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Temperature fluctuations and maternal estrogens as critical factors for understanding temperature-dependent sex determination in nature

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Abstract

Vertebrates with temperature-dependent sex determination (TSD) have justifiably received a lot of attention when it comes to the potential effects of climate change. Freshwater turtles have long been used to characterize the physiological and genetic mechanisms underlying TSD and provide a great system to investigate how changing climatic conditions will affect vertebrates with TSD. Unfortunately most of what we know about the mechanisms underlying TSD comes from laboratory conditions that do not accurately mimic natural conditions (i.e. constant incubation temperatures and super physiological steroid manipulations). In this paper, we review recent advances in our understanding of how TSD operates in nature that arose from studies using more natural fluctuating incubation temperatures and natural variation in maternal estrogens within the yolk. By incorporating more natural conditions into laboratory studies, we are better able to use these studies to predict how changing climatic conditions will affect species with TSD.

Graphical abstract

Keywords

Temperature fluctuations; sex ratios; estrogens; turtle

INTRODUCTION

The importance of temperature as a modulator of biological processes has been long recognized, but given the state of the global climate, there is an increased urgency to understand how current and future climatic conditions will affect organismal phenotypes, including physiological responses (Angilletta 2009). Temperature affects all organisms to

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some degree, but ectothermic vertebrates, including those with temperature-dependent sex determination (TSD), are considered highly vulnerable to the potentially detrimental effects of a changing climate (Janzen 1994, Paaijmans et al. 2013). Beyond concerns of climate change, species with TSD have served as model systems to investigate the effects of incubation temperatures on numerous offspring traits (Charnov and Bull 1977). TSD represents a relatively unique situation where wild populations of animals have been used to study the effect of temperature on physiological and genetic processes under controlled laboratory conditions, allowing us to decipher how temperature ultimately effects the phenotype, including sex, of the developing embryo (Janzen 1995, Booth 2006, Paitz et al. 2010).

Despite having this wealth of data from laboratory studies, we still lack a basic understanding of how TSD operates in nature, largely resulting from the use of conditions (e.g., constant incubation temperatures and supraphysiological steroid manipulations) that do not reflect conditions experienced in nature. Further, many studies manipulate single traits (e.g., temperature or steroids), but this approach has obvious limitations when trying to understand a complex phenomenon such as TSD. It is clear that our approaches have challenged our ability to anticipate how changing conditions might affect organisms with TSD, necessitating new and innovative thinking about to study this phenomenon in both the laboratory and the field. As part of this review, we consider historical studies to provide context for more recent work that focuses on increasing the biological realism of laboratory treatments, including those studies that examine multiple variables simultaneously. These recent studies clearly demonstrate the value of incorporating more realistic thermal profiles when approaching questions related to TSD, and are helping clarify how sex determination responds to more complex thermal conditions that are indicative of the field. Finally, we provide suggestions for future studies, highlighting new approaches that may prove fruitful for helping us understand how species with TSD are likely to respond to changing environmental conditions.

Constant temperature incubation studies are the foundation of TSD research

Constant temperature incubation studies have been critical for identifying and describing TSD. Starting with the first description of TSD in a vertebrate, the rainbow lizard (*Agama agama*) by Charnier (1966), a multitude of studies have split eggs, either through haphazard assignment or by clutch, between two or more incubators held at different constant temperatures, and the resulting sex ratios are recorded (Bull and Vogt 1979, Ewert and Nelson 1991, Ewert et al. 1994). For species with TSD, sex ratios predictably differ with incubation temperature. Further, the majority of constant incubation temperatures produce sex ratios that are almost completely male (“male producing temperatures”) or completely female (“female producing temperatures”), with a relatively narrow range of temperatures (1–2 °C) separating male and female producing temperatures, although this range can be wider in some species (see Hulin et al. 2009). This range, also known as the transitional range of temperature, produces mixed sex ratios and contains the pivotal temperature, or the temperature that produces a population wide 50:50 sex ratio (Mrosovsky and Pieau 1991,

Pieau 1996). Constant temperature studies have also revealed different patterns of TSD based upon which sex is formed at cool or warm incubation temperatures; TSD pattern 1a (cool males, warm females; Fig. 1A), TSD pattern 1b (cool females, warm males; Fig. 1A), and TSD pattern 2 (one sex is formed at cool and warm temperatures, the other sex is formed at intermediate incubation temperatures, resulting in two pivotal temperatures; Fig. 1B). From constant temperature studies we know that TSD is widespread in reptiles, occurring in all crocodilians and the tuatara, most turtles, and many lizards (reviewed in Valenzuela and Lance 2004). To date, there are no reports of TSD in snakes.

Constant temperature incubation studies have allowed for the characterization of many aspects of the sensitivity of the temperature response. For example, we know that the gonads, as they undergo sexual differentiation, are most sensitive to temperature during the middle third of embryonic development (Bull and Vogt 1981, Yntema and Mrosovsky 1982, Desvages et al. 1993), and that the window of sensitivity coincides with incubation temperature, being wider under cooler incubation conditions and narrower under warmer incubation conditions (Wibbels et al. 1991a). Despite turtle species varying widely in their geographic distributions, their reported pivotal temperatures are surprisingly similar both within populations of a species and across species, ranging from 28–30 °C for most species (Bull et al. 1982, Ewert et al. 1994, Rimblot et al. 1985, but see Hulin et al. 2009). Further, constant temperature studies have allowed us to probe the relationship between physiology and TSD (Crews et al. 1994, Freedberg et al. 2008, Paitz and Bowden 2009), and more recently the genetic underpinnings of TSD (Matsumoto and Crews 2012, Schroeder et al. 2016). Each of these metrics provides important insight into how an embryo might respond to a given thermal environment.

Estrogens are a mechanistic link between incubation temperatures and sex ratios

Since TSD was demonstrated in a number of reptiles, there has been a growing interest in determining what embryonic processes are influenced by incubation temperatures and impacting sex determination. Some of the earliest work on deciphering the physiological mechanisms underlying TSD demonstrated that estradiol is capable of reversing sex in most species with TSD (Pieau 1974, Bull et al. 1988, Wibbels et al. 1991b, Crews et al. 1994, Pieau et al. 1994). In these studies, the exogenous application of estradiol, typically at supraphysiological doses, to developing eggs resulted in the production of female hatchlings from incubation temperatures that typically produce male hatchlings. However, some recent work has produced somewhat counterintuitive results where the repeated administration of supraphysiological doses of estradiol results in the production of male hatchlings at female producing temperatures (Warner et al. 2014). The most common experimental approach was to place eggs at constant incubation temperatures that produce males and add various doses of estradiol to examine the effect on resulting sex ratios (Bull et al. 1988, Wibbels et al. 1991). From these studies we know that an interplay between incubation temperature and estradiol exists such that a lower dose of estrogen is required to produce female hatchlings when incubation temperatures are closer to the range that produce female hatchlings (Wibbels et al. 1991b, Crews et al. 1994). We also know that the period of development in

which sex determination is sensitive to estradiol manipulations coincides with the period when the developing gonads are sensitive to incubation temperatures, roughly the middle third of embryonic development (Wibbels et al. 1991a, Merchant Larios et al. 1997). Administration of estradiol early in development (before gonads are developing) or late in development (after gonads have differentiated) has less of an effect on resulting sex ratios than manipulations that take place during the middle third of development (Wibbels and Crews 1995). Additional support for the role of estrogens in TSD comes from pharmacological manipulations; blocking the production of estradiol by inhibiting aromatase (Crews et al. 1994, Dorizzi et al. 1994) or blocking estrogen receptors (Kohn et al. 2015) results in the production of male hatchlings regardless of incubation temperature. Ultimately, exogenous manipulations of estradiol signaling led to the conclusion that the production of estradiol was a vital process in triggering ovarian differentiation in species with TSD (Lance 2009).

As a whole, laboratory studies of TSD have established the range of incubation temperatures that tend to produce each sex for a large number of species, and that estrogens play a key role in signaling ovarian differentiation at female producing temperatures. The principle drawback to these constant temperature studies is that they are not very ecologically relevant given that, under most circumstances, temperatures fluctuate daily in nature. Embryos from most species will spend a portion of each day at male producing and a portion at female producing temperatures, which raises the question of how do the underlying mechanisms of TSD operate when temperatures routinely switch between those that produce males and those that produce females? Further, do maternally derived estrogens affect offspring sex ratios in species with TSD? The latter question relies on physiologically relevant concentrations of estrogens, and when coupled with more ecologically relevant thermal environments, may provide important clues as to how TSD operates in nature (Bowden et al. 2000, Bowden et al. 2014, Carter et al. 2017).

Maternally derived estrogens can affect sex in species with TSD

Eggs of all vertebrate species studied to date possess maternally derived steroids (Groothuis et al. 2005, Paitz and Bowden 2010) and these steroids have been shown to influence embryonic development in numerous taxa (Groothuis et al. 2005). Maternally derived estradiol is present in several species with TSD where sex determination is also known to be sensitive to exogenous estradiol (Conley et al. 1997, Bowden et al. 2000, Bowden et al. 2002, Elf et al. 2002). This led to the hypothesis that females may be able to manipulate offspring sex ratios by varying amounts of estradiol within their eggs (Bowden et al. 2000). Similar hypotheses exist for yolk androgens and their potential impact on sex determination, but the relationship (if any) between maternal androgens and resulting sex ratios is less clear (Radder 2007). A few studies in turtles with TSD have characterized natural patterns of variation in maternal estradiol. For both the red-eared slider (*Trachemys scripta*) and the painted turtle (*Chrysemys picta*), there is a dramatic increase in the amount of estradiol present within a female's second clutch of the nesting season compared to her first clutch of the nesting season (Bowden et al. 2000, Bowden et al. 2001, Paitz and Bowden 2009, Bowden et al. 2011, Carter et al. 2017, Carter et al. 2018). This consistent variation in maternal estradiol levels has been used to investigate the physiological mechanisms

underlying the potential effects of maternal estradiol (Paitz and Bowden 2013). Once eggs are laid and start to develop, levels of estradiol within the egg drop rapidly (Bowden et al. 2002, Elf et al. 2002, Paitz and Bowden 2009), with similar rates of decline happening at male and female producing incubation temperatures (Paitz and Bowden 2009). This drop in levels of estradiol results from maternal estradiol being metabolized (Paitz and Bowden 2008), with estrone sulfate being the primary metabolite formed during embryonic development (Paitz et al. 2012, Paitz et al. 2013). As a consequence of this metabolism, little to no estradiol reaches the embryo from the yolk (Crews et al. 1991, Paitz et al. 2012), rather estradiol almost exclusively reaches the embryo in the form of estrone sulfate (Paitz et al. 2012). Estrone sulfate is present in *T. scripta* eggs as a maternally derived compound and exhibits a positive increase with season (Paitz and Bowden 2013). Importantly, estrone sulfate is capable of inducing the production of female hatchlings when applied exogenously, and appears to be just as potent as estradiol in inducing the production of females (Paitz and Bowden 2011, Paitz et al. 2013). Steroid sulfatase, the enzyme responsible for converting estrone sulfate and estradiol sulfate back to estrone and estradiol respectively is present with *T. scripta* embryos and may modulate the effects of these two metabolites (Paitz et al. 2017). Taken together, this work demonstrates that several endogenous steroids and steroid metabolites are present, and they are capable of influencing sex determination. Two of these compounds, estradiol and estrone sulfate, are known to co-vary in a seasonal manner that could impact offspring sex ratios.

Fluctuating temperature incubation studies are the future of TSD research

While the progress made in understanding TSD was greatly aided by isolating and limiting the effect of temperature through holding temperature constant, it is undeniable that constant temperatures are not biologically realistic for most situations (Bowden et al. 2014, Carter et al. 2017, Carter et al. 2018). In the shallower nests of many freshwater turtle species, temperatures fluctuate daily in a near sinusoidal fashion (Fig. 2). In deeper nests, such as those of sea turtles, the depth of the nest will affect the degree of flux experienced (Tomillo et al. 2017), and eggs towards the top of the nest tend to experience greater daily temperature variation as is found in nests of the snapping turtle (*Chelydra serpentina*) (Telemeco et al. 2016). Yet, we have only a limited amount of information on how embryos respond to temperature fluctuations, in part because it is a challenge to isolate the effects of temperature on phenotype in nature, and, until recently, it has been difficult to mimic the thermal conditions of the field in the laboratory.

Early studies employed relatively simply temperature single or double temperature shifts that were primarily, used to help delineate the temperature sensitive period (Fig. 3A). While these simplistic shifts between male and female producing temperatures were successful in identifying the window of temperature sensitivity, embryos were exposed to relatively long periods at constant temperatures (Wibbels et al. 1991, Valenzuela 2001). Recognizing that conditions in the field were not constant, two alternative approaches have been used to simulate daily temperature cycles which are termed here alternating plateaus and sinusoidal waves (Figs. 3 B and C). Although both methods do expose developing embryos to daily temperature cycles, the way in which temperatures oscillate between male and female (or high and low) temperatures differs greatly. When exposed to alternating plateaus, embryos

are still primarily experiencing constant temperature conditions for much of development with only brief, and rapid transition periods between the thermal minima and maxima (Ashmore and Janzen 2003, Neuwald and Valenzuela 2011). In contrast, embryos exposed to sinusoidal waves spend the majority of time in transition between the thermal minima and maxima, with only brief periods at either extreme. Functionally, sinusoidal waves are much more similar to daily temperature cycles in nature (see Fig. 2), which should provide researchers a greater ability to mimic field conditions in the laboratory. Several studies have even used temperature traces from natural nests to program incubators to fully mimic natural nest temperatures (Pearson and Warner 2018, Thompson et al. 2018). Although still somewhat limited, an increasing number of studies using reptiles with TSD are incorporating daily temperature fluctuations, and predictably, many aspects of the phenotype respond differently to developing under fluctuating conditions relative to constant conditions including hatching success/offspring survival (Les et al. 2009, Warner and Shine 2011), incubation duration (Les et al. 2009), and body size (Les et al. 2007).

For species with TSD, one critically important component of fitness, sex, is among those traits that are responsive to temperature fluctuations. When compared to the thermal responsiveness of other traits, the response is relatively consistent with more female-biased sex ratios being produced in species with TSD pattern 1a under fluctuating conditions relative to constant temperature treatments of the same mean, and with increasing amplitude of fluctuations (Bull 1985, Georges et al. 1994, Les et al. 2007, Paitz et al. 2010, Carter et al. 2017). For example, incubating *C. picta* eggs at a constant 27.0 °C resulted in 100% males while eggs incubated at 27.0 ± 8 °C resulted in 100% females despite the fact both conditions had the same mean temperature (Paitz et al. 2010). While temperature fluctuations typically result in the increased production of females, extreme fluctuations can produce male-biased sex ratios (Neuwald and Valenzuela 2011). This is likely because embryonic development is retarded at thermal extremes, with a greater proportion of development taking place when temperatures are more moderate (Georges et al. 1994, Bowden et al. 2014).

Many recent studies are now focusing on how reptiles may respond to climate change, which is particularly important given that one of the major predictions of climate change models is that temperature variability, including fluctuations, will increase (Alexander et al. 2006, Vasseur et al. 2014). In addition to testing how species may respond to increased temperature fluctuations, many studies are also incorporating field-based components to examine natural variation in thermal conditions (Christie and Geist 2017), as well as long-term field data (Janzen 1994, Schwanz et al. 2010, Carter et al. 2018) to provide a more comprehensive understanding of how climate change may impact thermally sensitive species.

Increasing ecological and biological realism: heat waves, endogenous estrogens, and sex determination

The number of studies on species with TSD using sinusoidal temperature fluctuations in the laboratory are increasing, and the results from these studies clearly indicate that phenotypes

are differentially affected by temperature fluctuations (Les et al. 2007, Paitz et al. 2010). Given that many of these studies also find that sex is a key phenotypic factor that responds differently to constant and fluctuating conditions and is sensitive to estrogens, studies that simultaneously examine these environmental and maternal effects may provide critical information on how TSD operates in nature. Manipulations of estradiol in field incubated eggs have been shown to increase the production of females, but only in milder years since warm years already extremely female biased sex ratios (Warner et al. 2017). In a recent study, we examined how a wide range of temperature fluctuations ($26.5 \pm 2^\circ\text{C} - 29.0 \pm 3^\circ\text{C}$) affected sex ratios across the nesting season in *T. scripta* (Carter et al. 2017). In this species, late season clutches have approximately 10-fold more endogenous estradiol than early season clutches (Paitz and Bowden 2009). For fluctuating temperature treatments that produced mixed sex ratios, late season clutches were more female-biased than early season clutches, suggesting that those clutches have a greater predisposition towards developing as females, likely because of higher endogenous estradiol levels (Carter et al. 2017). Since this study included temperatures that produced all males or all females, it was possible to describe the transitional range of temperature under fluctuating conditions, approximately 1.5°C , and to determine sex ratio reaction norms for early and late season clutches (Carter et al. 2017). Pivotal temperatures were established from these reaction norms; early season clutches had a pivotal temperature that is approximately 0.4°C warmer than late season clutches (Carter et al. 2017). This study provides useful information for describing a number of temperature sensitive responses that are commonly reported for constant temperature studies, but heretofore not for fluctuating conditions; however, it applied the same fluctuating regime throughout development. Although sinusoidal fluctuations are much closer to what embryos would experience during development in the field, most nests experience variation in the magnitude of both temperature ranges and fluctuations across development (see Fig. 2). In addition to variation resulting from seasonal temperature shifts (i.e., temperatures increasing from early summer to late summer), more proximal weather events such as storms can exacerbate or dampen daily temperature fluctuations.

The next step in refining laboratory studies is to capture at least some of this stochasticity, while still allowing for a relatively controlled environment to better understand how temperature is affecting phenotype. To this end, we are now examining how brief exposures to elevated thermal conditions (i.e., heat waves) affects sex determination, and whether there are seasonal or population level differences in the response (Carter et al. 2018). In this study, the heat wave treatment ($29.5 \pm 3^\circ\text{C}$) was applied during the middle third of development for varying durations (3–30 days), with the remainder of development taking place at a cooler, male producing temperature ($27.0 \pm 3^\circ\text{C}$). The results from this work suggest that developing embryos are highly sensitive to heat waves, with an exposure duration of 5 days producing 16% females, and a 50:50 sex ratio produced with only 7.9 days of exposure (Carter et al. 2018). Although the populations did not vary in their sensitivity to the heat wave, late season clutches required approximately two fewer days of exposure than early season clutches to achieve a 50:50 sex ratio, presumably as a result of having higher yolk estradiol levels (Carter et al. 2018). The short duration required to produce females is surprising, and confirms that the use of the temperatures experienced during larger swaths of development (e.g., middle third of incubation) are likely to be misleading for sex ratio

predictions (Georges et al. 2004). Further, it suggests that the interplay between temperature and steroids that was previously demonstrated under constant thermal conditions using exogenous doses of steroids, persists when using more ecologically and biologically relevant treatments.

Next steps

Over the last 20 years, we have made significant strides forward in our understanding of how a variety of factors such as temperature and steroids affect sex determination, but most of this work has examined factors individually. Although we have long recognized the challenges of translating the complexity of the field into the laboratory, we are increasingly recognizing the necessity of moving towards more complex laboratory studies that examine the effects of multiple factors simultaneously on phenotype (e.g., Gutzke et al. 1987, Carter et al. 2018). This need is further exacerbated by the growing threat of global climate change. A recent study by Jensen and colleagues (2018) found that, for some populations of green sea turtles (*Chelonia mydas*) nesting along the great barrier reef, sex ratios appear to be so highly female biased that population persistence is in question. And, based upon their predictions, conditions favoring the production of females extend back approximately 20 years (Jensen et al. 2018). With most current predictions for change emphasizing the likelihood of increasing temperature fluctuations, in addition to increases in average temperatures, this would seem to be an obvious direction for research on species with TSD to focus, keeping in mind that sex is but one aspect of phenotype that is sensitive to temperature.

There are also a number of interesting approaches being used in non-TSD species that could prove useful in studies of TSD. A recent study by Carlo and colleagues (2018) using *Sceloporus undulatus* (a species with genetic sex determination) eggs simulated current and predicted warmer thermal conditions in the laboratory, while simultaneously examining unmanipulated and thermally elevated nests in the field. They found that eggs developing under elevated thermal conditions in either the laboratory or the field had decreased survivorship and there was a negative effect on hatchling body size (Carlo et al. 2018). While the effects on hatchling phenotype are similar to those reported for species with TSD, the approach taken by the authors, examining both lab and field manipulations that are in line with climate change predictions, is one that should be adopted more broadly to help identify traits that are most sensitive to predicted climate change. By simulating average field conditions in the laboratory, it is possible to examine how daily variation in thermal fluctuations affects phenotype (Pearson & Warner 2018). Such an approach can be used to examine how seasonal variation affects phenotype, as in Pearson and Warner (2018), or could be used to examine differences among years provided that long term field data are available.

While the basic biology underlying TSD is fascinating in its own right, it is nearly impossible to ignore the pressing need for novel approaches that will help us better appreciate the current and future status of these thermally sensitive species.

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Figure 1.

Generalized predicted responses of sex ratios produced when eggs are exposed to constant laboratory incubation conditions in species with TSD. (A) TSD patterns 1a (thick line) and 1b (thin line) both have one pivotal temperature that produces a 50:50 sex ratio, while (B) TSD pattern 2 has two pivotal temperatures.

Figure 2.
Thermal profile from a *T. scripta* nest during summer 2007. Temperature recordings were taken every 30 minutes. Figure adapted from Paitz et al. 2010.

Figure 3.

Diagrammatic representations of the types of temperature fluctuation treatments that have been used in the laboratory to study TSD. For simplicity, the responses here are for species with TSD pattern 1a. Panel A illustrates single or double temperature switches where eggs are initially exposed to either a male producing temperature (MPT) or female producing temperature (FPT) and then switched to the other temperature (single) and then back again (double) during embryonic development. Panels B and C illustrate alternating plateaus (B) and sinusoidal waves (C) where eggs cycle between MPT and FPT on a daily basis. The shaded box highlights the major difference between these latter two approaches with alternating plateaus having relatively rapid transitions and longer periods of constant temperatures and sinusoidal waves having longer transition periods.