

# Staying cool, keeping strong: incubation temperature affects performance in a freshwater turtle

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## Keywords

*Elusor macrurus*; hatchling; swimming performance; righting response; climate change; conservation physiology.

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## Abstract

It is unclear how predicted rises in ambient temperature associated with climate change will impact upon the survivorship of oviparous reptiles. Given that the incubation temperature can influence hatchling phenotype, understanding how elevated temperatures during development can affect the ability of hatchlings to undertake routine behaviours is important, especially for threatened species. Here we tested if raising mean incubation temperature above natural levels altered the physiology of hatchlings to an extent that behavioural function was impaired. Firstly, incubation temperatures were recorded from nests of the freshwater turtle (*Elusor macrurus*) in the wild, and the observed thermal range (26–31 °C) used to define the experimental protocol. Then, freshly laid *E. macrurus* eggs were collected and incubated at three constant temperatures (26, 29 and 32 °C). Embryos incubated at 32 °C had the lowest hatching success. Those that did hatch were smaller than the other groups and had a reduced post-hatch growth rate. On land, the ability of hatchling turtles to right themselves is critical, and the turtles incubated at 32 °C took 30-times longer to do this than those incubated at 26 °C. Once in the water, hatchling turtles must be able to swim effectively to evade predation and obtain food items. During swimming trials the 32 °C group exhibited a lower mean stroke force ( $10.5 \pm 0.3$  mN) and spent less time swimming ( $133.7 \pm 17.7$  s) compared with hatchlings incubated at 29 °C ( $13.4 \pm 0.4$  mN,  $281.3 \pm 25.7$  s) and 26 °C ( $15.7 \pm 0.5$  mN,  $270.8 \pm 28.5$  s). The results of the present study illustrate that even slight rises in the mean incubation temperature, over that observed in the wild, can impact upon a hatchling's performance.

## Introduction

Incubation temperature affects embryonic growth and development of oviparous animals (Deeming & Ferguson, 1991). If the incubation temperature falls outside the embryo's optimum thermal range for significant periods of time (low or high) embryonic malformation may occur, such as bone deformity, organ failure and dysfunction of the central nervous system (see Deeming & Ferguson, 1991; Burgess, Booth & Lanyon, 2006). Thermal conditions during incubation are therefore an important determinant of the embryos fitness, and birds generally incubate their eggs at close to constant temperatures with little variation among species (Deeming & Ferguson, 1991). Most reptiles do not provide parental care and their eggs are exposed to far more variation in incubation temperature than birds (Deeming & Ferguson, 1991). Many species of turtle do, however, bury their eggs in an underground chamber, which not only provides protection from predation but also buffers the eggs from diel variations in temperature and prevents desiccation (Miller & Dinkelacker, 2008).

The thermal buffering effect of chamber depth on egg incubation temperature is substantial, with one study

reporting diel variations of 40 °C at the surface are nullified at 50 cm depth (Booth, 2006). Generally, there is a linear relationship between the body size of the nesting female and the depth of the egg chamber (Booth & Astill, 2001), and because of this freshwater turtles generally lay shallower nests compared with marine turtles. Shallower nests will be more susceptible to variations in the environmental conditions, and therefore the effects of localized shifts in ambient temperature may first become apparent within freshwater turtle species.

Varying the temperature of incubation by only a few degrees centigrade has been shown to alter both the morphological and physiological traits of hatchling turtles, such as body size, amount of residual yolk upon hatching, growth rate (GR), diving ability and locomotor performance (Janzen, 1993; Bobyn & Brooks, 1994; Roosenburg, 1996; Booth, 2000; Steyermark & Spotila, 2001; Du & Ji, 2003; Booth *et al.*, 2004; Burgess *et al.*, 2006; Delmas *et al.*, 2007; Mickelson & Downie, 2010). However, it is difficult to draw solid conclusions from the literature due to large inter-study variation to the response of varying incubation temperature. This may be due for two main reasons: (i) the sex of many of the species examined is determined by incubation

temperature with the threshold temperature varying between species; (ii) the range of experimental temperatures are disparate in their relationship to the incubation temperature experienced in the wild nests. We argue that experimental trials need to be undertaken on freshwater turtle species where the influence of temperature sex determination can be removed, and secondly, that experimental results need to be relevant to what may be expected in the natural conditions.

If environmental conditions are unfavourable for a species, local extinctions may occur, and species with a limited geographic distribution will be more susceptible. Freshwater turtles are often confined by suitable habitats and many have small geographical ranges (Cann, 1998). Mary River turtle *Elusor macrurus* is a freshwater turtle, which is only found within a single river system in Queensland, Australia. The population has dramatically decreased over the past decades and the species is currently listed as endangered (IUCN red list 2008). Females lay rigid-shelled eggs in shallow nests (<20 cm depth) on open sandy banks with no vegetation cover (Cann & Legler, 1994). Climate records for the local area have shown a gradual increase in ambient temperature over the past three decades, which are particularly prevalent during the spring and summer months (~1 °C, QLD Government, 2009). This time correlates with the egg incubation period for *E. macrurus* and we hypothesized that alterations in hatchling physiology due to changes in the incubation temperature would be deleterious to the survival of the hatchling turtles.

The aims of the present study were to assess the range of mean incubation temperatures experienced by the eggs in the wild, and then to determine how different incubation temperatures may influence hatchling phenotype. We also wanted to assess how predicted rises in ambient temperature for the local area may impact upon the physiology and performance of the hatchlings.

## Materials and methods

### Study site and nest temperatures

Between October and December, the Mary River turtle *E. macrurus* nests on non-vegetated sandy banks along the Mary River, Queensland, Australia. Nesting occurs at night after a period of heavy rain (Cann & Legler, 1994), and for this study the nests were located the following morning during spring 2009. Upon locating nests, the egg chamber was exposed by hand, and a temperature data logger (2 cm diameter, Hobos<sup>®</sup> TidBit<sup>®</sup>, Onset<sup>®</sup>, Bourne, MA, USA) that logged temperature every 40 min was placed adjacent to the clutch at a depth which corresponded to the middle of the nest chamber. The sand was then replaced and the clutch covered to the original depth. The nests were monitored once a week for signs of hatching, and the temperature data logger was then removed once the hatchlings had exited the nest. Data were obtained for 16 nests that were laid on four river banks along a 30 km stretch of river.

### Egg incubation and hatchling morphology

Fifty-six *E. macrurus* eggs (from three different clutches) were collected 48 h after being laid in nests along the Mary River, Queensland, Australia. The eggs were weighed and randomly distributed into three containers (one per experimental treatment) 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) set at 26, 29 or 32 °C. The sand surrounding the eggs were sprinkled with water every 48 h throughout the entire incubation period, in order to maintain near constant water potential. The eggs were weighed every 14 days. Twenty-four hours after hatching, the turtles were removed from the incubators and each group 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 with a climate similar to the turtle's geographic location. Water and air temperatures varied with ambient conditions (between 20 and 28 °C) and were similar for all holding tanks. The hatchlings were fed three times a week on commercial turtle pellets and pre-frozen bloodworms.

Straight carapace length was measured with electronic callipers and body mass recorded with electronic scales upon hatching, 10 days after hatching (once they have completely absorbed the yolk sacs) and then every 14 days, and the GR (g/day), which is linear and it was calculated using the following equation:

$$GR = \frac{[\text{mass}_t] - [\text{mass}_{t_0}]}{t - t_0}$$

where  $t$  is time difference between measurements ( $t = 10$  days old;  $t_0 = 105$  days old).

### Righting response

The 'time to right' of the hatchlings is defined as the time from when the turtle started to move, after being placed upside down, to the moment it righted itself (Delmas *et al.*, 2007). A paper lined 51 plastic container was partitioned into quarters and a 10-day-old hatchling was placed into each quarter (four hatchlings per trial). Hatchlings (26 and 29 °C groups,  $n = 17$ ; 32 °C group,  $n = 10$ ) were placed inside the cells 45 min before the trial to allow them to acclimate to the experimental environment. The ambient temperature was maintained at  $26 \pm 0.5$  °C for the duration of the experimental period. Hatchlings were fasted for 24 h before experimentation. Each hatchling performed three righting events per replicate. The trials were recorded by a digital video camera (Sony DCR-HC52, North Ryde, NSW, Australia) and the time to right data were collected and analysed from the images. In some instances the turtle did not right itself after 30 min and due to ethical concerns the trial was aborted and the data were not used in the study. The study was repeated 2 weeks later, resulting in each turtle being tested six times.

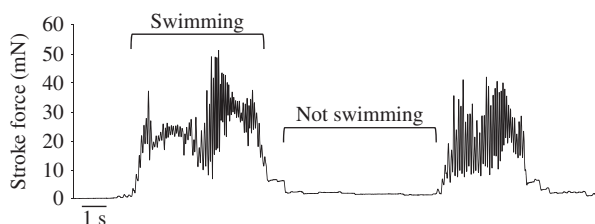
## Swimming performance

Swimming performance was assessed by examination of stroke frequency and force, and the proportion of time spent swimming. At 4 weeks of age 10 hatchlings were randomly selected from each treatment group and swam individually in a glass aquarium (41 × 26 × 35 cm) filled with 30 cm of freshwater at constant temperature (26 ± 0.5 °C). A Velcro patch (1 cm<sup>2</sup>) was glued to the carapace and a monofilament nylon line attached to the Velcro (Burgess *et al.*, 2006). The nylon tether was attached to a force transducer (MLT010, AD Instruments, Bella Vista, NSW, Australia) in the perpendicular plane, resulting in the measured force on the vertical plane regardless of the direction the turtle swam (for details see Burgess *et al.*, 2006). The force transducer was calibrated before each trial by suspending a known mass in the vertical plane. The force transducer was connected to a data acquisition system (Power Lab 2/20 connected to a ML110 Bridge amplifier) and the force sampled at a frequency of 100 Hz. Each hatchling swimming trial was replicated 14 days apart, and hatchlings were not fed for 18 h before swimming. Force recordings (Fig. 1) were analysed for the following variables: (i) mean stroke force: mean force recorded for each consecutive 30-s period over an 8 min recording period; (ii) total time spent swimming during the 8-min recording period; (iii) stroke frequency: determined by averaging the number of force peaks per second within each swimming event.

## Statistics

Straight carapace length, body mass and growth rate data from the three experimental groups were tested using multiple sample analyses of variance. The *F*-test was used to denote a significant difference between the means. If significant differences existed, multiple range tests were used to show which means were significantly different from each other. Kruskal–Wallis tests were used if the presence of outliers was detected. All data are presented as mean ± SE, and a difference between groups was deemed significant if  $P < 0.05$  (StatGraphics Plus 5.1).

The data for the righting response and mean stroke force were investigated using general linear mixed models (Zuur *et al.*, 2008). These data were log-transformed to standardize and homogenize residuals. Body mass was incorporated as a covariate and because measurements were taken repeatedly from the same individuals, turtle ID was included as a random effect. For the swimming performance data the interaction



**Figure 1** Force trace generated by a hatchling *Elusor macrurus* during a trial of the swimming performance experiment.

between incubation temperature and time was included in the model. All mixed models were performed in the R programming language (R Development Core Team 2010) using the nlme library of functions (Pinheiro *et al.*, 2010).

The effects of incubation temperature upon time swimming and mean stroke frequency were tested using a Multi-factor ANOVA, accounting for body mass as a covariate (StatGraphics Plus 5.1).

## Results

### Thermal profiles of nests

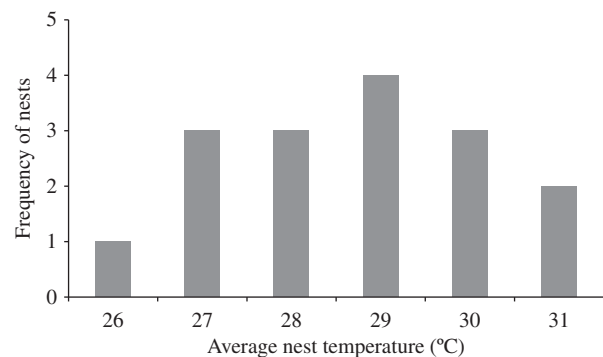
The average incubation temperature of *E. macrurus* eggs in the wild ranged from 26 to 31 °C (Fig. 2). Out of the 16 nests measured, 25% had a mean temperature of 29 °C, with one nest having a mean temperature of 26 °C and two nests a mean of 31 °C. Collectively the mean temperature for all nests measured over the entire incubation period was 28.5 ± 3.2 °C (mean ± SD).

### Egg incubation and hatchling morphology

There was no difference in the initial egg mass between the temperature treatments before incubation ( $F_{(2,53)} = 0.29$ ,  $P = 0.75$ ). The incubation period was significantly different for each treatment group with eggs incubated at higher temperatures hatching earlier (Table 1,  $F_{(2,41)} = 73.68$ ,  $P < 0.001$ ). Eggs incubated at 32 °C had a lower hatching success compared with 26 and 29 °C (Table 1). Furthermore, 10 days after hatching, hatchlings incubated at 32 °C were smaller than those from 26 to 29 °C ( $F_{(2,41)} = 7.24$  and 6.81, respectively,  $P < 0.05$ ; Table 1). Hatchlings from the 32 °C group remained smaller than their siblings incubated at lower temperatures for the remaining experimental period (~100 days), because of a slower post-hatch growth rate ( $F_{(2,41)} = 6.54$ ,  $P = 0.003$ ; Table 1).

### Righting response

Righting time for 10-day-old hatchlings was influenced by incubation temperature (Fig. 3). Hatchlings incubated at

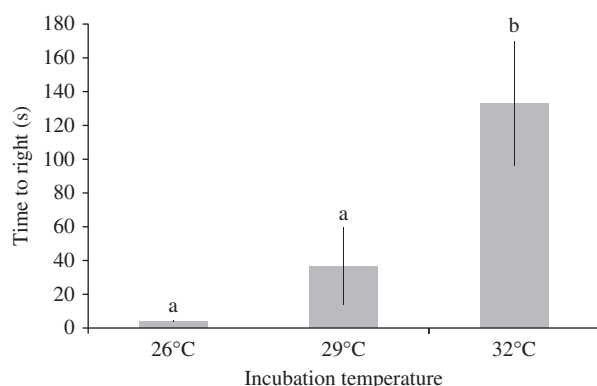


**Figure 2** The distribution of mean nest temperature for 16 *Elusor macrurus* nests during the 2009 nesting season. Data were collected from four nesting banks along a 30 km stretch of the river.

**Table 1** Summary data from eggs and hatchling *Elusor macrurus* incubated in captivity at three constant temperatures: 26, 29 and 32 °C

Incubation temperature	26 °C (n=17)	29 °C (n=17)	32 °C (n=10)
Incubation length (days)	70 ± 0.7 (68–72)*	52 ± 0.7 (50–54)*	42 ± 0.5 (41–43)*
Hatching success	89%	89%	56%
SCL (mm) upon hatching	34.9 ± 0.5	34.9 ± 0.3	32.7 ± 0.4*
Body mass (g) – upon hatching	7.38 ± 0.2	7.31 ± 0.1	6.91 ± 0.1*
Growth rate (g)/day - 105 days old	0.047 ± 0.003	0.041 ± 0.003	0.029 ± 0.004*

\*Indicates statistical differences ( $P < 0.05$ ) and numbers indicate the mean ± SE.



**Figure 3** Time for *Elusor macrurus* hatchlings (10 days old) to right themselves as they started to move after being placed upside down (time to right). Hatchlings were incubated at three constant temperatures (26 and 29 °C groups:  $n = 17$ ; 32 °C group:  $n = 10$ ). Bars height and error bars indicate the mean ± SE. Different letters indicate significant differences ( $P < 0.001$ ).

26 °C group righted themselves 10-times faster than hatchlings incubated at 29 °C, and 34-times faster than the 32 °C group ( $L = 30.57$ , d.f. = 2,  $P < 0.01$ ; Fig. 3). A repeat of the same experiment with the same individuals 14 days later showed the same response between groups ( $P < 0.05$ ). Body mass (g) was not significantly correlated with the time to right ( $L = 1.24$ , d.f. = 1,  $P = 0.26$ ).

### Swimming performance

In general, the behaviour of the hatchlings during the swimming trials was similar with the highest stroke force exerted during the first few seconds after entrance into the water after which the mean stroke force declined over approximately 2 min, and then remained relatively constant throughout the remainder of the trial (Fig. 4). The interaction between time and incubation temperature was significant for the model ( $L = 10.17$ , d.f. = 2,  $P < 0.01$ ). The mean stroke force during the first 30 s of the trial was significantly different between all three treatments ( $L = 6.43$ , d.f. = 2,  $P < 0.05$ ). Hatchlings incubated at 32 °C had a significantly lower mean stroke force than those incubated at 26 and 29 °C (ANCOVA LS adjusted means: 26 °C group =  $29.0 \pm 1.2$  mN, 29 °C group =  $29.7 \pm 1.0$  mN, 32 °C group =  $25.4 \pm 1.3$  mN;  $F_{(2,27)} = 71.43$ ,  $P < 0.01$ ; Fig. 4). This pattern was continued with the 32 °C group maintaining a significantly lower mean stroke force

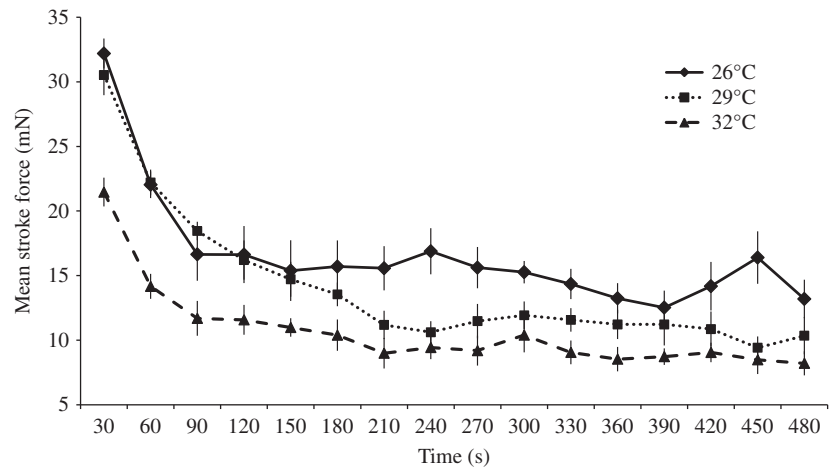
for the 8 min duration of the trial ( $L = 7.37$ , d.f. = 1,  $P < 0.01$ ; Fig. 4). Hatchlings incubated at 26 °C maintained a greater mean stroke force for the remaining trial period when compared with the other two groups ( $L = 4.27$ , d.f. = 1,  $P < 0.05$ ; Fig. 4). Body mass (g) was significantly correlated with mean stroke force during swimming performance trials ( $L = 14.41$ , d.f. = 1,  $P < 0.001$ ), but even when adjusted for differences in body mass, the hatchlings incubated at 32 °C still had a significantly reduced mean stroke force during the first 30 s of the swimming trial (Fig. 4) relative to the other two groups.

Hatchlings incubated at 32 °C spent significantly less time swimming during the 8 min trial (26 °C group =  $270.8 \pm 28.5$  s, 29 °C group =  $281.3 \pm 25.7$  s, 32 °C group =  $133.7 \pm 17.7$  s;  $F_{(2,27)} = 11.38$ ,  $P < 0.01$ ; Fig. 5a), but had a significantly greater stroke frequency per swimming event when compared with the other two groups (26 °C group =  $4.7 \pm 0.1$  Hz, 29 °C group =  $4.4 \pm 0.1$  Hz, 32 °C group =  $6.1 \pm 0.2$  Hz;  $F_{(2,27)} = 43.78$ ,  $P < 0.01$ ; Fig. 5b).

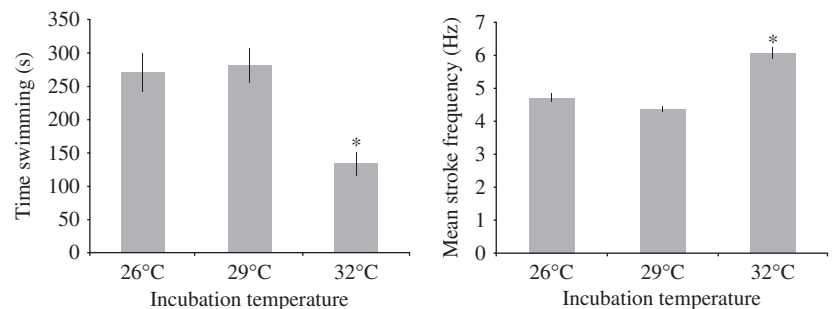
### Discussion

The present study showed conclusively that differences in incubation temperature of only a few degrees altered the phenotype and performance of hatchling Mary River turtles *E. macrurus*. The eggs were incubated at three experimental temperatures, two were within the range of natural nest temperatures, and one treatment was 1 °C warmer than the highest mean nest temperature recorded in the wild. Those hatchlings incubated at this higher than average nest temperature had a significantly poorer performance throughout experimental trials, and this physiological limitation upon behavioural function may have detrimental ecological implications for this species.

The *E. macrurus* embryos hatched earlier when incubated at warmer temperatures. This is a typical response in oviparous reptiles, resulting from an increased embryonic rate of development (Deeming & Ferguson, 1991). A consequence of a shorter period for incubation can be less yolk material being converted into tissue (Booth, 2000), and accordingly, *E. macrurus* hatchlings incubated at 32 °C had a significantly smaller body size upon hatching than those from the 29 °C group. Body size was similar between the 26 and 29 °C groups, suggesting that the thermal effects upon yolk conversion were only significant at the higher thermal range of the study. This result is opposite to that observed for the smooth softshell turtle *Apalone mutica* which



**Figure 4** Mean stroke force (mN) generated every 30 s over an 8 min trial period by *Elusor macrurus* hatchlings ( $n=10$ ) incubated at 26, 29 and 32 °C. Symbols indicate the mean  $\pm$  SE.



**Figure 5** Total time spent swimming (a) and stroke frequency per power stroking output (b), of *Elusor macrurus* hatchlings ( $n=10$ ) incubated at 26, 29 and 32 °C. Bars height and error bars indicate the mean  $\pm$  SE. \*Indicates statistical difference ( $P<0.01$ ).

hatched with a larger body size when incubated at 30 °C, compared with 28 °C and 26 °C (Janzen, 1993). Similar discrepancies can be observed throughout the freshwater turtle literature (Brooks *et al.*, 1991; Rhen & Lang, 1995; Roosenburg & Kelley, 1996; Janzen & Morjan, 2002; Ji *et al.*, 2003; Booth *et al.*, 2004) and may have arisen due to disparity in the relationship between the experimental thermal range and the optimum ambient temperature for the embryos. For example, the average thermal range of *A. mutica* nests in the wild is between 28 and 36 °C (Ewert, 1979), and therefore the experimental study undertaken by Janzen (1993) was within the lower portion of the nest thermal range.

After hatching, the growth rate of *E. macrurus* incubated at 32 °C remained significantly lower than those incubated at 26 and 29 °C. Turtles with high post-hatch growth rates would attain a larger size quicker, which may decrease predation risk, increase digestive breadth (i.e. handle and consume a greater range of prey items) and reach sexual maturity earlier (Perez, Collado & Ramo, 1979). Post-hatch growth rates have been used as an indicator of post-hatch fitness in Chelonians, but again the influence of incubation temperature shows contrasting inter-study results; with increased post-hatch growth occurring at the highest (Bobyn & Brooks, 1994; Booth *et al.*, 2004), the intermediate (Spotila *et al.*, 1994), and the lowest experimental incubation temperatures (Roosenburg & Kelley, 1996), and some studies finding that incubation temperature had no effect on post-hatch growth rate (Ji *et al.*, 2003).

As in most turtles, female *E. macrurus* lay their eggs in a nest buried on land, and the hatchlings must make their own way to water. On land, hatchling turtles are destabilized easily and frequently find themselves tipped upside down (Burger, 1976). This may increase mortality through predation or dehydration (Finkler, 1999; Steyermark & Spotila, 2001; Kolbe & Janzen, 2002). Experimental manipulation of *E. macrurus* hatchlings in the laboratory showed that the ability of a hatchling to right itself was substantially reduced when incubated at 32 °C. This effect was maintained over 3 weeks post-hatching, thus covering the period when they would be leaving the nest and moving by their own accord to the river. Despite the importance of righting behaviour to the survival of hatchling turtles, only a few studies have examined how it is influenced by incubation temperature. Hatchlings of the freshwater turtle *Graptemys ouachitensis* and *Trachemys scripta elegans* took a longer time to right themselves when incubated at 25 °C compared with 30 °C (Freedberg *et al.*, 2004). The sex of these species of freshwater turtle is, however, determined by incubation temperature with incubation at 25 °C producing all males and at 30 °C producing all females. Therefore, the extent by which temperature influences the hatchlings ability to right itself was potentially confounded by differences in sex. *Elusor macrurus* do not show temperature-dependent sex determination (Georges & McInnes, 1998), and therefore the effects of incubation temperature could be examined without the complicating influence of sex.

Once they have entered water, hatchling turtles need to move through the aquatic environment, acquire food and evade predators. These behaviours are likely to be influenced by the strength and stamina of swimming, and performance may influence survival. Hatchlings incubated at 26 °C maintained mean stroke force at a significantly higher level than hatchlings from 29 °C, and 29 °C were significantly higher than 32 °C over the 8 min swimming trial. The lower absolute force of the 32 °C group was in part caused by their smaller body size, but the reduction in mean stroke force remained significant even when differences in body mass were accounted for by using body mass as a covariate. Although the 32 °C group had a significantly higher stroke frequency per swimming bout than the other groups, this did not compensate for their lower mean stroke force and shorter length of the swimming bout. Comparing all previous studies that have examined the effects of incubation temperature upon swimming performance in turtles, there is general disagreement about its effect, with warmer incubation temperature increasing performance in some species but decreasing in others (Janzen, 1993; Du & Ji, 2003; Booth *et al.*, 2004; Burgess *et al.*, 2006).

The underlying physiological processes responsible for the significantly reduced performance in hatchling *E. macrurus* incubated at 32 °C are presently unclear. It has been proposed in alligators that one of the organs largely affected by incubation temperature is the hypothalamus, directly affecting the levels of different hormones released by this organ during embryogenesis (Deeming & Ferguson, 1988, 1989). This may lead to a number of physiological impairments to muscle physiology, tissue aerobic capacity, bone morphology, mitochondrial oxidation, metabolic efficiency or cardio-respiratory regulation (Kinney, Matsuura & White, 1977; Burggren & Shelton, 1979; White, Hicks & Ishimatsu, 1989; Wang & Hicks, 1996; Hicks & Wang, 2004; Guderley & Seebacher, 2011; Reed *et al.*, 2011). Further studies are required to identify, which of these are the most significant in disrupting hatchling performance.

Experimental studies can never exactly replicate the wild situation and it should be noted that the thermal regimes of the eggs in the laboratory were kept constant where as the temperature of nests in the wild fluctuated both daily and over longer time periods. It was not possible, however, to standardize the level of variability that the wild nests were exposed to because they varied widely between nests. This occurred due to nest depth, the soil composition, slope angle and nesting bank slope bearing and orientation. Here we simplified the protocol by incubating the eggs at constant thermal regimes and further comparative studies are required to explore the significance of fluctuating and constant incubation temperatures upon hatchling performance.

In the case of *E. macrurus*, the outlook does not appear good. Our study findings show that a mean incubation temperature of 1 °C above the warmest nest recorded during the 2009 nesting season reduced hatching success, hatchling growth rate and compromised righting and swimming abilities. The mean air temperature throughout the 2009 nesting season was not significantly different from that

recorded over the past decade for the area, but these years had a mean ambient temperature between 0.5 and 1.2 °C warmer when averaged over previous decades (Commonwealth Scientific and Industrial Research Organisation & Bureau of Meteorology, 2007). Although we do not illustrate a direct connection between warming of the environment and the decline in the *E. macrurus* population, our findings suggest that predicted increases in ambient temperature for the Mary River catchment (1.6–3.0 °C by 2070, QLD Government, 2009) may be detrimental to this species.

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