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REVIEW ARTICLE

The influence of daily temperature fluctuations during incubation upon the phenotype of a freshwater turtle

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Abstract

Incubation temperature influences the phenotype of the hatchling turtles. The aims of the present study were to investigate the daily fluctuations in temperature to which eggs of the freshwater turtle *Elusor macrurus* are exposed to in the wild and examine how these fluctuations may affect the phenotype and performance of the hatchlings. Eggs in the wild experienced an overall mean daily fluctuation of 5.7°C throughout the incubation period, but on particular days, the variation was as low as 2°C and as high as 22°C. Fifty-four eggs were collected from the wild and incubated in the laboratory at one constant (28°C) and two fluctuating (28 ± 3 and 28 ± 6°C) thermal regimes. Egg mass, incubation length and hatching success (89%) were similar for the 28 and 28 ± 3°C groups, whereas the 28 ± 6°C group only had a 5% hatching success, and the incubation length was 10 days longer. Upon hatching, there was no significant difference in body mass or straight carapace length between the 28 and 28 ± 3°C groups, and within the first 8 weeks of hatching, there was no significant difference in growth rate, self-righting time, crawling speed and swimming performance. A single survivor from the 28 ± 6°C group had a body mass that was 27% less compared with the other two groups and it did considerably poorer in all the performance tests. The study findings illustrated that daily fluctuations in incubation temperature up to 6°C had no effect upon hatchling *E. macrurus* phenotype, but there was a limit (12°C) by which the extent and recurrence of these fluctuations became detrimental. These thermal regimes are not yet apparent in the wild but will occur within the geographical range of this species according to climate change predictions.

Introduction

Most oviparous reptiles bury their eggs in underground chambers for incubation (Deeming & Ferguson, 1991; Deeming, 2004; Booth, 2006). This is an evolved behavioural strategy, which not only protects the eggs from predation but also buffers the temperatures and hydric regime that the clutch experiences during incubation (Miller & Dinkelacker, 2008). In freshwater turtles, research has demonstrated that shifts in mean constant incubation temperature of only a few degrees can significantly affect the phenotype of the hatchlings by altering morphology, physiology and locomotor performance (Janzen, 1993; Bobyn & Brooks, 1994*a*,*b*; Roosenburg & Kelley, 1996; Booth, 2000; Steyermark & Spotila, 2001; Du & Ji, 2003; Booth *et al*., 2004; Delmas *et al*., 2007; Micheli-Campbell *et al*., 2011). In the wild, however, the eggs are rarely exposed to constant temperatures, as the females of most freshwater species lay shallow nests where the variation in daily temperature is increased by the proximity of the clutch to the substrate surface (Booth, 2006). To date, little is known about the effects of such thermal regimes upon the phenotype of hatchling turtles.

The majority of empirical thermal incubation studies in freshwater turtles have focused upon species with temperature-dependent sex determination (Schwarzkopf & Brooks, 1985; Demuth, 2001; Les, Paitz & Bowden, 2007; Du, Shen & Wang, 2009). These studies have shown that daily fluctuations in incubation temperature produced a larger proportion of females when compared with the constant treatments. Alterations in other morphological and physiological traits were also observed and the extent of these responses varied widely between species. For example, fluctuating incubation temperatures resulted in a smaller body mass of hatchling *Chinemys reevesii* compared with those incubated at a constant temperature (Du *et al*., 2009). Swimming ability and immune response were improved in *Chrysemys picta* and *Trachemys scripta* when eggs were incubated under the fluctuating thermal regimes (Ashmore & Janzen, 2003; Les *et al*., 2007;

Les, Paitz & Bowden, 2009), whereas other species showed no significant alterations in hatchling performance between constant and fluctuating incubation temperatures (Du *et al.*, 2009). Consequently, in species with temperature-dependent sex determination, it is inconclusive to determine whether the differences in hatching phenotype occur as a direct consequence of the thermal fluctuation, or whether these differences result from inherent variation between the sexes.

The aim of the present study was to compare the effects of constant and fluctuating incubation temperatures upon the morphology, locomotor performance and growth rate (GR) of a species of freshwater turtle *Elusor macrurus* whose sex is not determined by temperature (Georges & McInnes, 1998). *E. macrurus* (Mary River turtle) lays rigid-shelled eggs in shallow nests (~20-cm depth) on steep sandy banks with no vegetation cover, and nesting events typically occur between late-spring (October) and early-summer (December; Cann & Legler, 1994). Because of these characteristics, we predicted that the *E. macrurus* eggs would experience large daily temperature fluctuations in the wild. We assessed the extent of these daily temperature fluctuations, and then through controlled laboratory manipulations, determined the influence of fluctuating incubation temperatures upon the hatchling phenotype. We hypothesized that hatchlings incubated under thermal regimes most similar to conditions experienced by the eggs in the wild would show improved performance over hatchlings that were not.

Material and methods

Nest temperatures in the wild

Elusor macrurus nests were located the morning following a night of heavy rainfall. The newly formed eggs were exposed by hand and a temperature data-logger (2-cm diameter, Hobos® TidBit® Onset®, Bourne, MA, USA) was placed adjacent to the clutch at a depth corresponding to the middle of the nest chamber. Each data-logger was programmed to record the temperature every 40 min. The sand was then replaced and the clutch covered to the original depth. The temperature logger was recovered once the hatchlings had exited the nest. Data were obtained for 16 nests laid in four separate river banks within an approximately 30-km stretch of river during the 2009/2010 nesting season.

Egg incubation and hatchling morphology

Fifty-four *E. macrurus* eggs, collected from four different clutches, were transported to The University of Queensland. In the laboratory, they were randomly distributed into three containers (one per experimental treatment, each containing four to five eggs from each clutch), which were filled with wet sandy soil collected from the nesting site. Each container was placed into a controlled temperature incubator (I-36VLC9 Intellus Ultra, Percival Scientific Inc., Perry, IA, USA); these were programmed to a constant (28°C) and two fluctuating temperature treatments (28 ± 3 and 28 ± 6°C; 24-h cycle). A

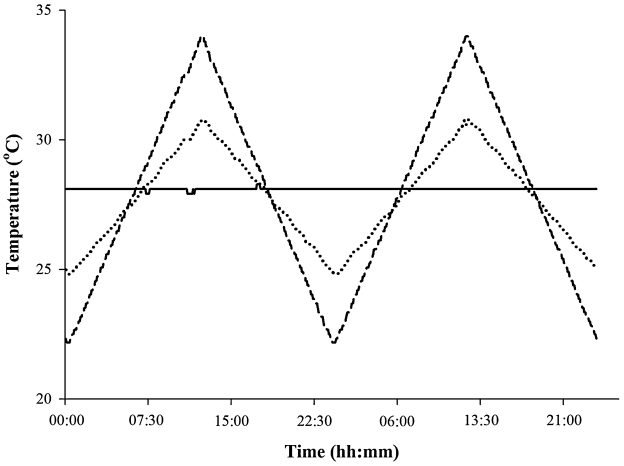

Figure 1 Programmed temperature profile (recorded by data-loggers) experienced by *Elusor macrurus* eggs (n = 18) in the laboratory after being collected from the wild (solid lines = 28°C; dotted lines = 28 ± 3°C; dashed lines = 28 ± 6°C).

temperature logger (Hobos® TidBit® Onset®) was placed inside each incubator (beside the eggs) to record and monitor incubation temperature regime (Fig. 1). In order to maintain near constant water potential, the sand surrounding the eggs were sprinkled with 3 mL of water every 48 h throughout the entire incubation period. The eggs were checked in a weekly basis (for the presence of blood vessels) and weighed every 14 days.

Twenty-four hours after hatching, the turtles were removed from the incubators and each group was placed into a separate tank containing gravel, shelters, basking platforms and water at 20-cm depth. The holding tanks were kept in an outdoor facility under ambient conditions similar to that experienced in the wild. Temperature loggers placed inside each holding tank (Hobos® TidBit® Onset®) showed that water and air temperatures varied between 20 and 28°C but were similar across all holding tanks. The hatchlings were fed three times a week a commercial turtle pellet diet and pre-frozen bloodworms. The same amount of food was delivered to each tank and it was ensured that all of the food was consumed.

Straight carapace length (SCL) was measured with electronic callipers and body mass was recorded with electronic scales upon hatching (t_0) and every 14 days thereafter until day 56 (t). GR (g day^{-1}) was the same between measurement periods, so a single GR was calculated for the 56-day growth period using the equation:

$$GR = \frac{[mass_t] - [mass_{t_0}]}{t - t_0}$$

Crawling speed

After hatching, the turtles were kept in the incubators for 24 h and, after this period, they had small spots painted on

different areas of their carapaces with non-toxic whiteout for identification (ID). Prior to being placed into their respective holding tanks, hatchlings were placed onto the centre of a hard surface, 50-cm diameter arena covered with sand. Hatchlings (28, 28 ± 3 and 28 ± 6°C) were placed in the arena in groups of two and covered with a dark box for 5 min. The box was then removed, and locomotion time was assessed as the time that the hatchlings started to move to the moment that they reached the edge of the arena. Each hatchling was tested twice, with an hour interval between trials, ambient temperature was constant (26 ± 0.5°C). Crawling speed was determined by the distance covered by the hatchling divided by time.

Righting response

The 'self-righting time' is defined as the time from when the hatchling first moved until the moment it righted itself after being placed upside down on its carapace (Delmas *et al.*, 2007). A 10-L plastic container was fabric lined (covering asymmetries on the surface), partitioned into eight cells of equal size (L 11 × W 11 × H 10.3 cm) and, at 7 days of age, a single hatchling was placed into each cell (eight hatchlings per trial). Hatchlings were positioned at the centre of the cells, which were ~13 times larger than the hatchlings in order to avoid their contact with the walls. The ambient temperature was maintained constant (26 ± 0.5°C) for the entire duration of the experiment. Hatchlings (28, 28 ± 3 and 28 ± 6°C) were fasted for 24 h prior to experimentation and placed inside the cells 45 min prior to each trial. Each hatchling performed three self-righting events per trial. The righting events were recorded by a digital video camera (Sony DCR-HC52, North Ryde, NSW, Australia) and the self-righting time determined by analysis of the recorded images. When turtles did not attempt to self-right within 30 min of being placed on their carapaces, the trail was abandoned and accounted as not-attempted.

Swimming performance

Swimming performance was assessed when the hatchlings were 4 weeks old (28, 28 ± 3 and 28 ± 6°C). Hatchlings were swum individually in a glass aquarium (41 × 26 × 35 cm) at constant temperature (26 ± 0.5°C). Swimming force was measured using a force transducer (MLT010, ADInstruments, Bella Vista, NSW, Australia) attached to the turtle with a monofilament nylon line and Velcro patch (see Burgess, Booth & Lanyon, 2006 for details). Before each trial, the force transducer was calibrated by suspending a known mass in the vertical plane and force was sampled at a frequency of 100 Hz (Power Lab 2/20 connected to a ML110 Bridge amplifier; see Micheli-Campbell *et al.*, 2011). To ensure that the post-feeding duration was equal for all turtles, swimming trails were always undertaken 18–21 h after a feeding event. Swimming trails were performed between 9:00 AM and 12:00 PM. The following variables were measured: (1) mean stroke force: mean force recorded for each consecutive 30-s period over a 10-min recording period; (2) total time spent swimming during the 10-min recording period; (3) stroke frequency: determined by averaging the number of force peaks per second within each swimming event.

Statistics

Multiple analyses of variance (ANOVAs) were used to analyse egg mass, while a *t*-test was performed to analyse SCL, body mass and GR data. The *F*-test was used to denote a significant difference between the means. Data obtained from the one individual incubated at 28 ± 6°C were not included into the analysis. All data are presented as mean ± SE, and a difference between groups was deemed significant if $P < 0.05$ (STATISTICA10, StatSoft, Inc., Tulsa, OK, USA).

Mixed model ANOVA was used for the analyses of the self-righting time, crawling speed, and swimming stroke frequency and force. Body mass was accounted as covariate for the tests, and time was incorporated as covariate for the stroke force data. Turtle ID was included as a random factor because the measurements were taken repeatedly from the same individuals. The effects of incubation temperature upon time swimming were tested using general linear model (analysis of covariance) incorporating body mass as covariate.

Results

Thermal profiles of *E. macrurus* nests in the wild

The temperature data collected from 16 *E. macrurus* nests showed considerable temperature variation within nests throughout the 3-month nesting season (October–December; Fig. 2a). Throughout the incubation period, the daily nest temperature showed a gradual increase from ~26 to ~30°C (Fig. 2a), with an overall average temperature for all the nests during this period of 28.5°C. The fluctuation in daily temperature experienced by the eggs in the wild ranged from 2 to 22°C (Fig. 2b); 40% of the measurement days exhibited a daily fluctuation between 4 and 6°C, 30% between 7 and 11°C, and 4% between 12 and 22°C. Over a 24-h daily cycle, the temperature profile was similar for all nests – with the coolest time of the day occurring around 08:00 and the warmest around 16:00. The 90th percentile showed that for each hour over the 24-h period, the temperature varied by ~6°C throughout incubation (Fig. 2c). The inter-nest median temperature throughout the incubation period ranged between 26 and 30°C, and the overall incubation temperature range also varied between nests (Fig. 2d).

Egg incubation and hatchling morphology

Elusor macrurus eggs initially placed into the different incubation regimes were of similar mass [$n = 54$; 8.82 ± 0.09 g, $F_{(2,51)} = 1.05$, $P = 0.36$], with egg mass remaining similar throughout the entire incubation period for eggs incubated at 28 and 28 ± 3°C. The eggs incubated at 28 ± 6°C, however, had a significantly reduced mass from the second week of development

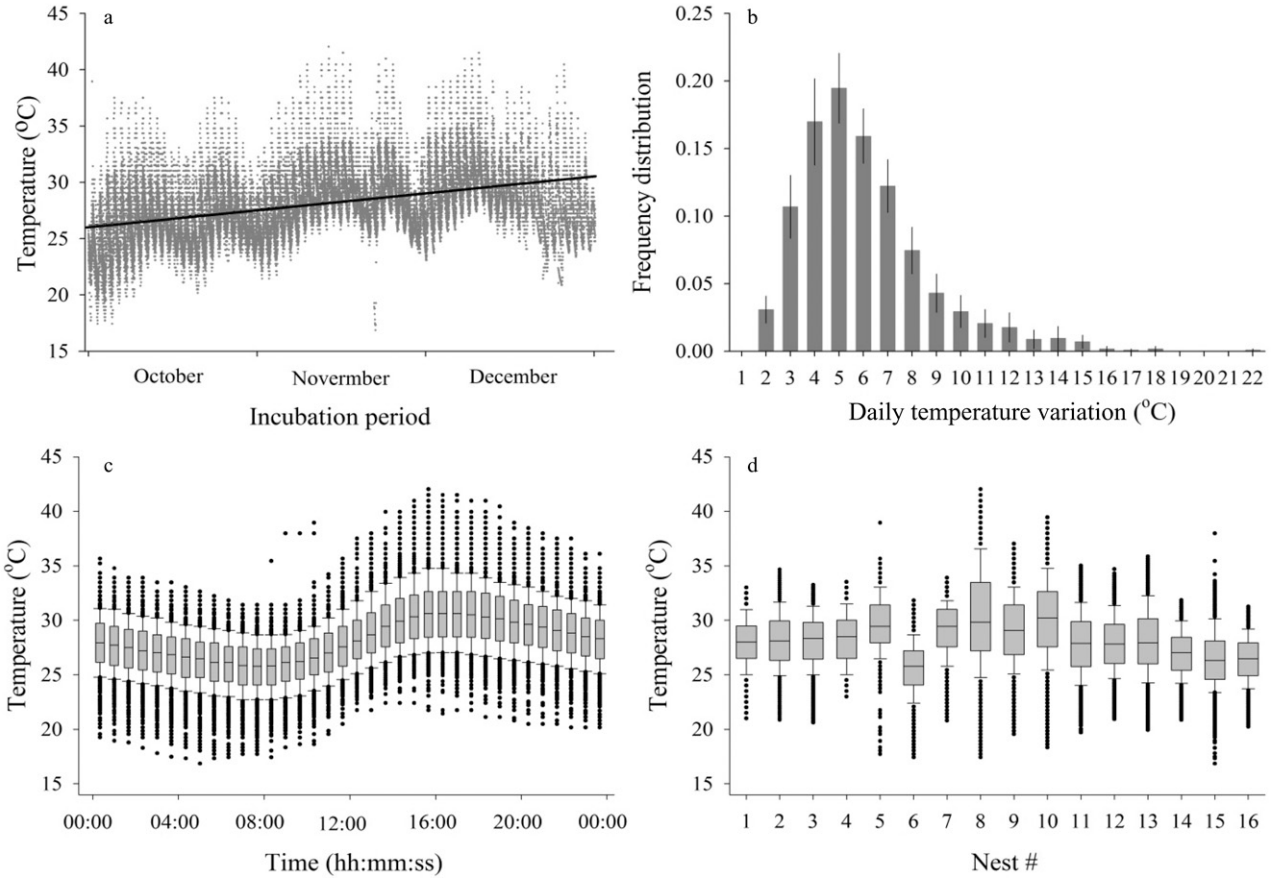

Figure 2 Thermal profile of *Elusor macrurus* nests in the wild ($n = 16$). (a) Overall data collected from October to December during nesting season showing the temperatures experienced by 16 *E. macrurus* nests in the wild (16 data points for each 40-min interval throughout the incubation period; solid line represents the trend line). (b) Frequency distribution of the daily variation experienced by the nests (bars height and error bars indicate the mean ± SE). (c) Box-and-whisker plot of the daily temperature profile data experienced by the nests. (d) Box-and-whisker plot of temperature data recorded from each nest during the incubation period.

[Fig. 3; $F_{(2,51)} > 6.24$, $P < 0.01$]. In this group, the embryos died at different developmental stages throughout the incubation period, with only four hatchlings breaking the egg shell and only one hatchling (5%) survived beyond 24 h (Table 1). Both constant (28°C) and lower fluctuating (28 ± 3°C) thermal regimes had the same hatching success (89%), and the hatchlings had a similar mean body mass [$F_{(1,30)} = 2.25$, $P = 0.12$], SCL [$F_{(1,30)} = 2.60$, $P = 0.07$] and post-hatch GR [$F_{(1,30)} = 2.24$, $P = 0.12$; Table 1]. Because only one individual hatched from the 28 ± 6°C treatment group, it was not possible to perform statistical analysis with these data; however, this hatchling had a smaller body mass, SCL and post-hatch GR in relation to the other hatchlings incubated at the other two thermal regimes (Table 1).

Crawling speed

After removal of the darkened box, the turtles typically stayed stationary for ~3–4 s and then started to crawl. Fortunately for measurement, all turtle movements occurred in a straight line towards the edges of the experimental arena. There was no difference [$F_{(1,30)} = 1.18$, $P = 0.84$] in the crawling speed exhibited by the 1-day-old hatchlings from constant (28°C) and fluctuating (28 ± 3°C) temperature treatment groups (Table 2). The one hatchling that survived from the group incubated at 28 ± 6°C failed to crawl when tested.

Righting response

There was no statistical difference in self-righting time between the constant (28°C) and fluctuating (28 ± 3°C) experimental groups [Table 2; $F_{(1,30)} = 2.51$, $P = 0.12$]. The only survivor from the 28 ± 6°C group was the only individual that did not attempt to right itself within the maximum trial time (30 min).

Swimming performance

During the first few seconds after being placed in the water, the hatchlings exerted their highest stroke force, which

declined sharply over the first 120 s, followed by a gradual decline throughout the rest of the trial period (Fig. 4). Both time [$F_{(1,638)} = 713.2$, $P < 0.01$] and body mass [$F_{(1,30)} = 11.24$, $P < 0.01$] had significant effects upon the stroke force exerted by the hatchlings, which was not, however, influenced by incubation thermal regime [Fig. 4; $F_{(1,30)} = 0.08$, $P = 0.77$]. Both groups achieved similar maximum stroke force during the first 30 s (28°C group = 121.0 mN; 28 ± 3°C group = 123.4 mN), and both groups had a similar decline in swimming force, with stroke force decreasing to approximately 50 mN after 4.5 min (Fig. 4). Stroke frequency during swimming was not affected by incubation thermal regime [Table 2; $F_{(1,30)} = 0.44$, $P = 0.51$], but was significantly affected by body mass [$F_{(1,30)} = 11.10$, $P < 0.01$]. The length of time that the hatchlings spent swimming during trials was not affected by body mass [Table 2; $F_{(1,30)} = 1.94$, $P = 0.17$] or incubation thermal regime [Table 2; $F_{(1,30)} = 0.95$, $P = 0.34$].

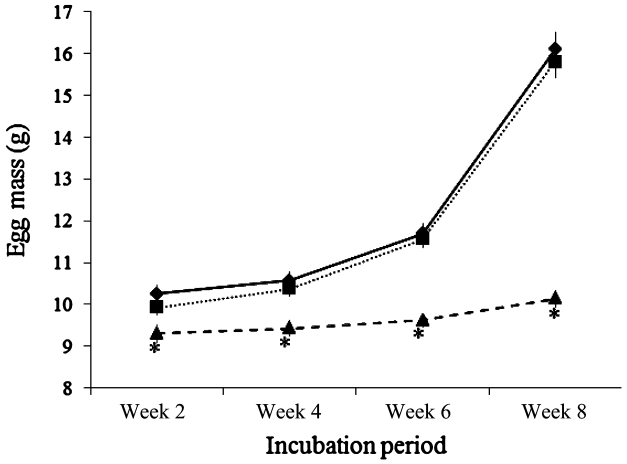

Figure 3 Mass during development of *Elusor macrurus* eggs ($n = 18$) during incubation in the laboratory. Symbols and error bars indicate the mean ± SE (solid lines = 28°C; dotted lines = 28 ± 3°C; dashed lines = 28 ± 6°C), and (*) indicates statistical differences ($P < 0.05$).

The one survivor from the 28 ± 6°C group had a similar swimming pattern; exerting its maximum stroke force during the first 30 s of the trial, followed by a period when such stroke force decreases substantially, until the last phase when the stroke force was maintained at a constant level (Fig. 4). However, the hatchling maximum stroke force was only 69.9 mN, and its constant stroke force was approximately 30 mN (Fig. 4). This hatchling spent 268.3 s swimming, which was about half the time spent swimming in comparison to the hatchlings from the other incubation regimes, but its stroke frequency of 31.1 Hz was greater than that exhibited by the hatchlings from the other two experimental groups (Table 2).

Discussion

The present study examined the temperature profiles of *Elusor macrurus* nests in the wild, and experimentally incubated eggs in thermal regimes that mimicked those experienced in natural nests. The extent of the daily temperature fluctuations varied across the entire nesting season and between nests, with mean temperature for all the nests varying by approximately 4°C throughout the nesting season. Approximately 66% of measured days showed a daily fluctuation in temperature of between 2 and 6°C, with higher daily temperature fluctuations only being experienced occasionally. Under controlled laboratory conditions, *E. macrurus* eggs incubated at a constant mean temperature of 28°C showed no significant difference in hatchling phenotype from those incubated at the same mean temperature but under a daily fluctuation of 6°C. A previous study showed that an increase in the mean constant incubation temperature from 29 to 32°C was detrimental to hatchlings of this species of turtle (Micheli-Campbell *et al.*, 2011). A limitation of this study was, however, that the eggs were incubated at constant thermal regimes that did not reflect the natural thermal variability experienced by eggs incubating in natural nests. The present study addresses this limitation by demonstrating that environmentally realistic fluctuations in daily nest temperatures are not as significant in influencing

Table 1 Summary data from eggs ($n = 18$) and hatchlings *Elusor macrurus* incubated in captivity at three thermal regimes: 28°C constant, 28 ± 3°C and 28 ± 6°C. The straight carapace length (SCL) and body mass data are upon hatching

Incubation temperature	28°C ($n = 16$)	28 ± 3°C ($n = 16$)	28 ± 6°C ($n = 1$)
Incubation length (days)	56 ± 0.2 (55–58)	57 ± 0.2 (55–59)	66
Hatching success	89%	89%	5%
SCL (mm)	32.4 ± 0.2 (31.1–33.3)	32.1 ± 0.3 (30.1–33.8)	27.6
Body mass (g)	7.2 ± 0.1 (6.7–8.1)	7.3 ± 0.1 (6.4–8.1)	5.3
Growth rate (g day^{-1}; over 56 days)	0.15 ± 0.01	0.15 ± 0.1	0.19

Table 2 Summary data from performance experiments upon hatchlings *Elusor macrurus*. Data are mean ± SE ($P > 0.05$)

Incubation temperature	28°C ($n = 16$)	28 ± 3°C ($n = 16$)	28 ± 6°C ($n = 1$)
Crawling speed (cm s^{-1})	2.3 ± 0.2	2.2 ± 0.2	–
Righting response (self-righting time; s)	27.8 ± 6.5	38.0 ± 9.1	–
Swimming performance (time swimming; s)	448.1 ± 32.8	470.7 ± 15.5	268.3
Swimming performance (stroke frequency; Hz)	23.9 ± 0.5	26.5 ± 0.5	31.1

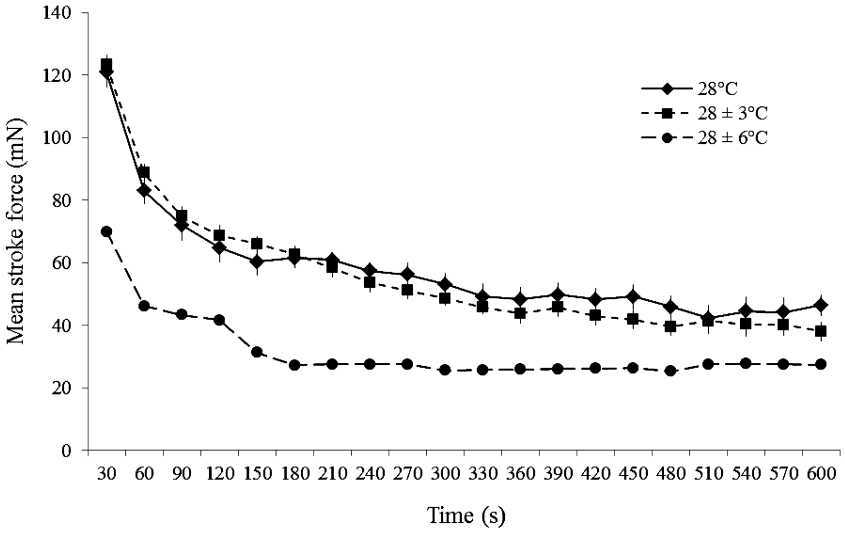

Figure 4 Mean stroke force (mN) generated every 30 s over a 10-min trial period by hatchlings *Elusor macrurus* incubated at three thermal regimes: one constant (28°C, n = 16) and two fluctuating (28 ± 3°C, n = 16; 28 ± 6°C, n = 1). Symbols and error bars indicate the mean ± SE ($P > 0.05$).

hatchling phenotype as absolute differences in the mean temperature (Micheli-Campbell *et al*., 2011).

A constant thermal regime of 32°C during incubation significantly reduced the GR, self-righting time and swimming performance of hatchling *E. macrurus*, as well as detrimentally affecting their morphology (smaller individuals; Micheli-Campbell *et al*., 2011). The results of the present study have shown that a fluctuating incubation thermal regime, which attained temperatures of up to 31°C for a short period each day throughout the entire incubation period, did not influence the phenotype of hatchling *E. macrurus*. However, this absence of effect only occurred within a certain temperature range and the incubation of hatchling *E. macrurus* under a 12°C daily fluctuation thermal regime, with temperatures reaching 34°C for a short period each day, was lethal for 95% of the embryos and phenotypically detrimental for those that hatched.

The eggs that were exposed to a 12°C daily temperature fluctuation did not gain weight to the same magnitude as the eggs from the other experimental groups. This pattern occurred from early stages of the developmental period and illustrated that the embryos exposed to a 12°C daily fluctuating temperature were converting the yolk material into tissue at a lower rate in comparison to the other experimental groups. Yolk consumption was not directly measured, but the eggs were checked on a weekly basis for the presence of blood vessels and weighed fortnightly. These measurements confirmed that the eggs were not dead, although they were not gaining weight at the same rate as the groups exposed to a reduced fluctuation in daily temperatures. Only four hatchlings from this group broke through the egg shell under their own volition, but only one was sufficiently strong to complete the process and survive. The timeline of embryo development has not been described for *E. macrurus*. However, for the freshwater turtle *Chelydra serpentina*, after 2 weeks of development, the embryos have a notochord and at least 14 pairs of somites, and the tail is beginning to take shape. The neural folds, forming the cranial structure, are completely fused by the 12th day of the development, and the embryo has a partially developed heart (Yntema, 1968). This phase in the development of the nervous and circulatory systems is likely to result in the embryos being highly sensitive to external thermal conditions (see Deeming & Ferguson, 1991). Studies of oviparous reptile embryogenesis have shown that excessively elevated or reduced incubation temperatures during embryo development cause abnormalities of the central nervous system and vertebral column, disrupt development of the hypothalamus and alter yolk absorption. In most cases, high embryo mortality results (Yntema, 1960; Vinegar, 1973, 1974; Webb *et al*., 1983; Ferguson 1985; Burger, Zappalorti & Gochfeld, 1987; Deeming & Ferguson, 1988, 1989; Birchard & Reiber, 1996). Therefore, a large number of developmental variables may be detrimentally affected by temperature during the egg incubation period in reptiles. In the present study, defining which embryogenesis stage and/or morphological structures were responsible for the high mortality in the 28 ± 6°C group was inconclusive. The embryos died across a range of developmental stages and it was not possible to define which mechanisms were responsible for the malformations and developmental abnormalities observed for *E. macrurus* embryos exposed to 12°C daily temperature fluctuation.

Data collected from nests in the wild showed that *E. macrurus* eggs were exposed to daily fluctuations in temperature of 12°C and above for ~4% of the time. However, such variations were not experienced by the embryos on a regular basis during the developmental period. The median nest temperatures recorded here combined with findings from a previous study (Micheli-Campbell *et al*., 2011) strongly suggest that *E. macrurus* eggs in the wild are being exposed to median temperatures approaching the upper thermal threshold for the species. Occasionally, *E. macrurus* nests have been found where the whole clutch has not developed, and more regularly where a few eggs from the clutch do not develop (Micheli-Campbell, pers. obs.).

Although this study suggests that elevated temperatures during incubation may be responsible for some of the mortality of embryos, monitoring of nests temperature and hatching success over multiple years is required to confirm this hypothesis. The urgency for these data is highlighted by the 23 Global Climate Models (IPCC, 2007), which predicted a 12-fold increase in the number of days reaching temperatures above 35°C over the next 60 years in the Mary River catchment (Queensland Government, 2009). This scenario may result in increases of both mean and daily fluctuations in ambient temperature. The *E. macrurus* population has crashed from that of only a few decades ago (Flakus, 2002) and the species is currently listed as endangered (IUCN, 2011). As a consequence of the low population, the gene pool of the species has a limited capacity to respond to environmental shifts. Moreover, the population is geographically restricted to the Mary River catchment and therefore unable to relocate to a more optimal environment if conditions become unfavourable. Based on the findings of the present study, we argue that the recruitment of hatchling *E. macrurus* into the wild may be compromised because of greater fluctuations in the daily thermal regime during incubation as well as increases in the average temperature (as shown by Micheli-Campbell *et al*., 2011). These effects may be especially pronounced if the embryos experience these conditions at the beginning of the developmental period.

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