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RESEARCH PAPER

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Environmentally induced phenotypic plasticity explains hatching synchrony in the freshwater turtle *Chrysemys picta*

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Abstract

Environmentally cued hatching allows embryos to alter the time of hatching in relation to environment through phenotypic plasticity. Spatially variable temperatures within shallow nests of many freshwater turtles cause asynchronous development of embryos within clutches, yet neonates still hatch synchronously either by hatching early or via metabolic compensation. Metabolic compensation and changes in circadian rhythms presumably enable embryos to adjust their developmental rates to catch up to more advanced embryos within the nest. Hatchlings of the North American freshwater turtle *Chrysemys picta* usually overwinter within the nest and emerge the following spring, but still hatch synchronously via hatching early. Here, we used rates of oxygen consumption and heart rate profiles to investigate the metabolic rates of clutches of *C. picta* developing in conditions that result in asynchronous development to determine if compensatory changes in metabolism occur during incubation. Embryos hatched synchronously and displayed circadian rhythms throughout incubation, but exhibited no evidence of metabolic compensation. Phenotypic traits of hatchlings, including body size and righting performance, were also not affected by asynchronous development. We conclude that less developed embryos of *C. picta* hatch synchronously with their clutch-mates by hatching early, which does not appear to inflict a fitness cost to individuals. The ultimate mechanism for synchronous hatching in *C. picta* could be for hatchlings to ensure an optimal overwintering position within the center of the nest. Consequently, immediate fitness costs will not hinder hatchling survival. The geographic location, as well as environmental and genetic factors unique to populations, can all influence hatching behavior in turtles through phenotypic plasticity. Hence, synchronous hatching is an adaptive bet-hedging strategy in turtles, but the mechanisms to achieve it are diverse.

Highlights

Our study demonstrates that synchronous hatching in *Chrysemys picta* results from environmentally induced phenotypic plasticity. We monitored heart rates and respiration rates during embryonic development to reveal that neonates can hatch 2–3 days earlier than expected without metabolic compensation or identifiable physiological consequence. Thus, synchronous hatching is likely a bet-hedging

adaptive strategy in turtles, and the mechanisms to achieve it are diverse, suggesting a likelihood of convergent evolution of hatching behavior in turtles.

KEYWORDS
Circadian rhythms, convergent evolution, embryonic development, environmentally cued hatching (ECH), metabolic compensation, turtle

1 | INTRODUCTION

Phenotypic plasticity is the interaction between environmental factors and genotype, which results in the expression of an environment-specific phenotype and can impact fitness and survival (Scheiner, 1993; West-Eberhard, 1989, 2003). Variability in the environments experienced by embryos during development influences adaptive phenotypic plasticity (Scheiner, 1993; Scheiner & Lyman, 1991), altering the behavior, morphology, and physiology of the embryo (Doody, 2011). Plasticity in developmental response to changes in the environment stimulates an expression of traits that reduce immediate threats to their survival, such as hatching behavior, morphology, and performance (mobility; Doody, 2011; Shine, 2004).

Reptilian embryos can alter the timing of their hatching in response to environmental conditions through phenotypic plasticity within boundaries set by incubation temperature, which is known as environmentally cued hatching (ECH; Colbert, Spencer, & Janzen, 2010; McGlashan, Spencer, & Old, 2012; Spencer & Janzen, 2011; Spencer, Thompson, & Banks, 2001). There are three forms of ECH: early, synchronous, and delayed hatching (Doody, 2011; Spencer & Janzen, 2011). These mechanisms are not mutually exclusive, and some species show multiple forms of ECH (Warkentin & Calswell, 2009, Doody, 2011). Timing of hatching can affect the survival of the offspring both immediately (risk of predation) and in the future (resource availability, size, and performance of an individual; Brinkhof, Anton, Hage, & Simon, 1993; Colbert et al., 2010; O'Donoghue & Boutin, 1995; Sheldon, Kruuk, Merilä, & Crespi, 2003; Spencer & Janzen, 2011; Tucker, Paukstis, & Janzen, 2008). The ultimate mechanisms of ECH in reptiles are still poorly understood, but the proximate mechanisms yield variation in the developmental stage at hatching, and/or a reduction in the variation in incubation time, which may increase an individual's chance of survival through a tradeoff between the risks and benefits of hatching (Gomez-Mestre, Wiens, & Warkentin, 2008; Warkentin & Calswell, 2009).

Temperature significantly affects all biological processes from the molecular level to organism-level processes, including metabolism and growth (Rome, Stevens, & John-Alder, 1992; Johnston, Vieira, & Hill, 1996; Packard, Packard, Miller, & Boardman, 1988; Wilhoft, 1958). Embryonic development and incubation period are sensitive to temperature changes, with warmer temperatures accelerating growth and developmental rate and therefore shortening incubation periods (Booth, 1998; Deeming & Ferguson, 1991; Monaghan, 2008). In the nests of many freshwater turtles, a vertical thermal gradient exists because of the diel fluctuations of temperature, and seasonal variation in temperatures (Telemeco et al., 2016; Thompson, 1988, 1997; Thompson, Packard, Packard, & Rose, 1996). The temperature thus affects eggs differently depending on their position in the nest (Georges, Limpus, & Stoutjesdijk, 1994), with eggs at the top of the nest generally experiencing warmer temperatures than eggs at the bottom, and these differences may be as high as 6°C (Thompson, 1988, 1997). Such thermal variation within the nest has the potential to cause embryos to develop at different rates (asynchronous development) during the incubation period. Despite such variation in developmental rate between embryos, offspring of many species still hatch synchronously (Colbert et al., 2010; McGlashan et al., 2012; Spencer et al., 2001; Thompson, 1989).

Synchrony can occur when embryos delay hatching until stimulated by an environmental cue (Doody et al., 2001; Doody, Stewart, Camacho, & Christian, 2012; Webb, Choquenot, & Whitehead, 1986), or when less developed embryos, either adjust their developmental rate through metabolic compensation (McGlashan et al., 2012) or hatch at an earlier developmental stage (Colbert et al., 2010; Spencer & Janzen, 2011). Previous experimental studies have shown that embryos that are initially less developed (i.e., incubating at cooler temperatures) advance their hatching date by 2–3 days to hatch at approximately the same time as their warmer incubated siblings (Colbert et al., 2010; McGlashan et al., 2012; Spencer et al., 2001). Under these conditions, embryos were seen to increase their developmental rates in the final third of incubation to "catch up" to the more advanced embryos, likely with a cost (increased heart and respiration rates, and reduced yolk sac volume), known as metabolic compensation (McGlashan et al., 2012). Precocial species exhibit a decline in metabolic rate several days before hatching (Booth, 2000; Booth & Astill, 2001; Du, Zhao, & Shine, 2010; McGlashan et al., 2012; Thompson, 1989). This period is considered a "resting stage" where (a) tissue growth is essentially complete, (b) sensory, neuromuscular, and thermoregulatory systems mature (Thompson, 1989; Vleck, Hoyt, & Vleck, 1979; Vleck, Vleck, & Hoyt, 1980; Webb et al., 1986), and (c) any necessary acclimation can occur with minimal metabolic cost (McGlashan et al., 2012). Metabolic compensation is essential to survival as any reduction in developmental time can adversely affect the growth and performance of hatchlings (Janzen, 1993; Vince & Chinn, 1971; Warkentin, 1995). Remarkably, metabolic compensation does not incur a reduction in hatchling size and locomotor performance (McGlashan et al., 2012; McGlashan, Loudon, Thompson, & Spencer, 2015).

Several factors have likely driven the evolution of synchronous hatching in turtles. Synchronous hatching allows hatchlings to

emerge from a nest together, thereby increasing their chance of survival by swamping predators, or reducing their exposure to prey switching generalist predators (Bradbury, Campana, Bentzen, & Snelgrove, 2004; Santos et al., 2016; Sih & Moore, 1993; Tucker et al., 2008; Warkentin, 1995, 2000). Embryos can hatch early and synchronously in response to a predatory attack (Vitt, 1991; Warkentin, 1995, 2000), or when the risk of predation is reduced (Bradbury et al., 2004). Environmental cues may also stimulate synchronous hatching, as with pig-nosed turtles (*Carettochelys insculpta*), which can delay their hatching until an environmental trigger (hypoxia) stimulates hatching (Doody et al., 2001, 2012; Webb et al., 1986). Painted turtles (*Chrysemys picta*) hatch synchronously apparently through early hatching, which could be an overwintering strategy to improve their positioning within the nest to increase their chance of survival until emerging the following spring (Colbert et al., 2010). In contrast, Murray river short-necked turtles (*Emydura macquarii*) and eastern long-necked turtles (*Chelodina longicollis*) both hatch synchronously as a predator avoidance strategy (Tucker et al., 2008) through metabolic compensation, which is evident through an increased rate of oxygen consumption ($\dot{V}O_2$), and altered heart rate patterns during development (McGlashan et al., 2012, 2015). Circadian rhythms in heart rate also appear during development, and embryos might be able to detect the heart rate rhythm of their clutch-mates to communicate developmental stage, and thus respond to perceived differences by metabolically compensating (Aubret, Blanvillain, Bignon, & Kok, 2016; Loudon, Spencer, Strassmeyer, & Harland, 2013; McGlashan et al., 2015).

Offspring in many populations of freshwater turtles in the northern hemisphere overwinter in the natal nest, where hatchlings delay emergence until the following spring (Gibbons, 2013). *C. picta* frequently overwinter in the natal nest, yet they exhibit synchronous hatching (Colbert et al., 2010). It is not yet known whether *C. picta* also uses metabolic compensation to hatch early. For *C. picta*, winter is a critical period for hatchling survival as temperatures within the nest can drop below −10°C, and mortality because of freezing approaches 100% when temperatures fall to −14°C (Packard & Packard, 2004; Packard, Fasano, Attaway, Lohmiller, & Lynch, 1997). Ensuring an optimum overwintering position in the nest, where conditions are ideal for reducing energy expended or reducing the chance of mortality from freezing is a significant benefit of hatching early (Colbert et al., 2010; Spencer & Janzen, 2014). Still, early hatching *C. picta* has fitness costs, with a reduction in performance exhibited for up to 9 months after hatching (Colbert et al., 2010). This reduced performance suggests that embryos are missing an important stage of development, which may be vital for precocial species (Peterson & Kruegl, 2005; Vleck et al., 1979).

Synchronous hatching may be an adaptive "bet-hedging" strategy, with the mechanisms to achieve it differing among turtle species (Colbert et al., 2010; Doody et al., 2001; McGlashan et al., 2012). Metabolic compensation in Australian chelids enables embryos to complete development, which prevents them from being disadvantaged when they emerge from the nest (McGlashan et al., 2012). Metabolic compensation can be costly in terms of the yolk reserves necessary for post-hatching growth and development (McGlashan et al., 2012; Radder, Shanbhag, & Saidapur, 2002, 2004). Because neonates of *C. picta* typically remain in the nest overwinter, we would not expect to see metabolic compensation in this species, as using yolk reserves before hatching could be detrimental to their survival in the nest (Willette, Tucker, & Janzen, 2005). The aim of this study was to determine that synchronous hatching occurs in *C. picta* by hatching early and not through metabolic compensation. If less advanced embryos within asynchronous clutches metabolically "catch up" to more advanced embryos, then we predict an increase in $\dot{V}O_2$ and heart rate compared with synchronous clutches. We investigated metabolic rate by measuring $\dot{V}O_2$ and heart rate profiles to detect variation among embryos in controlled conditions of synchronous and asynchronous incubation. Phenotypic and performance measures were also used to reflect developmental variation.

2 | MATERIALS AND METHODS

2.1 | Egg collection and incubation

Eggs of *C. picta* were collected from freshly built nests at the Thomson Causeway Recreation Area, Thomson, IL, from the June 4–7, 2012. Eggs were excavated from nests within 5 hr of oviposition and were placed in plastic containers with moist soil. Ten clutches (with an average of 11 eggs per clutch) were obtained over a 2-day period and transported to Iowa State University. All eggs were uniquely marked using an HB pencil, weighed (g), and placed in plastic containers ($200 \times 100 \times 50\ mm^3$), half buried in a 1:1 mixture of vermiculite and deionised water (−150 kPa; Colbert et al., 2010; Spencer et al., 2001). To account for evaporation and maintain water potential of vermiculite, containers were weighed and rehydrated weekly. To counteract potential thermal gradients within incubators, the position of the containers were also rotated weekly (Janzen, 1993).

2.2 | Incubation method

The treatment (asynchronous) group and the control (synchronous) group were set up using protocols similar to Spencer et al. (2001) and Colbert et al. (2010). Both the treatment and control groups contained five clutches, each with nine eggs, for a total of 45 eggs per treatment. To establish asynchrony within the five asynchronous clutches, three of the nine eggs from each clutch were incubated at 30°C (asynchronous-more advanced), with the remaining six eggs (asynchronous-target) incubated at 26°C for the first 7 days of incubation. After 7 days, the asynchronous-target eggs were reunited with the three asynchronous-more advanced eggs. The asynchronous-more advanced eggs were positioned between the asynchronous-target eggs (within 10 mm; Figure 1). All containers were then incubated at 28°C until hatching. The remaining five clutches in the control (synchronous) group were arranged using the same procedure to control for egg movement in the experimental setup. Three

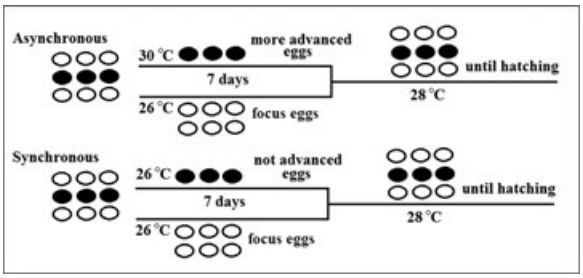

FIGURE 1 Eggs from each clutch were divided and incubated separately for 7 days, and then returned to the same containers to continue incubation at 28°C. Asynchronous group: three more advanced eggs (black) were incubated at 30°C, and six eggs (white) were incubated at 26°C for 7 days. Synchronous group: three not advanced eggs (black) were incubated at 26°C, and six eggs (white) were incubated at 26°C for 7 days. All eggs were then placed in 28°C incubators until the completion of incubation

eggs (synchronous-not advanced) were incubated at 26°C for the 7-day period. The remaining six eggs (synchronous-target) were also incubated at 26°C so that all nine eggs were still at the same developmental stage when reunited (Spencer et al., 2001). After eggs were reunited, containers were incubated at 28°C until hatching. Each clutch was incubated in a separate container throughout the experiment. Heart rates and $\dot{V}O_2$ of target embryos from asynchronous and synchronous groups were monitored weekly, and containers were opened every 2–3 days to allow fresh air exchange throughout incubation until the commencement of pipping/hatching.

2.3 | Oxygen consumption

The rate of oxygen consumption ($\dot{V}O_2$) was measured from Week 4–8 of the 8-week incubation period. One target egg from each clutch (five asynchronous and five synchronous) was selected for O_2 analysis each week. $\dot{V}O_2$ of each egg was measured twice within a 24 hr period and the average of both measures was used. Oxygen (%) was measured in a closed system using an S-3A oxygen analyzer (AEI Technologies, Pittsburgh, PA). The analyzer was first calibrated (20.94% O_2) using 50 cc of atmospheric air injected using a syringe and syringe pump (Vleck, 1987). Airtight chambers were constructed from 500 ml metal paint cans and sealed with lids that were fitted with a two-way stopcock. Eggs were placed on moist cotton balls (to keep them from desiccating) in the opened chamber and allowed to come to thermal equilibrium in the 28°C incubator approximately 5 min before sealing. The lid was sealed and an initial (F_I) air sample was taken from the outflow port with a syringe fitted with a stopcock. The chamber pressure was allowed to equilibrate before the outflow port was sealed and containers were carefully placed in the 28°C incubator. A final (F_E) sample was taken at the conclusion before the egg was returned to its original incubator container. To measure $\dot{V}O_2$, eggs remained in the airtight chambers for 1–5 hr, depending on the stage of incubation. A blank control was in place to account for any leakage or spontaneous change in O_2 not caused by the egg. Temperature and air pressure were recorded at the beginning of the experiment to allow correction to standard temperature pressure volume of each can. F_I and F_E samples were then put through the O_2 analyzer via two 3 ml syringe barrels, one with silica gel and the other with a combination of ascarite and soda lime to remove CO_2 and moisture from the sample. Fractional O_2 concentrations of F_I and F_E samples were analyzed, and $\dot{V}O_2$ was calculated using the equation $\dot{V}O2 = V\frac{(FI-FE)}{t(1-FE)}$ where V represents the dry gas volume in the chamber and t represents time between F_I and F_E samples (Vleck, 1987; Vleck et al., 1979, 1980)

2.4 | Heart rate measurements

Heart rates (bpm) were measured from Weeks 3–8 of incubation to determine heart rate profile, circadian rhythm, and metabolic compensation. Heart rates were used to infer metabolic rate (Du, Radder, Sun, & Shine, 2009; Wallace & Jones, 2008) of embryos and identify any difference between asynchronous and synchronous groups. One target egg from each clutch was removed from its incubator container and immediately placed in a Buddy digital egg monitor system (Avian Biotech, U.K.) in complete darkness. Buddy monitors provide a noninvasive method to sense and amplify cardiovascular signals of an embryo using variations of infrared radiation intensity that is absorbed and reflected by arterial vasculature in the egg (Lierz, Gooss, & Hafez, 2006). Because of the potential heating effect on the embryo from the Buddy system, care was taken not to exceed a maximum time of 10 min (Sartori, Taylor, Abe, & Crossley, 2015). Three readings were taken once heart rates had stabilized (within 2 min; McGlashan et al., 2015; Sartori et al., 2015). Heart rates were calculated as the mean of the three readings, which were taken at 30 s intervals. Eggs were not disturbed during the recording period, thus any embryonic movement could be detected and distinguished from heart rate readings, in which case we waited for movement to cease and heart rate to stabilize before recording again (McGlashan et al., 2015). Each week a different egg was used to record heart rate. The heart rate of each egg was taken at four time intervals over a 24-hr period (0500, 1100, 1700, and 2300 hr) once a week to identify circadian rhythms and the average daily heart rate for the weekly profile.

2.5 | Pipping and post-hatching measurements

To determine incubation period, eggs were inspected for signs of pipping (initial perforation of the eggshell with caruncle) twice daily (Gutzke, Paukstis, & Packard, 1984). The incubation period was measured as time in days from oviposition until pipping. Neonates were allowed to hatch unassisted, after which they were weighed (g), straight carapace length and width, straight plastron length and width were measured (mm), and righting ability was recorded within 12 hr of hatching. To measure righting ability, all hatchlings were placed on their back (carapace), and the time taken to right themselves was measured, as were latency to begin moving and total active righting time. All tests were terminated after 180 s. Measurements of righting ability were used as an index of

neuromuscular development (Colbert et al., 2010; Peterson & Kruegl, 2005; Vleck et al., 1979, 1980).

2.6 | Data analysis

Clutch was included as a random effect in all models and where treatment effects were significant, post-hoc Tukey tests were used to determine where pairwise differences occurred. Incubation period was analyzed in all four independent variables (asynchronous-more advanced, asynchronous-target, and synchronous-more advanced synchronous-target) using a linear mixed model (PROC MIXED; SAS, version 9.3, SAS Institute, Cary, NC) and egg mass was used as a covariate. Embryonic $\dot{V}O_2$ and heart rates were also compared among weeks and among asynchronous-target and synchronous-target eggs using PROC MIXED. Egg mass and week of incubation were included in the model as covariates.

Circadian rhythms were determined through daily heart rate fluctuations, which were calculated as a difference from the mean diel rate at each time point. Peaks were aligned with time of day in both asynchronous and synchronous groups to determine circadian rhythms. Differences from the mean diel rate were analyzed between asynchronous and synchronous groups each week and at each time point as a repeated measure using PROC MIXED with egg mass as a covariate. The differences between heart rates at each time point (i.e., −6hr to peak) within treatment groups were analyzed using a one-way analysis of variance.

Phenotypic variation in carapace length and width, plastron length and width, and hatchling mass were compared among asynchronous and synchronous groups using a multivariate analysis of variance with egg mass as a covariate (PROC GLM). Hatchling righting ability was analyzed using PROC MIXED with hatchling mass as a covariate. A binomial test was used to assess propensity to right of hatchlings (PROC GLIMMIX).

All $\dot{V}O_2$, heart rate, and growth data analyzed with PROC MIXED were log transformed before analysis to ensure linearity in the models. Examination of residuals ensured that all assumptions of univariate parametric statistics were met. In the multivariate analysis, the assumption of multivariate normality could not be directly tested because of sample size limitations. Thus, Pillai's Trace was used as a test statistic because it is robust to violations of multivariate normality (Scheiner, 2001). Statistical significance was determined at the 0.05 Type I error level, and data are presented as the mean ± standard error of mean.

3 | RESULTS

3.1 | Incubation period

Of the 89 eggs, 11 failed to hatch (2 asynchronous-target eggs, 4 synchronous-target eggs, 3 asynchronous-more advanced eggs, and 2 synchronous-not advanced eggs). Hatching synchrony occurred within clutches in both asynchronous (treatment; $t_{73} = 1.84$, $p = 0.2641$) and synchronous groups (control; $t_{73} = 0.04$, $p = 1$), with incubation period averaging 54 ± 0.3 and 55 ± 0.3 days, respectively (Figure 2). The incubation period of eggs in asynchronous or synchronous clutches was significantly affected by treatment ($F_{3,73} = 4.30$, $p = 0.0076$). Pairwise comparisons showed that asynchrony was established, as the incubation period of asynchronous-more advanced and synchronous-not advanced eggs differed by 2 days ($t_{73} = 2.84$, $p = 0.0293$). Asynchronous-more advanced eggs also hatched earlier than synchronous-target eggs and synchronous-not advanced eggs within clutches ($t_{73} = 3.25$, $p = 0.0091$), but incubation period did not differ between asynchronous-target eggs and synchronous-target eggs ($t_{73} = 1.90$, $p = 0.2361$).

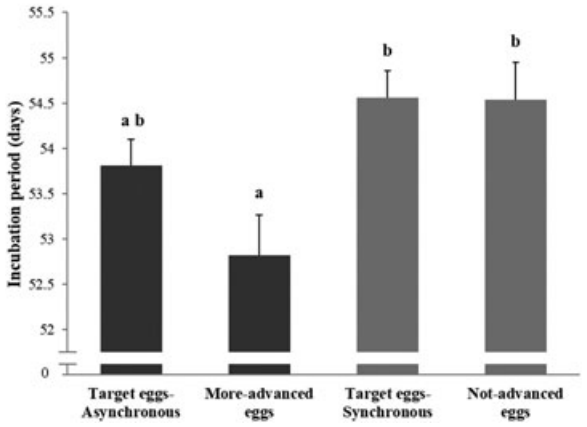

FIGURE 2 Mean incubation period of eggs in asynchronous and synchronous groups, ±SE, $N = 78$. Letters indicate significant pairwise differences among weeks in both asynchronous and synchronous groups, and identical letters indicate no difference. SE: standard error of mean

3.2 | Oxygen consumption

Treatment and control groups did not differ in $\dot{V}O_2$ at any time point during incubation ($F_{1,30} = 0.01$, $p = 0.9149$; Figure 3), but there was a time effect ($F_{4,30} = 3.33$, $p = 0.0226$). $\dot{V}O_2$ increased with egg mass

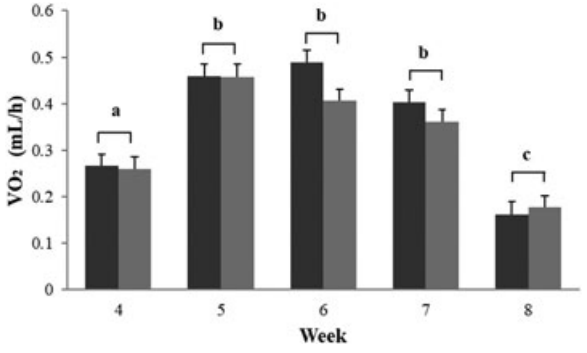

FIGURE 3 LSMeans of oxygen consumption (mL/hr) of embryos in asynchronous-target (dark gray) and synchronous-target (light gray) eggs from Week 4–8 of incubation, ±SE, $n = 10$, $N = 50$. Letters indicate significant pairwise differences among weeks in both asynchronous-target and synchronous-target eggs. SE: standard error of mean

($F_{1,30}$ = 15.78, p = 0.0004), and there was an interaction effect between egg mass and time ($F_{4,30}$ = 0.27, p = 0.0432) but not between egg mass and treatment ($F_{1,30}$ = 0.12. p = 0.7289), treatment and time ($F_{4,30}$ = 0.27 p = 0.8969), or egg mass, treatment, and time ($F_{4,30}$ = 0.28, p = 0.8860). $\dot{V}O_2$ of asynchronous-target eggs peaked at 0.48 ml/hr in Week 6 of incubation and declined to 0.16 ml/hr before hatching in Week 8. Synchronous-target eggs peaked at 0.45 ml/hr in Week 5, and then gradually declined to 0.17 ml/hr in Week 8. $\dot{V}O_2$ increased from Week 4–5 within each group (t_{30} = −5.15, p < 0.0001) and declined from Week 7–8 of incubation (t_{30} = 6.77, p < 0.0001).

3.3 | Heart rate

Heart rates did not differ significantly between asynchronous-target eggs and synchronous-target eggs at any time point during incubation ($F_{1,36}$ = 1.19, p = 0.2819; Figure 4). They were further unaffected by egg mass and by interactions of treatment and egg mass. Heart rates did vary among weeks of incubation ($F_{5,36}$ = 5.55, p = 0.0007), gradually decreasing from 96 (±2.6) to 60 (±2.6) bpm in asynchronous groups and from 100 to 67 bpm in synchronous groups from Week 3–8 of incubation. There was no interaction effect of egg mass and time, treatment and time, nor egg mass, treatment, and time. Heart rate in both treatment groups declined from Week 6–7 (t_{47} = 3.71, p = 0.0069) and Week 7–8 (t_{47} = 3.60, p = 0.0094) of incubation.

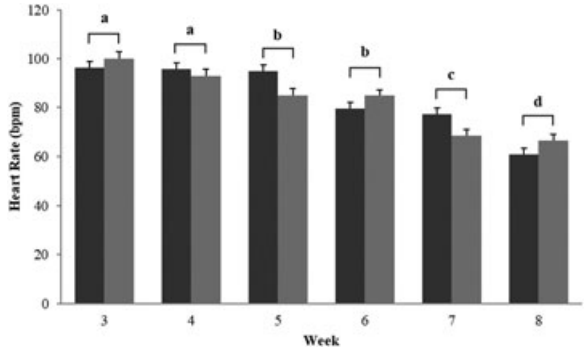

FIGURE 4 LSMeans of heart rate (bpm) of embryos in asynchronous-target (dark gray) and synchronous-target (light gray) eggs from Week 3–8 of incubation, ±SE, n = 10, N = 60. Letters indicate significant pairwise differences among weeks in both asynchronous-target and synchronous-target eggs

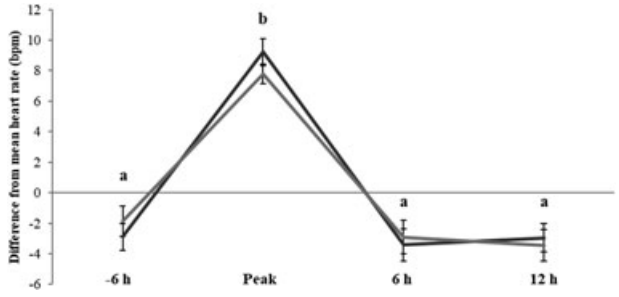

FIGURE 5 The difference from mean heart rate of asynchronous-target (dark gray) and synchronous (light gray) groups over a 24 hr period across weeks 3–8 of incubation. The x-axis represents the duration (hr) away from the peak heart rate and the y-axis represents the difference from the mean heart rate (bpm), ±SE, N = 62. Letters indicate significant pairwise differences among 6 hr time periods in both asynchronous and synchronous groups. SE: standard error of mean

Circadian rhythms in heart rates were present, with a clear peak in heart rates throughout the day in both asynchronous-target eggs (−6 hr to peak $F_{1,14}$ = 18.92, p = 0.0007; peak to 6 hr − $F_{1,14}$ = 20.41, p = 0.0005; Figure 5) and synchronous-target eggs (−6 hr to peak − $F_{1,14}$ = 5.59, p = 0.03; peak to 6 hr − $F_{1,14}$ = 24.69, p = 0.0002). Individuals showed minimum and maximum heart rates over a 24 hr period that varied up to 6 bpm, but they were not correlated with the time of day in both asynchronous and synchronous groups. There was no significant difference between asynchronous and synchronous groups at each time point; −6 hr ($F_{1,30}$ = 0.22, p = 0.64), peak ($F_{1,30}$ = 0.00, p = 0.99), 6 hr ($F_{1,30}$ = 1.39, p = 0.25), nor 12 hr ($F_{1,30}$ = 0.01, p = 0.94). There was a modest week effect ($F_{4,30}$ = 2.80, p = 0.044) and egg mass by week interaction effect ($F_{4,30}$ = 3.38, p = 0.021) during the peak time point, but further pairwise tests revealed no significant differences between weeks. No other main effects or interaction effects were statistically significant at any time point.

3.4 | Post-hatching measurements

Multivariate analysis of all measured phenotypes (carapace length and width, plastron length and width, and mass) post-hatching revealed that hatchling size was not affected by the interaction between treatment and egg mass (Pillai = 0.2142, $F_{15,174}$ = 0.89, p = 0.5742). Treatment alone also did not affect hatchling size (Pillai = 0.165, $F_{10,114}$ = 1.03, p = 0.4265), with egg mass alone explaining the majority of variation in body size (Pillai = 0.647, $F_{5,56}$ = 20.54, p < 0.0001; see Table 1).

Performance and neuromuscular development, as assessed by righting ability, were not affected by treatment group. There was no significant difference between any of the treatment groups in ability to right successfully ($F_{2,65=1.3}$, p = 0.2783). Latency to right, total time to right, and active righting time also were not affected by treatment group (Table 2).

4 | DISCUSSION

Here, we show that *Chrysemys picta* neonates hatch synchronously regardless of developmental stage, with less developed embryos hatching at the same time as their more advanced clutch-mates, with no indication of metabolic compensation. Asynchronous-target eggs hatched within 1 day of the asynchronous-more advanced eggs with which they were incubated with, and this was consistent with the synchronous group, where synchrony was expected. The incubation period of asynchronous-more advanced eggs was shortened

TABLE 1 Mean ± standard error of morphometric traits for asynchronous-more advanced eggs (n = 11), asynchronous-target eggs (n = 27), synchronous-not advanced eggs (n = 13), and synchronous-target eggs (n = 25) in *Chrysemys picta*

	Asynchronous-more advanced	Asynchronous-target eggs	Synchronous-not advanced	Synchronous-target eggs
Carapace length (mm)	24.8 ± 0.6	25.6 ± 0.3	25.6 ± 0.7	25.6 ± 0.4
Carapace width (mm)	22.4 ± 0.5	22.9 ± 0.2	22.6 ± 0.6	22.8 ± 0.4
Plastron length (mm)	24.8 ± 0.6	24.7 ± 0.4	24.7 ± 0.7	24.8 ± 0.5
Plastron width (mm)	16.8 ± 0.4	17.3 ± 0.2	17.3 ± 0.5	17.2 ± 0.3
Mass (g)	4.8 ± 0.3	5.0 ± 0.2	4.9 ± 0.3	5.0 ± 0.2

TABLE 2 Mean ± standard error of righting ability for asynchronous-more advanced eggs (n = 8), asynchronous-target eggs (n = *19*), synchronous-not advanced eggs (n = 8), and synchronous-target eggs (n = *14*) in *Chrysemys picta*

Righting time	Asynchronous-more advanced	Asynchronous-target eggs	Synchronous-not advanced	Synchronous-target eggs
Latency	68.8 ± 17.8	40.2 ± 11.6	24.36 ± 17.8	74.2 ± 13.5
Total time	100.8 ± 26.0	98.5 ± 16.9	61.1 ± 26.0	102.8 ± 19.7
Active	42.7 ± 21.7	58.3 ± 12.2	10.2 ± 18.8	43.8 ± 14.2

Note. Righting ability was assessed as latency to right, total time to right, and active righting time (s).

compared with the synchronous-not advanced eggs because of the variation in incubation temperature at the beginning of the experiment. The difference indicated that the asynchronous setup (Figure 1) did in fact result in a substantial difference in incubation period, like that detected in a prior study (Colbert et al., 2010).

There was no evidence of metabolic compensation inferred through heart rate and $\dot{V}O_2$ of developing target eggs in both the asynchronous and synchronous clutches, as these measures were similar between treatments at all time points. $\dot{V}O_2$ peaked during Weeks 5 and 6 of incubation and declined in the final 2 weeks, as was expected based on previous studies (Booth & Astill, 2001; Booth, Thompson, & Herring, 2000; Du, Thompson, & Shine, 2010; McGlashan et al., 2012; Thompson, 1989), but this was not significant between asynchronous and synchronous-target eggs. Embryonic heart rates were also similar between target eggs in both asynchronous and synchronous clutches, and these gradually decreased from Weeks 3–8 of incubation, similar to the pattern seen in some semi-precocial birds (Cain, Abbott, & Rogallo, 1967; Tazawa, 2005; Tazawa, Hiraguchi, Kuroda, Tullett, & Deeming, 1991; Tazawa, Kuroda, & Whittow, 1991) and snapping turtles (*Chelydra serpentina*; Birchard & Reiber, 1996). The decline in metabolic rate during the resting period (80%–90% of development) before hatching indicated by both heart rate and $\dot{V}O_2$ has also been detected in embryos of *C. longicollis* (McGlashan et al., 2015), *C. insculpta* (Webb et al., 1986), *E. macquarii* (McGlashan et al., 2012; Thompson, 1989), and the American alligator (*Alligator mississippiensis*; Thompson, 1989). The resting period is when thermal acclimation has occurred, and the effects of temperature are relatively less compared with early development; thus, metabolic costs for growth are not usually as high (Birchard & Reiber, 1995, 1996). Hence, if metabolic compensation was occurring, it would be expected during this period, as seen in *E. macquarii* (McGlashan et al., 2012). Synchronous hatching with no evidence of metabolic compensation suggests that less advanced embryos are hatching at an earlier developmental stage, with the more advanced eggs in the clutch, and possibly missing out on the resting stage of development (Colbert et al., 2010).

Circadian rhythms were present in developing *C. picta* embryos despite an absence of environmental cues, such as photoperiod. Over a 24 hr period, heart rates increased and reached a "peak," indicating high developmental activity, and then dropped, suggesting low developmental activity or daily "resting." These rhythms are not synchronized to the time of day among eggs, and there is no indication of synchrony within clutches when compared from week to week, as seen in *C. longicollis* and *E. macquarii* (Loudon et al., 2013; McGlashan et al., 2012). The mean weekly heart rate reflects metabolic rate of embryos, and deviations from the mean rate throughout the day suggest positive or negative compensatory changes in embryonic metabolism (McGlashan et al., 2015). Asynchronous groups did not exceed the potential for periods of higher peak daily rate or reduced resting periods relative to synchronous groups. There was no significant difference in any deviation from the mean between asynchronous and synchronous groups at each time point, implying the absence of metabolic compensation via diel heart rate fluctuations.

Hatchlings were not obviously disadvantaged by hatching early, in either growth or development. The lack of differences in body size or mass of hatchlings between treatments, as well as their ability to right themselves, indicates that hatching early does not substantially affect development. Embryos that were developmentally behind during incubation hatched synchronously with more advanced embryos. There was no evidence to indicate metabolic compensation, nor any phenotypic or performance differences at the time of hatching, suggesting that there is potential for small shifts in hatching

timing without observable metabolic or phenotypic costs. Although previous studies have found that synchronous hatching affects the performance of neonates, a difference in the length of time used to experimentally establish asynchrony might be the cause. Colbert et al. (2010) kept more advanced embryos at a warmer temperature for 11 days. Siblings were most likely too underdeveloped and, when stimulated to hatch synchronously by their more advanced clutch-mates, were ultimately disadvantaged in performance. Comparisons of the two studies on *C. picta* indicate that temporal differences in temperature extremes that result in a greater variation in developmental rates may also be biologically relevant.

In terms of biological significance, climatic extremes could affect incubation conditions (Walther et al., 2002) and therefore the level of asynchrony between clutch-mates. Eggs at the top of the nest experience greater temperature fluctuations throughout the day and are warmer for more than 75% of the day when compared with eggs at the bottom (Shine & Elphick, 2001; Telemeco et al., 2016; Thompson, 1988, 1997; Thompson et al., 1996). Air temperature, substrate thermal conductance, and chamber dimensions can also influence nest temperatures (Thompson, 1988). Each factor influences the extent to which temperature differences within a nest might yield variation in embryonic metabolism and growth. Natural conditions in nests could stimulate greater differences among clutch-mates than elicited experimentally, and thus result in neonates with reduced performance. Further studies are needed to determine the upper limit of synchronous hatching in natural conditions. The final 10% of incubation is when hatching synchrony can take place once hatching competence is achieved (Doody, 2011; Gomez-Mestre et al., 2008). The asynchronous design in this study was 12% of the total incubation period, but in a study by Colbert et al. (2010), the setup was 20% of the total incubation period. Nevertheless, both this study and the study by Colbert et al. (2010) demonstrated that synchronous hatching occurs in *C. picta* regardless of developmental differences at the beginning of incubation and the potential costs that may arise from hatching early. If the difference in development is small, then synchrony will not incur performance costs, but if the difference is great, then hatchling recruitment in some years may decline because more individuals are underdeveloped at hatching.

Although temperature is a major determinant of the rate of embryonic development in ectotherms (Booth, 1998; Deeming & Ferguson, 1991; Monaghan, 2008), hatching time is nonetheless plastic (Doody, 2011; Packard & Packard, 2000; Spencer & Janzen, 2011; Warkentin, 2011). Abiotic and biotic forces unique to species and populations drive synchronous hatching, with environmental cues (e.g., heart rate, movement, and embryo–embryo communication) within the nest potentially triggering metabolic compensation or early hatching (Doody, 2011). Of particular interest are the physiological mechanisms and endocrine responses, such as how hormones might alter development and behavior. Thyroid hormones can induce changes to metabolism or hatching behavior independent of temperature without any metabolic or post-hatching cost (McGlashan, Thompson, Van Dyke, & Spencer, 2017; O'Steen & Janzen, 1999). Increased thyroid hormone concentrations strongly correlate with the final stages of embryogenesis (Dimond, 1954; Shepherdley et al., 2002) and could be important for hatching behavior (McGlashan et al., 2017).

Embryonic development is complex, with egg position and thermal gradients in a nest influencing the developmental rate of embryos, and potentially causing asynchronous clutch conditions (Deeming & Ferguson, 1991; Thompson, 1988, 1997). The environment in which embryos develop can also influence hatching behavior. By overwintering in the nest, hatchlings presumably experience a more favorable environment and reduced risk of predation when they emerge in the following spring (Gibbons, 2013; Gibbons & Nelson, 1978; Wilbur, 1975).

Synchrony by hatching early could ensure an optimal overwintering position in the nest for *C. picta* to reduce the chance of mortality from freezing, dehydration, and starvation from loss of energy reserves (Costanzo, Dinkelacker, Iverson, & Lee, 2004; Costanzo, Lee, & Ultsch, 2008; Spencer & Janzen, 2014). Winter nest temperatures are variable (Weisrock & Janzen, 1999) and could influence the overall energy expenditures of neonates in the nest. During winter, metabolic demands are generally low but maternally sourced yolk is used during this time, and the warmer the winter, the more energy is used (Muir, Dishong, Lee, & Costanzo, 2013; Willette et al., 2005). In *C. picta*, where warmer incubation conditions produce more females and cooler temperatures produce more males, males in these cooler overwintering nests use less yolk than females in these same nests, and the converse is true for females from warmer nests (Spencer & Janzen, 2014). Thus, retaining yolk energy stores without metabolic compensation is imperative in preparation for atypical overwintering conditions, which can result in up to a 50% loss of energy (Spencer & Janzen, 2014). Although yolk sac volume was not measured here and does prove a limitation, this study looked at pipping times as the indicator for the neonates' readiness to hatching. As we wanted to ensure neonates hatched completely, unassisted, we did not measure yolk sac volume as there may be potential bias due to variation in when they extricate themselves from the eggs confines.

The timing of hatching is a life-history trait influenced by the tradeoff between the risk and benefit of hatching, including growth and development, hatchling energetics, and predator avoidance (Baker, Costanzo, Iverson, & Lee, 2013; Carr & Hirth 1961; Doody et al., 2001, 2012; Santos et al., 2016; Webb et al., 1986). Whether emergence is triggered immediately after hatching or several months later could depend on differing selective pressures, including geographic location, environmental and/or genetic factors unique to populations (Breitenbach, Congdon, & Sels, 1984). The phenomenon of synchronous hatching appears uniform in turtles, but the mechanisms to overcome the effect of variable temperatures on developmental rates vary considerably. Pleurodiran turtles metabolically compensate during incubation to hatch early and ensure synchronous hatching (McGlashan et al., 2012, 2015). Within cryptodires, at least one species delays hatching to synchronously

emerge when environmental conditions are right (Doody et al., 2001, 2012; Webb et al., 1986), whereas *C. picta* differ altogether by hatching early with no evidence of metabolic compensation during embryonic development and no evidence that hatchlings had a reduction in size or performance. The physiological mechanism underlying early hatching in *C. picta* might be the same mechanism that is seen to induce early hatching, with no metabolic or performance costs, in *E. macquarii* (McGlashan et al., 2017), but further studies on how the endocrine system facilitates embryonic development are required. In particular, thyroid hormones might prove to be the mechanism underlying many aspects of ECH in turtles. Developmental plasticity allows embryos to hatch synchronously despite the variation in temperature, with various abiotic and biotic driving forces for individual species. In sum, synchronous hatching is an adaptive bet-hedging strategy in turtles characterized by diverse mechanisms to achieve it.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

This manuscript is jointly authored. J. K. M. conceived the idea and, along with R.-J. S., F. J. J., and M. B. T., designed the methodology. J. K. M. collected the data and carried out data analysis. All four authors contributed critically to the writing of the manuscript and gave final approval for the publication.

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