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# Embryonic communication in the nest: metabolic responses of reptilian embryos to developmental rates of siblings

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Incubation temperature affects developmental rates and defines many phenotypes and fitness characteristics of reptilian embryos. In turtles, eggs are deposited in layers within the nest, such that thermal gradients create independent developmental conditions for each egg. Despite differences in developmental rate, several studies have revealed unexpected synchronicity in hatching, however, the mechanisms through which synchrony are achieved may be different between species. Here, we examine the phenomenon of synchronous hatching in turtles by assessing proximate mechanisms in an Australian freshwater turtle (*Emydura macquarii*). We tested whether embryos hatch prematurely or developmentally compensate in response to more advanced embryos in a clutch. We established developmental asynchrony within a clutch of turtle eggs and assessed both metabolic and heart rates throughout incubation in constant and fluctuating temperatures. Turtles appeared to hatch at similar developmental stages, with less-developed embryos in experimental groups responding to the presence of more developed eggs in a clutch by increasing both metabolic and heart rates. Early hatching did not appear to reduce neuromuscular ability at hatching. These results support developmental adjustment mechanisms of the ‘catch-up hypothesis’ for synchronous hatching in *E. macquarii* and implies some level of embryo–embryo communication. The group environment of a nest strongly supports the development of adaptive communication mechanisms between siblings and the evolution of environmentally cued hatching.

**Keywords:** synchronous hatching; heart rate; developmental costs; turtle; *Emydura macquarii*; metabolic compensation

## 1. INTRODUCTION

In most oviparous species, incubation temperature is a major environmental factor influencing embryonic development. High temperatures accelerate embryonic development and reduce incubation period; conversely, low temperatures reduce developmental rates and increase incubation period [1]. However, other environmental factors may be more significant to actual hatching times. Timing of birth has implications for animals that produce many offspring and assessing environmental cues is important in species that synchronize birth [2]. Factors such as order of ovulation and disparate thermal microenvironments of eggs promote intra-clutch variation in incubation period [1], making synchronous birth or hatching an environmental variable. Yet, synchronous hatching through the alteration of incubation occurs in many oviparous taxa, including invertebrates [3], fishes [4], amphibians [5], crocodylians [6], squamates [7], turtles [8,9] and birds [10].

The life-history patterns of freshwater turtles provide an opportunity to test environmentally cued hatching in oviparous organisms. Turtles ovulate eggs simultaneously and embryonic development is arrested at the gastrula stage until oviposition [11]. Eggs are generally deposited in several layers within a shallow nest and environmental gradients alter developmental rates throughout

incubation [12]. Specifically, eggs near the top of a nest experience higher temperatures (up to 6°C higher), which results in shortened incubation periods relative to eggs near the bottom of the chamber [13]. Thus, hatching synchrony should not occur within a freshwater turtle nest because incubation times should differ significantly between eggs in different positions of the nest. Despite potential differences in developmental rates, both an Australian and a North American freshwater turtle exhibit synchronous hatching through ‘catch-up’ mechanisms, whereby less-developed siblings within a nest simply either hatch earlier or increase development rates (independent of temperature) in response to cues from siblings [8,14].

As an ultimate explanation, predator avoidance is believed to drive the evolution of synchronous hatching [8,14]. Group hatching and emergence decrease individual energy expenditure when evacuating from the nest [15] and may also swamp potential predators upon emergence. Swamping decreases the number of turtles at risk of predation through the *per capita* dilution of individual predation risk [8,14], as well as possibly confusing predators [16]. Although these factors would promote the evolution of synchronous hatching, or proximate mechanisms to detect sibling hatching or developmental cues, premature hatching would be selected against if a decrease in motor skills, relative to other siblings, results in increased mortality or risk of predation. Freshwater turtles, such as *Chrysemys picta*, appear to hatch prematurely rather than delay hatching to hatch synchronously

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with their siblings, but hatching and emergence are not closely linked [17]. In species where hatching and emergence are closely linked (e.g. *Emydura macquarii*), the trade-off between the benefits of synchronous hatching and the costs are primarily embedded in the secondary development period of incubation (figure 1). This period is associated with maturation of the neuromuscular system, whereas the primary development period (up to the peak metabolic rate) is associated with organ and tissue development [8,14]. Figure 1 is a theoretical metabolic profile for freshwater turtle eggs during incubation (based on *E. macquarii* eggs [15]). Respiration rates in reptiles and precocial birds generally drop by up to 25 per cent before hatching occurs [18–20], but hatching can occur at any time after peak metabolism. The fall in metabolism prior to hatching in some species is associated with the secondary development period, which is variable in length. Varying the length of this period may have both short-term and long-term costs, but if embryos do not adjust their developmental rate throughout incubation, they can only significantly shorten the secondary development period (figure 1), which could result in adverse effects on survival [14].

The aim of this experiment is to test the two competing proximate mechanisms of the catch-up hypothesis that facilitate synchronous or early hatching in freshwater turtles. The mechanisms by which turtles may adjust metabolism could be linked to compensatory changes in temperature in fluctuating environments. Although Booth [21] failed to demonstrate any developmental compensation after transferring freshwater turtle eggs from lower and higher constant temperatures midway through the incubation period, daily fluctuations and thermal inertia may provide a greater opportunity for subtle diurnal adjustments of developmental rate. Thus, it is important to evaluate competing mechanisms of the catch-up hypothesis under both constant and fluctuating temperature environments.

## 2. MATERIAL AND METHODS

### (a) *Study species*

*Emydura macquarii* inhabits slow-moving or stagnant waters of the Murray Darling Basin and some river basins of coastal New South Wales and southeast Queensland [22,23]. *Emydura macquarii* produce 10–30 eggs per year in relatively shallow terrestrial nests, and they emerge *en masse* to deposit eggs during or after rain from October to December [24]. Temperature differentials between the top and bottom of nests may be as much as 6°C [13].

### (b) *Egg collection*

Using funnel traps, female *E. macquarii* were captured from the Murray River, Albury, New South Wales (36°03' S, 146°56' E) from 11 to 15 November. Turtles were palpated to determine if they were gravid [8] and if so, they were given a subcutaneous intramuscular injection of 2 ml of oxytocin in the thigh [8] and were then placed in enclosed containers until oviposition. All eggs were given an identifiable number according to their clutch and egg position using a soft pencil (HB) [14]. Twenty clutches were used for this study (see electronic supplementary material for egg maintenance during incubation).

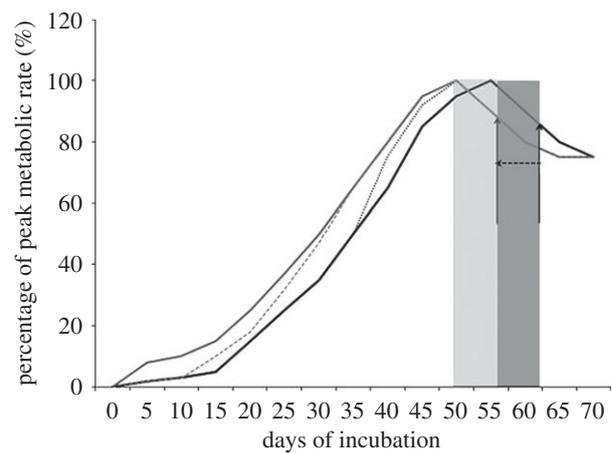


Figure 1. Stylized metabolic profile of turtle embryos (eggs) developing together with different rates of development throughout the incubation period. Solid black line represents eggs developing normally and the solid grey line represents the profile of eggs that have been incubated separately at warmer temperatures for the first week of development before being united with normally developing eggs. The grey dashed line represents eggs that are stimulated to increase their developmental rates early in development and hatch at a similar developmental stage as the stimulus eggs. The black dotted line represents eggs that are stimulated to increase their developmental rates later in development and hatch at a similar developmental stage as the stimulus eggs. The light grey region represents the secondary developmental stage of the stimulus eggs and the dark grey region represents the secondary developmental stage of normally developing eggs. The arrows at the end of each secondary development phase represent the normal hatching times for each group. For eggs that do not increase development in response to stimulus eggs, premature hatching by reducing the secondary development phase (dashed arrow) can also ensure synchronous hatching with more advanced eggs. Scenarios are based on the metabolic and hatching profiles of *E. macquarii* [12].

### (c) *Experimental design*

To assess hatching synchrony in *E. macquarii*, protocols similar to Spencer *et al.* [8] and Colbert *et al.* [14] were used. Specifically, we induced developmental asynchrony among clutch mates and then reunited eggs at a common incubation temperature and monitored their hatching times (electronic supplementary material, figure S1). Experimental treatments were designed to test whether less advanced embryos were either hatching prematurely or potentially adjusting developmental rates throughout the incubation period.

To establish developmental asynchrony, half of each clutch (six eggs) were incubated at 26°C and the other half were incubated at 30°C for 7 days. Turtle embryos developing at 31°C spend approximately half the time in each [25] developmental stage than embryos developing at 26°C [26]. After this period, eggs held at 30°C were removed from their containers and reunited next to their clutch mates that were at 26°C (electronic supplementary material, figure S1). The control was treated similar to the experimental groups, however, developmental asynchrony was not established between eggs, because both halves of the divided clutch were held at the same temperature (26°C) for the first 7 days of incubation.

Clutches were then incubated at either a constant 26°C (electronic supplementary material, figure S1a) or a fluctuating temperature regime (electronic supplementary material,

figure S1*b*). The fluctuating temperature regime involved a 24 h temperature cycle, whereby clutches spent 6 h at each of the following temperatures ( $-20^{\circ}\text{C}$ ,  $-26^{\circ}\text{C}$ ,  $-30^{\circ}\text{C}$ ,  $-26^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$ ) until the end of incubation. This temperature treatment allowed us to investigate whether metabolic compensation ( $\dot{V}\text{CO}_2$  and heart rate) occurred as temperatures changed throughout the day. Temperature segments were used in the analyses, rather than temperature *per se*. For example, the  $20\text{--}26^{\circ}\text{C}$  temperature segment means that the current incubation temperature is  $20^{\circ}\text{C}$  and the next incubation temperature is  $26^{\circ}\text{C}$ . Similarly, the  $30\text{--}26^{\circ}\text{C}$  temperature segment means that the current incubation temperature is  $30^{\circ}\text{C}$  but the next incubation temperature is  $26^{\circ}\text{C}$ .

For statistical analyses of each variable measured, we compared eggs that were initially incubated at  $26^{\circ}\text{C}$  (and not moved) in the experimental groups with the corresponding eggs of control groups at  $26^{\circ}\text{C}$  (and not moved) using two-tailed *t*-tests. The only difference between experimental and control groups is that more advanced eggs ( $30^{\circ}\text{C}$ ) were located in the experimental containers. Each time period (week) and fluctuating temperature segments were subjected to separate statistical analyses to compare  $\dot{V}\text{CO}_2$  and heart rate throughout the incubation period.

#### (d) Metabolic and heart rates

Carbon dioxide production ( $\dot{V}\text{CO}_2$ ;  $\text{ml h}^{-1}$ ) of all eggs was measured using closed system respirometry. A Qubit (S500) respirometer (Kingston, ON, Canada) was used to measure carbon dioxide production (see electronic supplementary material for methodology). The experimental design meant that at least four eggs from each treatment were measured at any given time. Eggs were measured only once during the incubation period and only one egg from each clutch was assessed in any given week. Weekly metabolic profiles were developed for each treatment. For the fluctuating temperature regime, metabolic profiles for each treatment were determined at four time intervals: 400 h ( $30^{\circ}\text{C}$ ;  $26\text{--}30$  segment), 1000 h ( $26^{\circ}\text{C}$ ;  $30\text{--}26$ ), 1600 h ( $20^{\circ}\text{C}$ ;  $26\text{--}20$ ) and 2200 h ( $26^{\circ}\text{C}$ ;  $20\text{--}26$ ) throughout incubation. From weeks 5–9 (hatching),  $\dot{V}\text{CO}_2$  was measured weekly for most temperature segments, however, in week 7 only one and not all temperature segments were completed before hatching occurred in week 9.

Heart rates were recorded using the Buddy digital egg monitor system (Avian Biotech, UK), which is a non-intrusive method for measuring heart rates of embryos in eggs [27]. The system uses infrared transmitters and sensors amplifying the cardiovascular signal of an embryo within the egg by 20 000 times. Eggs were individually placed in the monitor for 30 min. A digital camera recorded heart rate at 5 min intervals. Weekly profiles of heart rates were developed, as well as profiles for each time interval in the fluctuating temperature regime. Heart rates (b.p.m.) were calculated as an average for each egg over the 30 min period. Only one egg from each clutch was measured in any given week.

#### (e) Pipping

Incubation period was measured as the number of days from initial egg collection until pipping. Pipping (when the eggshell is first slit) is better than hatching as an index of the end of the incubation period, because it shows less variability than hatching [28].

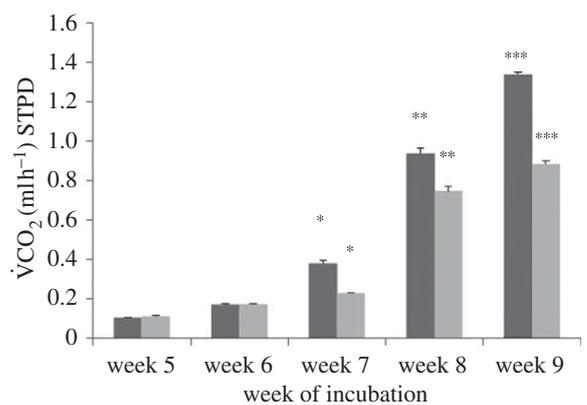


Figure 2. Metabolic profiles of turtle eggs throughout the incubation period. The graphs show average of experimental (dark grey) and control (light grey) eggs from weeks 5 to 9.  $\dot{V}\text{CO}_2$  was higher in experimental eggs only in week 7 ( $*t_{10} = 2.6$ ;  $p = 0.03$ ), week 8 ( $**t_{10} = 3.2$ ;  $p = 0.009$ ) and week 9 ( $***t_{10} = 3.8$ ;  $p = 0.003$ ) ( $\pm$ s.e.).

#### (f) Post-hatching development and growth

Righting trials were performed on all neonates within 12 h of hatching using the same methods as that of Colbert *et al.* [14] to assess whether a developmental cost was associated with the alteration in incubation periods, which would indicate the mechanism by which synchrony was achieved (see electronic supplementary material). We also measured the length, width and height of any external yolk sac from each individual as an index of energy expenditure. The volume of a cube ( $L \times W \times H$ ) was used as a crude measure to calculate the volume of yolk. Hatchlings were then weighed and measured using callipers to record straight carapace and plastron length, after which hatchlings were placed in small plastic containers with enough water to fully submerge for 7 days. Hatchlings were initially individually marked with an enamel paint pen applied to their carapace, but within 30 days, individual notches were applied to their marginal scutes [29] using dissecting scissors. After 7 days, turtles were moved to four 70 l aquaria with up to 50 turtles in each tank (see electronic supplementary material). All turtles were weighed and measured every 40–50 days.

## 3. RESULTS

### (a) Metabolic compensation

#### (i) Constant temperature regime

Metabolic compensation did not occur until week 7 of incubation, when  $\dot{V}\text{CO}_2$  levels in experimental treatment groups were significantly higher than control groups for the duration of incubation (figure 2).  $\dot{V}\text{CO}_2$  levels in the experimental groups were up to 67 per cent higher than the controls during this period. Similarly, heart rates did not significantly differ between experimental and control groups until week 8 of incubation, when heart rates of experimental groups were higher than control groups for the duration of incubation (figure 3).

#### (ii) Fluctuating temperature regime

Metabolic differences between experimental and control groups in the fluctuating temperature regime were similar to those detected in the constant temperature regime. Metabolic compensation was restricted to the last third of incubation, as  $\dot{V}\text{CO}_2$  did not significantly differ

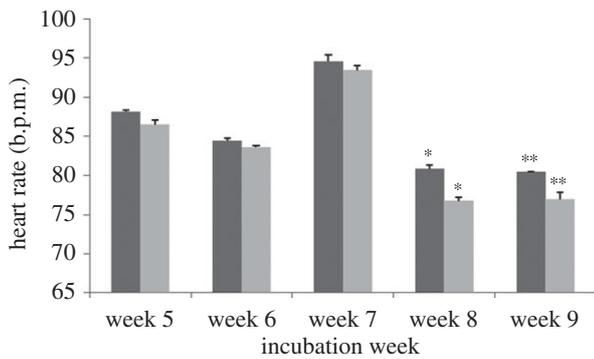


Figure 3. Heart rates (b.p.m.) of eggs at 26°C in the experimental (dark grey) and control (light grey) groups from weeks 5–9 (week 8:  $*t_4 = 3.7$ ;  $p = 0.02$ ; week 9:  $**t_4 = 3.5$ ;  $p = 0.03$ ) ( $\pm$  s.e.).

between experimental and control groups until week 7. Metabolic compensation did not depend on temperature or changes in temperature, with all temperature segments, except the 30–26°C segment, displaying increased  $\dot{V}CO_2$  in the experimental groups compared with the control groups (electronic supplementary material, figure S2). Significant differences in  $\dot{V}CO_2$  between experimental and control groups were observed from week 7 in the 20–26°C ( $t_{10} = 2.3$ ;  $p = 0.05$ ).

Heart rates of eggs in the fluctuating temperature regime were not significantly different between experimental and control groups in week 6 except in the 30–26°C segment, where eggs from experimental groups were approximately 5 b.p.m. higher than control groups. By week 8, heart rates were generally higher in experimental than in control groups, however, only in the 20–26°C segment, heart rates were significantly different ( $*t_4 = 4.6$ ;  $p = 0.01$ ). On average, eggs in experimental groups had heart rates 3–12 b.p.m. higher than in control groups.

#### (b) Hatching

Experimental group embryos pipped earlier than their controls (figure 4), although synchronous hatching did not occur in both the constant and fluctuating temperature regimes. Less-advanced embryos in the experimental groups began pipping up to 50 h (4 days) earlier than control turtles (figure 4). In both the fluctuating ( $t_8 = 2.7$ ,  $p = 0.02$ ) and constant ( $t_8 = 4.8$ ,  $p = 0.001$ ) temperature regimes, neonates from control groups had 55–150 per cent larger yolk sac volumes at hatching than neonates from experimental groups.

#### (c) Post-hatching development

Neuromuscular development (as assessed by righting time) was not affected by hatching early. Neonates from the experimental groups performed (righting ability at hatching) as well as, if not better than, their more advanced clutch mates and neonates from control groups (electronic supplementary material, figure S3). In the constant temperature regime, turtles from the experimental treatment group were able to right themselves on average, 5 s earlier than the more advanced turtles from the same clutch ( $t_8 = 0.62$ ,  $p = 0.5$ ). Similarly, early hatching turtles in the fluctuating temperature regime flipped, on average, 4 s earlier than the more advanced hatchlings from the same clutch (electronic supplementary material, figure S3;  $t_8 = 6.6$ ,

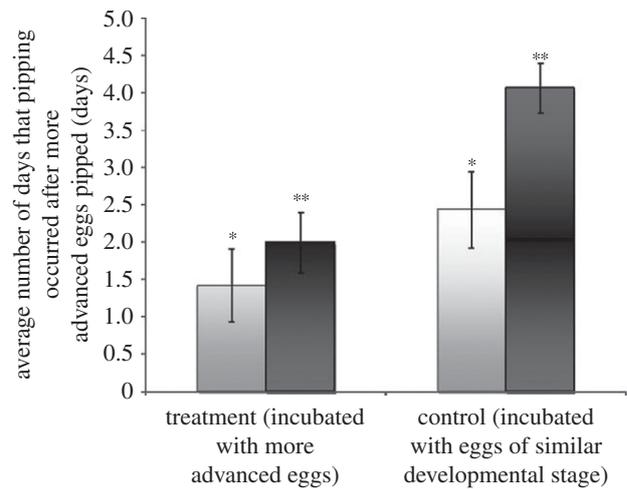


Figure 4. Mean number of days that pipping occurred in the constant (light grey) ( $t_8 = 2.4$ ;  $p = 0.04$ ) and fluctuating (dark grey) ( $t_8 = 5.0$ ;  $p < 0.001$ ) temperature regimes after developmentally accelerated eggs pipped (days) ( $\pm$  s.e.).

$p = 0.001$ ). In both fluctuating and constant temperature regimes, individuals from experimental and control groups did not significantly differ in average size at five months of age ( $p > 0.27$ ).

## 4. DISCUSSION

Incubation temperature primarily determines incubation times in reptiles but other environmental factors or cues appear more influential on hatching time, which is phenotypically plastic within boundaries set by incubation temperature. Actual cues for hatching in turtles derive from a range of biotic and abiotic factors. Predation or flooding is common trigger for spontaneous hatching in many species [9]. Spontaneous hatching can occur because many species complete development and enter embryonic aestivation until conditions are optimal, or the embryos are threatened [30]. Early or spontaneous hatching is unlikely to occur when eggs develop at different rates and hatching cues occur well before individuals, or the entire clutch, has completed development. Hence early hatching, which occurs in both *E. macquarii* [8] and *C. picta* [14], is less common in nature. Some frogs hatch early and at smaller sizes in response to predators [5,30].

Environmental cues, such as flooding and predation, are pulse events that require an immediate response by embryos, but if the cues are more subtle and constant throughout development, embryos may be able to adjust rates of development to facilitate hatching. Modification of hatching times occurs in turtles. Although synchronous hatching does not necessarily occur, turtles consistently hatch earlier than expected when in the presence of more advanced embryos [8,14]. The assumption is that reptiles have limited capacity to regulate metabolic processes independent of temperature, because they are ectothermic and thermoconformers. Hence, increases in metabolic rates and embryonic development above ambient temperature are presumably improbable and early, or synchronous, hatching is achieved through incomplete development [14]. However, this study clearly demonstrates that turtles have the ability to increase metabolism independent of incubation temperature.

Both metabolic and heart rates of embryonic *E. macquarii* increased in both the fluctuating and constant temperature regimes in response to the developmental stage of neighbouring eggs. These results support the fourth scenario (dotted line) proposed in our graphical model of the 'catch-up' hypothesis for synchronous hatching (figure 1). This scenario proposes that less advanced embryos in a clutch only increase metabolic rates late in development, in response to more advanced eggs. Differences in  $\dot{V}CO_2$  and heart rate between experimental and control groups in the current study do not manifest until the last third of incubation.

The secondary development period, or last few days of incubation, in reptiles and precocial birds is where organogenesis is complete and neuromuscular activity is increasing to prepare the neonate for complex movement immediately after hatching. This period has been proposed as the phase of incubation that can be shortened if necessary to reduce incubation period for group emergence from the nest. Japanese quail chicks (*Coturnix coturnix japonica*) with accelerated development take 1–2 h more to stand than normal chicks [20], and neuromuscular function of less-developed embryonic painted turtles (*C. picta*) is reduced for up to 9 months after hatching [14]. However, increases in metabolic rate in *E. macquarii* translated directly into increased rates of embryonic development because (i) more yolk reserves were used by experimental groups and (ii) neuromuscular developmental tests (righting ability) at hatching were not significantly reduced in experimental groups (electronic supplementary material, figure S3). Short-term costs of increasing developmental rates, as opposed to shortening the secondary development period, include a reduction in yolk reserves that may be vital for the initial few weeks of neonatal development and survival.

Selection for early hatching without reduced capabilities may be stronger in *E. macquarii* compared with *C. picta* because the Australian turtle emerges from the nest sooner than the North American turtle. Some populations of *C. picta* can spend 9 months overwintering within a nest [17] and selection may favour individuals that minimize mobilization of yolk reserves during incubation because they are required to survive overwintering. Hence, both the ultimate and proximate (i.e. increased developmental rates versus premature hatching) mechanisms for synchronous hatching in *C. picta* are different from that of *E. macquarii*. Tucker *et al.* [31] suggest that predator swamping is not an explanation for synchronous hatching in freshwater turtles, rather that synchronous emergence of turtle hatchlings from nests maximizes individual survival by minimizing exposure to prey-switching predators. Predator-avoidance at hatching would not be a strong evolutionary selective force in *C. picta* because there is a clear disconnect between hatching and emergence times, whereas reduced running, escaping or swimming ability when *E. macquarii* emerge from the nest may manifest in increased mortality.

But how does a reptile actively regulate metabolism independent of temperature? Given an embryo's inability to thermoregulate behaviourally, slight physiological increases in metabolic (and thus, developmental) rate are important mechanisms available to accelerate embryonic development and growth in reptiles. The proximate mechanisms responsible for the ontogenetic shift in heart rates and metabolism are unknown, but may relate

to maturation of cardiovascular control in late-term embryos, including both hormonal and nervous regulatory systems [32]. One such factor is thyroid hormone (TH), which plays a crucial role in growth, development and function of most vertebrate tissues, such as brain, bone, fat and skeletal muscles [27,33]. TH influences tissue accretion and differentiation in a foetus through both metabolic and non-metabolic mechanisms [33]. Critically, TH affects a variety of metabolic pathways, especially energy metabolism, and also plays a role in mammalian and reptilian metabolic responses to temperature acclimation [34]. TH is a key regulator of postnatal growth and the complex process of transition from allantoic to pulmonary respiration [35,36], yet the thyroid also is one of the earliest endocrine organs to differentiate and the metabolic adjustment in our experimental groups may lie with changes in TH.

The cues behind increased metabolism and synchronous hatching in turtles are not known. TH plays a role in the length of the incubation process of chickens and the production of TH in embryos is induced under hypoxic conditions [35,36]. Both  $CO_2$  levels and heart rates have been proposed as possible cues [8]. The increase in metabolic rates during the last third of development indicates that both physiological mechanisms are plausible explanations for a potential cue. Eggs were not placed in contact with each other and differences in heart rate might only be detected by embryos during the last phase of incubation when heart beats strengthen. Similarly,  $CO_2$  levels in a nest may only reach high concentrations during the last phase of incubation as the embryo develops and metabolism increases. Increasing  $CO_2$  levels within a nest may indicate imminent hatching and may be a cue for TH production and increased development of less-advanced embryos. Both factors are not mutually exclusive and the combination of endocrine and gas exchange cues may be responsible for synchronous or early hatching in turtles.

In conclusion, temporal and spatial synchrony for a wide range of behaviours is a common phenomenon; from calling and metamorphosing frogs to schooling in fish and even to flowering events in plants. The sheer magnitude and diversity of such synchronous events and other plausible links to fitness have led many to consider these behaviours to be unquestionably adaptive [37]. The Australian freshwater turtle, *E. macquarii*, is able to communicate the developmental status among clutch mates within a nest, which in turn metabolically compensate and adjust developmentally to hatch at times similar to their more advanced siblings. Yet in other species of turtle, synchronous or early hatching occurs concurrently with associated developmental costs that are not observed in *E. macquarii* [14]. The potential ubiquity of synchronous hatching within Testudines is supported by the presence of this phenomenon in two species as distantly related as *C. picta* (Megaorder Cryptodira, hidden-necked turtles) and *E. macquarii* (Megaorder Pleurodira, side-necked turtles; [17]). But it is unlikely that synchronous hatching represents a simple case of convergent evolution because both the ultimate and proximate mechanisms for synchronous hatching vary considerably between the two species. Although a single, group formation-driven origin of synchronous hatching in turtles cannot be

ruled out, the classic sea turtle example of group emergence of neonates may not be as prevalent as commonly perceived.

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