluctuations from the field in the laboratory. The advantage of the former approach is that animals experience the full range of environmental variables, and when coupled with an approach such as cross fostering, it may provide valuable information (e.g., Mitchell et al. 2013). The advantage of the latter approach is that it is possible to isolate the effects of temperature to a much greater extent without the confounding effects of variation in maternal choice of nest site, which can affect other factors that impact nest temperatures. Is it possible, however, to adequately mimic in the laboratory the thermal environment of the field? Ultimately, designing complementary field and laboratory experiments is likely to yield the most complete understanding of the effects of temperature on development.

In nature, incubating reptilian eggs are subjected to daily thermal fluctuations, with few reptiles providing parental care during incubation. However, females may preferentially select nest sites that maximize the female’s fitness based on the effects of the magnitude of thermal fluctuations on the offspring’s phenotype (Shine and Harlow 1996). The shape of the fluctuations is approximately sinusoidal, with increasing temperatures during the day and decreasing temperatures during the night (Shine and Elphick 2001; Warner and Shine 2008b; Paitz et al. 2010b). However, the magnitude and extent of the fluctuations can be affected by local environmental conditions, season, and vegetation cover (Schwartzkopf and Brooks 1987; Janzen 1994; Shine and Elphick 2001; Paitz et al. 2010b). For example, daily temperature fluctuations can be dampened by storm events (Warner and Shine 2008b). Across the incubation period, fluctuations may decrease in magnitude as daytime temperatures increase to a lesser extent than do nighttime temperatures during the summer in temperate regions (Ashmore and Janzen 2003). Fortunately, these sort of environmental conditions can be readily mimicked in the laboratory (Fig. 2A–C). Many reptiles deposit their eggs in subterranean nests varying from the relatively shallow nests of smaller freshwater and terrestrial turtles and lizards to the deeper nests produced by larger-bodied freshwater turtles and sea turtles. Depth of nest also affects temperature fluctuations, with eggs from shallow nests experiencing more uniform fluctuations throughout the nest than those from deeper nests where fluctuations are likely dampened for eggs further down in the nest relative to those located nearer the surface (Booth and Astill 2001; Booth 2006). Beyond temperature, there are a number of other abiotic factors that can influence a nest’s environment including hydric conditions and the composition of the soil (Gutzke et al. 1987; Packard et al. 1987). Collectively, the thermal environment experienced by developing embryos is complex and may be subject to changes across the developmental period and may vary with nest architecture.

**Modeling thermal effects on the development of reptiles**

Although studies under constant temperature provide important baseline information on traits that are likely to respond to temperature, they may not accurately represent the response of the trait when exposed to fluctuations in temperature. This is in part due to the fact that temperature can directly affect developmental rate. For any given species,
there is a range of temperatures over which development can proceed, which is bounded by the low thermal limit (LTL) and the high thermal limit (HTL). Extended exposure to temperatures below the LTL or above the HTL can result in embryonic death; however, short-term exposure can be tolerated in many cases. Between the low and HTLs lies the optimal developmental range (ODR) (Fig. 3). Operationally, embryos can develop when exposed to constant temperatures within the ODR, but could not survive continuous exposure to temperatures outside the ODR. The relationship between developmental rate and temperature is approximately linear within the ODR (Georges et al. 1994), but is non-linear outside the ODR (Sharpe and DeMichele 1977; Georges et al. 2005).

Efforts to model the relationship between temperature and sex ratios have determined that, for nests in the field, mean incubation temperature alone is not a good predictor of the nestlings’ sex ratio (Bull 1985; Georges 1989). The constant temperature equivalent (CTE) model takes the effects of daily temperature fluctuations into account when predicting nestlings’ sex ratios and has been supported by empirical data (Georges et al. 1994; Les et al. 2007; Paitz et al. 2010b). Further, the CTE model has been modified to account for the non-linear effects of temperature on development for temperatures that exceed the ODR (Georges et al. 2005), and a recent modification of the CTE model allows for temperature to increase over the incubation period (Telemeco et al. 2013). The CTE model is based on the premise that developmental rates positively scale with temperature, at least within the ODR. If an embryo is exposed to daily sinusoidal temperature fluctuations, half of the incubation period will be spent developing above the mean, and half below. However, because of the direct effect of temperature on developmental rate, more than half of development would occur while the embryo is experiencing temperatures above the mean due to the increase in developmental rate at higher temperatures (Bull 1985). The CTE is the estimated temperature at which half of embryonic development occurs above the mean and half occurs below. Temperature fluctuations that exceed the ODR modify the CTE because of the non-linear responses of developmental rate either to further increasing temperature above the HTL, or the cessation of development that occurs below the LTL (Georges et al. 2005; Les et al. 2009).

What do we know about the effects of temperature fluctuations on phenotype?

As was the case with incubation conducted at constant temperature, temperature fluctuations have been found to affect a variety of phenotypic characters, but the effects are not consistent and depend upon the species under study and the nature of the fluctuations employed. Often, studies involving fluctuating temperatures examine phenotypic responses to changing amplitudes around a stationary mean, as well as a direct comparison to eggs incubated at the mean temperature without fluctuation, allowing for comparisons to the larger body of literature on constant temperatures. From these studies characters such as body size (Les et al. 2007, 2009), immune function (Les et al. 2009), and locomotion (Shine and Harlow 1996; Ashmore and Janzen 2003) are known to respond to thermal fluctuations.

Both hatching success and duration of incubation are also affected by temperature fluctuations. In a recent study by Warner and Shine (2011), eggs from the jacky dragon (Amphibolurus muricatus), incubated at one of two constant temperatures (25°C or 28°C), or temperatures that fluctuated either ±4°C or ±8°C about that constant temperature, had altered durations of incubation. For eggs that fluctuated around 25°C, duration of incubation was shortened with increasing thermal flux compared with their constant-temperature counterparts, whereas duration was lengthened with increasing thermal flux for eggs that fluctuated around 28°C compared with their constant-temperature counterparts. A similar reduction in duration of incubation was found in the red-eared slider turtle (Trachemys scripta) when temperatures fluctuated around the LTL (23±3°C) when compared with constant 23°C.
(Les et al. 2009). By contrast, duration of incubation increased in the smooth softshell turtle (*Apalone mutica*) when temperature fluctuated closer to the HTL (30.5 ± 4°C) compared with constant 30.5°C (Ashmore and Janzen 2003). The effects on hatching success are typically limited to fluctuations that exceed the ODR, with temperatures more than or less than the ODR resulting in lower hatching success (Les et al. 2009; Neuwald and Valenzuela 2011).

By far the most consistent effect of temperature on offspring phenotype under either constant or fluctuating conditions is on sex determination in species with TSD. As with the other phenotypic characters discussed above, how temperature affects sex will depend both on the mean and the amplitude of temperature fluctuations, and whether fluctuations exceed the ODR. In general, as the amplitude of temperature fluctuations increase, there is a tendency for greater production of females, at least in species with Pattern Ia TSD (Bull 1985; Georges et al. 1994; Neuwald and Valenzuela 2011). In studies in which we compared incubation under constant conditions with that under temperatures that fluctuated around that same mean temperature, fluctuations reliably pushed the sex ratio toward females (Les et al. 2007), and in the painted turtle (*Chrysemys picta*), we reported a shift from 0% females at 27°C to 100% females at 27 ± 8°C (Paitz et al. 2010b). Surprisingly, in this same study, no other phenotypic characters were significantly affected by temperature fluctuations (Paitz et al. 2010b). Similar large shifts in sex ratios with temperature fluctuations have been observed in other species (Georges et al. 1994).

In species with Pattern II TSD, there is less information on how fluctuations in temperature might affect phenotypes, including sex. In a study of *A. muricatus*, it was found that increasing fluctuations around 25°C increased the proportion of males formed while increasing fluctuations around 28°C increased the proportion of females formed (Warner and Shine 2011). Although these patterns were consistent with the predictions of the CTE model, the match between predicted sex ratios and observed sex ratios was not as strong as has been reported in species with Pattern I TSD. This lack of fit may be due, in part, to the fact that the CTE model was developed for Pattern I species.

Somewhat paradoxically, a recent study in *C. picta* found that elevated fluctuations about a female-producing mean temperature (31 ± 5°C) resulted in the production of male offspring (Neuwald and Valenzuela 2011). *Chrysemys picta* displays Pattern Ia TSD with males produced at lower temperatures and females at higher ones under constant thermal conditions (Ewert and Nelson 1991). When they applied both the CTE model and the modified non-linear CTE model, the predicted values were 32.5°C and 27.2°C, respectively. Under constant conditions, these temperatures would produce all females (32.5°C) or all males (27.2°C), which closely matches their reported sex ratios. This study serves as a prime example of how deviations from the ODR can produce dramatic effects on development; when temperatures reached 36°C, exceeding the HTL, a lower proportion of development occurred than when temperatures reached 26°C, within the ODR for this species. It further suggests that even when developmental conditions deviate from the ODR, the CTE models predict sex ratios reasonably well.

**Does the frequency of temperature fluctuations affect phenotype?**

We know that thermal fluctuations during incubation alter both the range of temperatures and the amount of time experienced by embryos at any given temperature, and that variation in the amplitude (thermal range) of fluctuations can differentially affect phenotype; however, no study has yet examined how fluctuation frequency might affect development. Altering fluctuation frequency manipulates how time spent at male-producing or female-producing temperatures is divided over the course of incubation, while holding average temperature, range, and the total amount of time spent at male/ female temperatures the same among groups. Moreover, manipulating fluctuations independent of the average temperature or amplitude helps disentangle whether altered phenotypes observed from fluctuating incubation temperatures are a result of experiencing a broader range of temperatures, or an effect of thermal instability per se. We examined the effect of fluctuation frequency on the incubation period and on the phenotype of the offspring of *T. scripta*.

Clutches of *T. scripta* eggs (*n* = 19) were collected from females or freshly excavated nests at the Banner Marsh Fish and Wildlife Area, IL, in June 2013 and artificially incubated at one of three randomly assigned incubation regimes in a split-clutch design. All incubation regimes maintained the same average temperature and amplitude of 28.7 ± 3°C (CTE 29.4) (Georges 1994), exposing embryos both to male-producing and to female-producing temperatures during development. The incubation regimes only differed in their fluctuation frequency with a normal frequency (24-h cycle), a hypo-flux treatment (48-h cycle), and a hyper-flux treatment (12-h cycle) (Fig. 4). Eggs were collected...
under IDNR permit NH13.2084 and research was conducted following the IACUC guidelines of Illinois State University.

Hatching success was high overall (87%) and did not differ among incubation treatments (chi-squared test; Hyper-flux: 90%, Normal 85%, Hypo-flux 85%; \( P = 0.47 \)). Average incubation period was 57 days and was significantly different among treatments (ANOVA, clutch as random effect; \( F_{(2,197)} = 25.14; \ P < 0.0001 \)) with the hypo-flux treatment extending incubation by \( \sim 1 \) day compared with the other treatments (Tukey–Kramer post hoc; Hyper:Hypo \( t_{(197)} = -5.65, \ P < 0.0001 \); Hyper: Normal \( t_{(197)} = 1.02, \ P = 0.36 \); Hypo:Normal \( t_{(197)} = 6.43, \ P < 0.0001 \)). Hatching size (mass, plastron length, and carapace length) did not differ among incubation treatments (ANOVA, clutch as random effect; in all cases, \( P > 0.3 \)). Most notably, fluctuation frequency did not significantly alter sex ratios (percent female: Hyper-flux: 84%, Normal 95%, Hypo-flux 88%; Fisher’s exact test; \( P = 0.07 \)).

While we know that thermal fluctuations differentially affect reptilian phenotype compared with incubation at constant temperature (Les et al. 2007, 2009; Ashmore and Janzen 2003; Warner and Shine 2011), our data suggest that thermal fluctuation frequency does not significantly influence phenotype, either morphology or sex, at least when temperatures are within the ODR. While incubation period was extended by \( \sim 1 \) day in the Hypo-flux treatment, this extension is not likely biologically meaningful as turtles in our population overwinter in the nest before emerging the following spring. All three incubation regimes produced female-biased sex ratios despite fluctuating into and out of both male and female producing temperatures for equivalent durations (Paitz et al. 2010b). However, it appears that fluctuation frequency does not influence sex ratios and is therefore not likely an important effector of TSD. Moreover, our results suggest sex determination and thermally sensitive phenotypic characters are more sensitive to the cumulative amount of development completed at male or female temperatures rather than the frequency of movement above and below the threshold, consistent with temperature models (Georges 1994). Our research adds to the body of work indicating that the amplitude of the fluctuation is important in phenotypic development (Ashmore and Janzen 2003; Les et al. 2007, 2009; Warner and Shine 2011), but also indicates that the frequency of the fluctuation is not.

**Where do we go from here?**

Research utilizing natural incubation conditions that consider thermal fluctuations will advance our understanding of TSD, its adaptive significance, and conservation of species with TSD. Recognizing how organisms may be able to respond to changing thermal environments will be key to predicting the effects of climatic change on thermally sensitive species. In response to changing thermal environments, oviparous reptiles would appear to have a limited number of options to modify the conditions of incubation for their offspring; shift selection of nest sites toward cooler (Janzen 1994; Morjan 2003; Doody et al. 2006; Mitchell et al. 2008) or warmer (Neuwald and Valenzuela 2011) microclimates, change nesting phenology (Mitchell et al. 2008; Telemeco et al. 2013), make shifts in range, and modify the pivotal temperature (Booth 2006). These latter two options would presumably require longer periods of time to achieve, and thus may not be tractable strategies in the face of rapid climatic shifts. However, when considering how temperature fluctuations may factor into the response to climatic change, the potential effects on phenotype are less clear (Booth 2006). For example, temperature fluctuations that are currently predicted by models of climatic change (Walther et al. 2002; Boer 2009; Neuwald and Valenzuela 2011) may result in a dampened phenotypic response, provided that the

![Fig. 4](https://academic.oup.com/mcb/article-abstract/54/5/830/2797778)
fluctuations extend beyond the ODR. This can result from nests experiencing lower CTEs than previously predicted and may produce males under warmer conditions (Neuwald and Valenzuela 2011). So where might we focus our efforts to move forward? Below we suggest a few avenues for further study that would provide needed information both on responses to temperature and on the potential for physiological processes (such as the effect of estrogens on sex determination), to alter that response. We then consider the utility of our current characterizations of the patterns of TSD in reptiles, whether the CTE model is still useful for predicting the effects of incubation temperature on sex ratios, and how we might interpret the effects of extreme temperatures on phenotype.

**Comprehensive field-temperature data and realistic laboratory simulations**

Although more studies are employing temperature fluctuations in the laboratory, in order to better understand how variable thermal conditions are in the field, we need more comprehensive collection of temperature data from more locations. Temperature data for nests would be particularly informative for species with a broad latitudinal range. Until recently, data loggers that could be placed into individual nests were expensive and often not easy to launch in the field. To get around this, studies often relied on temperatures of the soil surface or air collected at nearby monitoring stations as a proxy for the condition in nests (Ackerman and Lott 2004). Unfortunately, these data cannot be localized to any specific nest, as they provide only limited information for understanding incubation in the field. Now that low-cost data loggers are available (e.g., iButtons, Maxim Integrated), it is possible to collect data on the temperatures of many nests within or among populations, and within and among years. Once available, these data would be of great benefit for laboratory studies attempting to mimic field conditions, and they would also provide useful basic information on thermal conditions during development.

**Integrating physiological mechanisms with temperature fluctuations**

While it is important to explore how changing thermal conditions affect phenotype, we have a limited understanding of the mechanism(s) by which temperature exerts its effects. Here, we focus on one well-described endocrine mechanism known to influence sex determination (i.e., estrogens), and suggest that work is needed to explore how estrogenic effects, both gonadal and maternal in origin, might be impacted by temperature fluctuations. From previous research, we know that, under constant incubation conditions, the application of estrogens to eggs during the TSP is sufficient to produce females at otherwise male-producing temperatures (Bull et al. 1988) while production of males at otherwise female-producing temperatures is most often accomplished through inhibitors of aromatase (the enzyme responsible for the production of estradiol) (Wibbels and Crews 1994; Warner and Shine 2011). For *T. scripta*, there is a synergism between estradiol and temperature such that less estradiol is required to produce females as temperatures approach the TRT compared with cooler, male-producing temperatures (Wibbels et al. 1991). This lead to the hypothesis that female-producing temperatures induce the expression of aromatase in developing gonads and this, in turn, triggers ovarian development (Crews and Bergeron 1994; Ramsey and Crews 2009). How might a mechanism such as this operate in natural nests where embryos are consistently experiencing both male-producing and female-producing temperatures? Is aromatase being turned on and off on a daily basis? Recent work by Matusmoto et al. (2013) demonstrated that female-producing temperatures result in the demethylation of key genes necessary for aromatase expression. This suggests that in order for aromatase to be induced, leading to the production of estradiol, the time spent in the female-producing range (i.e., above the temperature threshold for the occurrence of demethylation) must have a cumulative effect over time. Individuals that fail to spend sufficient time above the temperature threshold do not achieve sufficient demethylation and thus develop as males. Consistent with this cumulative-demethylation hypothesis, we report here that the frequency with which embryos cross back and forth between male-producing and female-producing temperatures does not affect sex determination. Future work on the role of demethylation in TSD should examine this process under more naturalistic fluctuating temperatures.

In addition to the gonadal production of estradiol, maternally derived estradiol is present in eggs at oviposition (Bowden et al. 2000; Elf et al. 2002) and can influence sex determination at intermediate temperatures (Bowden et al. 2000; Lovern and Wade 2003; Radder et al. 2009; but see Radder 2007). Seasonal variation, during which estradiol levels are 10× higher in second clutches compared with first clutches (Paitz and Bowden 2009; Bowden et al. 2011), seems to be the critical factor driving the
effect of maternal estradiol on sex determination (Bowden et al. 2000). We have demonstrated that this maternally derived estradiol is subject to in ovo metabolism very early in development (Paitz and Bowden 2008, 2011; Paitz et al. 2012) when estradiol is converted to estrone sulfate (Paitz and Bowden 2013). Importantly, estrone sulfate is both maternally derived (showing the same seasonal increase as maternally derived estradiol) (Paitz and Bowden 2013) and capable of reversing sex when applied exogenously (Paitz and Bowden 2013). Pairing the data on the physiological mechanisms underlying the effects of maternally derived estradiol on sex determination with the data on how temperature influences the gonadal production of estradiol, highlights a critical role for estradiol in TSD. Unfortunately, none of these processes have been investigated under more naturalistic conditions of incubation. If we are to understand how TSD operates in nature, future work must examine whether or not these processes operate similarly under fluctuating conditions.

**How should we characterize TSD?**

Do we need to move beyond the Type I versus Type II characterization for species with TSD? We would argue that the current scheme is still useful in that it identifies species as having one or more developmental ranges that produce a particular sex. It is now apparent that the simple description of Type Ia TSD as a scenario where females develop in warm nests is not accurate; extreme high temperatures slow development (Georges 2005), and thus can decrease the production of female hatchlings under conditions that traditionally would be thought to produce exclusively female offspring (Neuwald and Valenzuela 2011). However, in species with Type I TSD, there is functionally one range that produces females and another range that produces males. Type II species have two ranges that can produce a single sex, and another range that either produces the opposite sex, or a mix of both sexes. An example of this would be *A. muricatus*, where there is no temperature that produces only males (Warner and Shine 2011). Nonetheless, knowing and characterizing the temperature ranges that are likely to produce males versus females is useful, and still holds predictive power.

**How should we model the effect of incubation temperatures on resulting sex ratios?**

Do we need to move beyond the CTE model (Georges 2005), especially when dealing with extreme temperatures (Neuwald and Valenzuela 2011) or species with Type II TSD (Warner and Shine 2011)? We would argue that the CTE model is largely supported by empirical data and that the fundamental principle of determining the proportion of development that occurs within the temperature range(s) of male versus female is still vital to predicting sex ratios from temperature profiles. The model accurately predicts that extreme high temperatures will slow development and lead to the production of males because presumably more development is taking place within cooler male-producing temperatures (Neuwald and Valenzuela 2011). There are some inherent difficulties in estimating developmental rates at extreme temperatures because these temperatures are fatal to eggs over longer exposure periods. Despite this, the CTE model provides a reasonable estimation of sex ratios for incubation conditions that reach extreme temperatures. With regards to species with Type II TSD, it has been argued that the CTE model is not useful for predicting sex ratios (Warner and Shine 2011). While we concur that this is true *per se*, we would argue that there is no reason to expect the CTE model “in its current form” to predict sex ratios in a species that has two temperature ranges that produce one sex (or mostly one sex). Since the CTE is essentially the median temperature at which half of development occurs above and half of development occurs below, it can be easily applied to species with Type I TSD to determine whether or not more development occurred in the male range or the female range. We suggest that this model can be modified for species with Type II TSD to quantify the proportion of development that occurs between the two pivotal temperatures. If this value is >50%, we would predict the sex that is normally produced at intermediate temperatures to be produced. Unfortunately, developing and empirically testing a modified CTE model is beyond the scope of this article, but a model of this nature would be a valuable addition to the field. Until a modified CTE model that quantifies the proportion of development that occurs within the intermediate range is tested, we think it is premature to disregard the usefulness of CTE model for species with Type II TSD.

**How do we interpret effects elicited by fluctuating into extreme temperatures?**

Models of global climate change often predict an increased frequency of extreme temperatures (Easterling et al. 2000), so it will be important to understand how extreme temperatures influence development. As it pertains to incubation, we define
extreme temperatures as those outside of the range of constant temperatures that can sustain successful development (Les et al. 2009). Fluctuating into extreme temperatures beyond the ODR during incubation may elicit unexpected effects on offspring phenotype, including sex determination in species with TSD. Data suggest that extreme high temperatures (probably most relevant to incubation studies) can result in a deceleration of growth rates (Georges 2005; Les et al. 2009). This decreased growth at extreme temperatures has been used as an explanation as to why we might see sex ratios start to shift back toward the sex that is produced at cooler temperatures (Neuwald and Valenzuela 2011). While we agree that this is the most likely explanation, extreme incubation temperatures may alternatively cause a reversal of the mechanisms underlying TSD to produce the opposite sex. For example, there are reports in _C. picta_ of females being produced at extremely low temperatures (Gutzke and Paukstis 1984; Schwartzkopf and Brooks 1985) despite the fact that this species has been classified as having Type Ia TSD (Ewert and Nelson 1991). These findings suggest that it is plausible for the mechanisms underlying TSD to switch at temperature ranges where it is difficult to support embryonic development. While testing the effects of fluctuating into extreme temperatures on the mechanisms of TSD may prove difficult because of increased mortality, there may be additional physiological ramifications for embryos that fluctuate within the extreme thermal ranges underscoring the importance of such research. _In vitro_ studies (e.g., Matsumoto et al. 2013) will enhance our understanding of how climate change (via extreme thermal fluctuations) may alter the phenotype of species with TSD, since _in ovo_ studies are often not possible.

**Final thoughts**

We’ve long recognized that laboratory studies are often a poor representation of what is going on in the field (see, e.g., Gutzke et al. 1987), and given recent technological advances, a move toward greater complexity of laboratory studies of incubation is warranted. In particular, the availability of inexpensive data loggers and programmable incubators has made it feasible to carry out large-scale assessment of field conditions and then to mimic field temperatures in the laboratory. It is clear that we need to move beyond studies based on constant temperature if we are to understand how phenotypes can respond to the complex environments they are likely to experience in nature.

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