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Temperature Does Not Affect Hatch Timing in Snapping Turtles (*Chelydra serpentina*)

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ABSTRACT.—**Many oviparous species rely on hatching cues to ensure hatchlings maximize their survival, given the external environmental conditions. In nature, these cues are traditionally environmental (e.g., temperature) or social (e.g., communication between embryos). Examples of both are common throughout ectothermic taxa, particularly reptiles. In the present study, we explored the role of temperature in hatch timing in Snapping Turtles (*Chelydra serpentina*). We allowed embryos to incubate in wild nests for the majority of embryonic development, then isolated embryos in the lab, and maintained them at 24°C until they reached Yntema stage 25. At this developmental stage, external morphological differentiation is complete and yolk resorption begins. We then incubated embryos until pipping across a range of constant but biologically relevant temperatures (20, 23, 25, 28, or 30.5°C). To test whether thermal variance acts as a hatching cue, we also included a treatment in which temperature fluctuated diurnally around a stationary mean (25 ± 4°C). We found that the timing of egg pipping was not related to temperature treatment, thermal fluctuation, or sex of the embryo. Thus, contrary to traditional understanding, temperatures in the range studied do not affect the duration of the final embryonic stage in *C. serpentina* embryos, and a definitive hatching cue in this species is yet unknown.**

Plasticity in hatch timing can significantly increase survival for hatchlings of oviparous reptiles by reducing predation pressure on individuals, increasing group cooperation, prey switching, or conferring the ability to exploit suitable environmental conditions (Carr and Hirth, 1961; Arnold and Wassersug, 1978; Booth, 2002; Spencer and Janzen, 2011). Many oviparous reptiles exhibit cued hatching, whereby a social or environmental cue that suggests a preferred hatch window is detected by embryos and triggers hatching (Warkentin, 2011). Numerous environmental factors have been implicated as cues for hatching across oviparous reptiles, and hatching cues for some species have been established. Crocodilians are well-known to use vocalization as a cue for hatching (Whitehead and Seymour, 1990; Vergne and Mathevon, 2008). Two-toed Amphiumas (*Amphiuma means*) hatch in response to egg inundation with water (Gunzburger, 2003), and Delicate Skinks (*Lampropholis delicata*) can hatch in response to predation risk (Doody and Paull, 2013).

The ability to modify time of hatching is also widespread in turtles (Ewert, 1991; Doody et al., 2001, 2004). For example, Pig-nosed Turtles (*Carettochelys insculpta*) exhibit synchronous hatching when nests fill with river water (via a hypoxic cue), and also hatch earlier when mechanical vibrations are present (Georges et al., 2008; Doody et al., 2012). Embryos of Murray River Turtles (*Emydura macquarii*) hatch synchronously through a yet-unknown cue, wherein less advanced eggs in a clutch hatch much earlier than expected in order to match their advanced siblings (Spencer et al., 2001). This "catch up" phenomenon has also been observed in embryos of Painted Turtles (*Chrysemys picta*), though the mechanisms in this species are unknown (Colbert et al., 2010). Further, vocalizations have been noted in late embryos of Leatherback Turtles (*Dermochelys coriacea*), suggesting that sound may play a role in hatching synchrony (Ferrara et al., 2014).

Despite temperature's prominent role in many aspects of oviparous embryonic development, including development rate (Gillooly et al., 2002; Rollinson et al., 2018), and thus incubation time, the role of temperature as a hatching cue is unknown in many species (Spencer and Janzen, 2014). Temperature may affect hatch timing either by increasing the rate of metabolic function in embryos, or by serving as a direct cue to hatch. As the rate of physiological processes in ectotherm embryos are largely influenced by temperature (Gillooly et al., 2002), embryos may simply hatch sooner if they experience warm temperatures during the period of yolk resorption. Alternatively, temperature may act as a cue unrelated to development rate. For example, declining or low temperatures at the end of the activity season may signal to embryos that hatching should occur immediately because winter is approaching.

The present study aimed to determine whether temperature affects hatch timing in a population of Snapping Turtles (*Chelydra serpentina*) near this species' northern range limit. We standardized morphological age of wild-incubated embryos prior to the experiment, then focused on the relationship between temperature and pipping date only during the final stage of the embryonic phase, i.e., when the embryo has completed external morphological differentiation (Yntema, 1968), but has not yet hatched. The focal population in Algonquin Provincial Park, Ontario, Canada, is under a developmental time constraint where, in many years, there is insufficient thermal energy for embryos to develop completely and embryo mortality through freezing is common in the fall (Edge et al., 2017). Because embryos experience a time constraint, we hypothesized that embryos would exhibit accelerated hatch timing under warm conditions, which, in the wild, could allow embryos to emerge from the nest sooner.

MATERIALS AND METHODS

Study System and Experimental Setup.—We used *C. serpentina* embryos from a northern population located in Algonquin Provincial Park, Ontario, Canada (45.8372°N, 78.3791°W). Seven females laid clutches between 18 June and 23 June 2017. Shortly after laying, eggs from each nest were excavated and numbered with a fine-tip pencil in the order they were removed. We measured, weighed, and reburied eggs in their natural nest cavity within 24 h, replacing them in approximately their original lay order. We also placed an iButton DS1921G temperature logger

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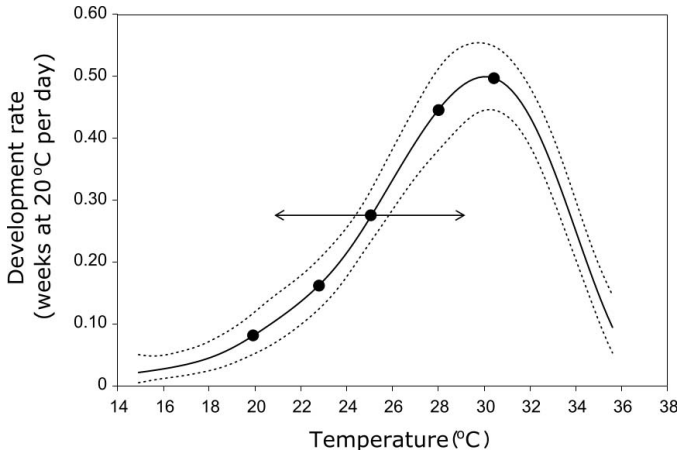

FIG. 1. Thermal performance curve for embryonic development in *Chelydra serpentina* from Algonquin Provincial Park, Ontario, Canada, with the location of treatments plotted along the development rate curve. Modified from Rollinson et al. (2018). Development is expressed as the number of weeks of development that would otherwise occur at a temperature of 20°C; in other words, 1 d spent at a constant temperature of 28°C is equivalent to the amount of development that occurs over about 0.4 wk (2.8 d) at 20°C. Arrows represent the range of temperatures experienced in the temperature fluctuation treatment, with a mean temperature of 25°C.

(Maxim Integrated, San Jose, California, USA) in the center of each nest. The nests incubated naturally until 14 September 2017. We then excavated approximately two-thirds of eggs from each clutch, and moved them to the University of Toronto, where we maintained them at 24°C in Echotherm Chilling Incubators (Torrey Pines Scientific, Carlsbad, California, USA), until the experiment began.

The animals used herein contributed data to this experiment, as well as to a long-term study on natural primary sex ratios for the focal population of *C. serpentina* (Massey, unpubl. data), and thus, the thermal histories for each nest were not manipulated to be similar.

Temperature Treatments.—We established five constant-temperature treatments (20, 23, 25, 28, or 30.5°C) and one temperature treatment that fluctuated on a diurnal cycle (25.1 ± 4°C). The goal of the fluctuating treatment was to mimic daily temperature variation, allowing us to explore the possibility that *C. serpentina* may hatch preferentially under fluctuating rather than constant temperature conditions. Importantly, the temperature fluctuations were chosen based on the thermal performance curve (TPC) for development rate in this population (Rollinson et al., 2018). The fluctuations were centered around the midpoint of the TPC's linear portion (Fig. 1), such that the sum of daily temperature (between 29.1 and 21.1°C) had the same predicted effect on developmental advancement as the constant 25°C treatment. Given that the late embryonic phase could still allow for some manner of development, or another temperature-dependent process such as yolk resorption, controlling for development rate across the two 25°C treatments enabled us to determine the influence of temperature variation alone on hatch timing without differential development rates confounding our results. We placed iButton DS1921 G temperature loggers (Maxim Integrated) in each treatment incubator in identical containers to the embryos to monitor incubation temperatures hourly throughout the experiment.

Experimental Design.—We randomly assigned eggs to a treatment once a sample egg in the clutch had reached Yntema stage 25; Yntema stage 26 represents hatching (Yntema, 1968). We based development stage assessments on morphological comparisons between our embryos and photographs delineating each developmental stage described by Yntema (1968), using the state of four characters: carapace, eyes, digits, and pigmentation. If characters suggested different stages, we took the average of the four characters. We considered all embryos in a given clutch (i.e., from a given female) to be the same stage of development. Therefore, dissecting one embryo and estimating its Yntema stage provided an estimate of the Yntema stage of the entire clutch.

The nests of *C. serpentina* are large and have a thermal gradient, which is generally thought to increase the development rate of the bottom-most eggs relative to eggs at the top of the nest. It was therefore possible that using only a few embryos to characterize the developmental stage of a clutch at the beginning of the experiment imposed a bias in our analyses. Because we sequentially numbered all eggs used in this experiment, we were able to use egg number as a proxy for burial depth in our analyses, allowing us to control for the possibility that eggs obtained from deeper in the nest were more developmentally advanced.

Within each temperature treatment, we incubated each subset of embryos (i.e., belonging to a given clutch) in a 7.62 by 5.08–cm cylindrical plastic container half-filled with moist vermiculite. We moistened each container daily using a spray bottle, such that the vermiculite in each container was qualitatively similar in appearance and texture, and that eggs were smooth and undented. However, we did not take measures of moisture loss (e.g., daily weighing of containers).

Once in treatments, we monitored eggs daily for pipping. We recorded hatch date for each embryo, and we removed hatched individuals from containers immediately.

Statistical Analyses.—We conducted all statistical analyses in the R environment (R Core Development Team, 2017). We constructed linear mixed-effects models using the R package lme4 (Bates et al., 2015), with clutch ID as a random intercept, and examined the effect of temperature as a grouping variable. We also examined the effects of sex and egg depth on hatch timing. We used normal approximations to obtain parameter-specific P values for each treatment temperature, egg depth, and sex. P values < 0.05 were considered to be statistically significant.

RESULTS

Eggs were incubated at 24.0°C for an average of 26 d before staging determined they had entered Yntema stage 25. Embryonic mortality over the course of the experiment was approximately 10% (9 out of 86 embryos failed to hatch). Dead embryos were not included in statistical analyses. None of the hatched embryos had noticeable deformities or apparent abnormalities. Of the 77 embryos utilized, 59 were reliably sexed post-hatch. Embryos possessing a mix of male and female characteristics (e.g., ovotestes) were excluded from all sex-based analyses, but still included in purely temperature-based analyses. Of the reliably sexed embryos, 45 were females and 14 were males.

The time period from Yntema stage 25 to pipping across all treatments ranged from 3 to 15 d, with a mean incubation duration of 9 d. Hatch date was approximately normally distributed, and neither a log-transformation, nor a square-root transformation changed our conclusions. Neither incubation temperature nor sex of the embryo had a significant effect on

TABLE 1. Parameter estimates from the linear mixed-effect model predicting the effects of temperature and egg depth on hatch timing (n = 77 hatched turtles) in *Chelydra serpentina* from Algonquin Provincial Park, Ontario, Canada. "20°C" is the reference category.

Parameter	Effect type	Estimate[a]	SE[b]	t value	P
Maternal ID	Random	2.24	1.50	–	–
Residual	Random	5.36	2.32	–	–
Intercept	Fixed	9.91	0.922	10.7	<0.001
23°C	Fixed	−0.231	1.08	−0.213	0.832
25°C	Fixed	−1.31	0.923	−1.42	0.156
25.1 +/− 4°C	Fixed	−1.11	1.00	−1.11	0.268
28°C	Fixed	−0.831	1.07	−0.774	0.439
30.5°C	Fixed	−1.10	1.10	−1.00	0.317
Egg depth	Fixed	−0.00188	0.0308	−0.0610	0.951

[a] Parameter estimates for random effects are variance estimates.
[b] Standard error estimates for random effects are standard deviation estimates.

TABLE 2. Parameter estimates from the linear mixed-effect model predicting the effect of sex on hatch timing (n = 59 sexed turtles) in *Chelydra serpentina* from Algonquin Provincial Park, Ontario, Canada, with "male" as the reference category.

Parameter	Effect type	Estimate[a]	SE[b]	t value	P
Maternal ID	Random	1.65	1.28	–	–
Residual	Random	3.47	1.86	–	–
Intercept	Fixed	8.76	0.590	14.9	<0.001
Sex (female)	Fixed	0.430	0.615	0.699	0.480

[a] Parameter estimates for random effects are variance estimates.
[b] Standard error estimates for random effects are standard deviation estimates.

hatch timing (Tables 1, 2; Fig. 2). Adding egg depth as a covariate in our model did not explain significant variation in hatch timing, suggesting that differences in hatch timing because of differences in burial depth were not substantial (Table 1). Further, there was no interaction between sex and temperature treatment ($P = 0.30$), suggesting both sexes hatched at the same time regardless of temperature.

DISCUSSION

Our study supports the novel finding that hatch timing during the final embryonic stage in *C. serpentina* is independent of temperature. Incubation temperatures, both constant and fluctuating, had no significant effect on hatch timing of *C. serpentina* embryos (Table 1; Fig. 2). These results also provide evidence that the sex of an embryo has no bearing on its hatching time (Table 2).

As temperature overwhelmingly governs the rate of physiological processes in embryos, one might expect that warmer temperatures may lead to faster hatching, perhaps by increasing the rate of yolk resorption. Under this scenario, one would assume embryos do not have an external hatching cue, and simply pip when differentiation is complete and yolk is resorbed. Contrary to expectations, hatching did not occur sooner in warmer treatments. Although we did not collect data on yolk size at hatching in the present experiment, others have noted that yolk size at hatching is highly variable, and due largely to the incubation regime experienced throughout the entirety of development, rather than in the final embryonic stage (Reece et al., 2002; Burgess et al., 2006). It is therefore likely that temperature does not act as a cue for hatching via differential yolk metabolism, or yolk sizes would always be consistent at hatching. Further, our findings are consistent with those of Andrews (2004) who found that, although temperature greatly influences embryonic development rates throughout overall incubation period, it has very little effect on the metabolism of late-stage embryos in particular.

We also explored the possibility that a thermal cue unrelated to developmental rate can trigger hatching in final-stage embryos of *C. serpentina*. We found that, when controlling for developmental rate, short-term thermal fluctuations within this range do not affect hatch timing. However, the possibility of both cold temperatures (<20°C, outside of the range we tested) and a decreasing thermal mean acting as hatching cues cannot

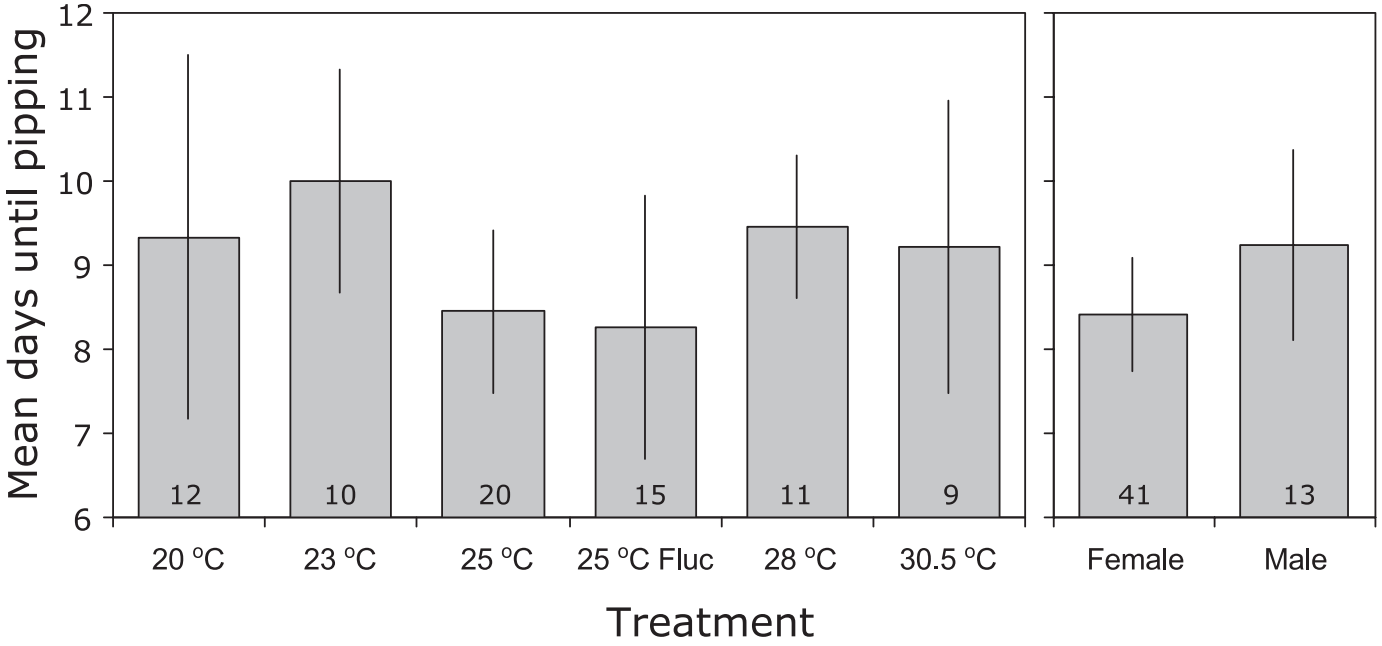

FIG. 2. Mean number of days from Yntema stage 25 to hatching (Yntema stage 26) for each temperature treatment, and by sex, in *Chelydra serpentina* from Algonquin Provincial Park, Ontario, Canada. Error bars represent 95% confidence intervals. Sample sizes in each treatment are the values within bars.

be precluded by this study. Embryos of *C. serpentina* take at least 2 mo to develop at their thermal optimum of 30°C (Yntema, 1968); therefore, cooling ambient temperatures in early fall (Bobyn and Brooks, 1994) should intersect with the occurrence of late-stage embryonic development in Algonquin Provincial Park. Given this biological context, we suggest a decreasing thermal mean or exposure to cool temperatures may yet be potential thermal cues for hatching in *C. serpentina*. Furthermore, other putative environmental cues such as mechanical perturbations, vocalizations, drastic changes in moisture, or chemical signals that were not isolated in the laboratory may act as hatching cues for *C. serpentina*.

Our inclusion of sex as a potential influence on hatch timing was to account for the possibility that sexes may experience different hatch times during the final embryonic stage. In Algonquin Provincial Park, *C. serpentina* has a characteristic pattern of temperature-dependent sex determination, such that low (<22°C) and high (>28°C) incubation temperatures produce primarily females, and intermediate (24–26°C) temperatures produce primarily males (Massey et al., 2018). Theory suggests that temperature-dependent sex determination could be selectively advantageous when individual fitness depends on the interaction of sex and the incubation environment (Charnov and Bull, 1977); in the case of *C. serpentina*, it may be advantageous for males to reach maturity sooner and with a larger body size. We might then expect males to hatch sooner after reaching Yntema stage 25, in order to take advantage of the remaining growing season and maximize their body size more quickly, especially in limiting northern climates (Bobyn and Brooks, 1994). However, in the present study, we found no significant difference in hatch timing between males and females. Temperatures experienced throughout the incubation period, rather than those only from Yntema stages 25–26, are likely responsible for any sex-specific, fitness-optimizing effects (Janzen, 1995; Rhen and Lang, 1995).

In chelonians, moisture is a significant source of variation in incubation time (Packard et al., 1987; Janzen et al., 1990; McGehee, 1990). Higher moisture levels increase the rate of embryonic growth and lipid uptake in *C. serpentina,* while also lengthening the overall incubation period (Morris et al., 1983; Packard et al., 1988). Therefore, a potential source of error in the present study is lack of standardization of moisture levels. Although we maintained moisture for each egg in a qualitative manner (i.e., ensuring the vermiculite was a consistent texture, and that eggs were visually similar and undented), we did not take quantitative measurements of moisture levels, nor did we control for moisture uptake while eggs were naturally incubating in the field. In future investigations of hatch timing, we recommend that eggs be kept in a standardized moisture setting for the entirety of incubation.

In some turtles, hatching can be triggered by vibrations, and possibly vocalizations (Vijaya, 1983; Doody et al., 2001; Georges et al., 2008; Ferrara et al., 2014). However, vibrations have not been reported as a hatch cue for *C. serpentina*, and vocalizations are not known to occur in embryos of this species. If these factors do affect hatch timing in *C. serpentina,* it is possible that the mechanical vibrations and noises from the incubator machinery may have affected hatching through overwhelming any embryo vocalizations and vibrations or through producing confounding noises and mechanical disturbances. Given that incubators are typically noisy and reliant on moving mechanical components, such as fans, at this time we cannot propose a realistic alternative that would isolate these factors in future experiments.

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