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RESEARCH ARTICLE

Tropical flatback turtle (*Natator depressus*) embryos are resilient to the heat of climate change

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ABSTRACT

Climate change is threatening reproduction of many ectotherms by increasing nest temperatures, potentially making it more difficult for females to locate nest sites that provide suitable incubation regimes during embryonic development. Elevated nest temperatures could increase the incidence of embryonic mortality and/or maladaptive phenotypes. We investigated whether elevated nest temperatures reduce hatching success in tropical flatback turtles (*Natator depressus*) nesting in the Gulf of Carpentaria, Australia. Egg incubation treatments began at 29.5°C and progressively increased in temperature throughout incubation, up to maxima of 31, 32, 33, 34 and 35°C. Elevated nest temperatures did not reduce hatching success or hatchling body size relative to control temperatures (29.5°C), but did speed up embryonic development. A combination of sudden exposure to high temperatures during the first 2 weeks of incubation (>36°C for 48 h) and prolonged warming throughout incubation (from 29.5–35°C) did not reduce hatching success. We also recorded an unusually high pivotal sex-determining temperature in this flatback turtle population relative to other sea turtle populations: an equal ratio of male and female hatchlings is produced at ~30.4°C. This adaptation may allow some flatback turtle populations to continue producing large numbers of hatchlings of both sexes under the most extreme climate change scenarios. Some tropical populations of nesting flatbacks may possess important adaptations to high-temperature incubation environments, which are not found in more southerly temperate populations.

KEY WORDS: Embryonic development, Hatching success, Lethal thermal limits, Marine turtle, Metabolic heating, Thermal mortality, Thermal tolerance

INTRODUCTION

Most physiological functions of ectotherms depend on temperature, suggesting that the vast majority of terrestrial species may be vulnerable to the effects of contemporary climate change (Deutsch et al., 2008; Doody and Moore, 2010; Weber et al., 2012). The effects of temperature on life history can vary with life stage, such that adult and embryonic stages have different habitat requirements and physiological tolerances (Telemeco et al., 2013a; Howard et al., 2014; Pike, 2014). In these instances, the embryonic stages of oviparous species, which often do not provide parental care during incubation, may be most vulnerable to climate change. This is because the eggs are unable to physically move and embryos thus remain susceptible to the temperatures experienced by the nest-site that the mother selected. The maximal thermal tolerance that eggs can withstand is often highly conserved among taxa, suggesting that the impacts of climate change will depend on whether current nest temperatures are pushed beyond the point at which important biological responses occur (e.g. the pivotal sex-determining temperature, or the temperature at which survival is reduced or phenotypic abnormalities occur).

Sea turtles are the most widely distributed reptile taxa (James et al., 2006; Pike, 2013) and the different life stages may be found in widely disjunct geographical areas. Climate warming will simultaneously impact nesting and egg incubation areas, coastal foraging grounds and oceanic habitats (Hawkes et al., 2009; Witt et al., 2010). The embryonic stage of sea turtles must occur on land because the amniotic eggs need to exchange gas across the shell membrane to survive through to hatching (Ewert, 1985). Because eggs are in the nest for long periods before hatching (~50–65 days), the effects of climate change are anticipated to be most dramatic during this phase of their life history (Hawkes et al., 2009; Fuentes et al., 2011; Pike, 2014). Warmer nest temperatures could therefore lead to reductions in hatching success, and if temperatures rise above the pivotal sex-determining temperature, the feminisation of hatchling sex output (reviewed by Jourdan and Fuentes, 2013). These strategies could all influence the ability of females to select nest sites that provide optimal incubation conditions, and therefore have important effects on hatchling production and long-term population dynamics.

Elevated nest temperatures can inhibit successful development of turtle embryos, leading to phenotypic abnormalities or death (Packard et al., 1988; Du and Ji, 2003; Maulany et al., 2012; Telemeco et al., 2013a). In freshwater turtles, high-temperature incubation can lead to central nervous system dysfunction, which can reduce hypothalamus development and yolk absorption (Micheli-Campbell et al., 2012). Even if the embryo manages to develop successfully, high temperatures within the nest can reduce oxygen levels and muscle coordination, both of which can inhibit ascent to the surface after hatching (Matsuzawa et al., 2002; Segura and Cajade, 2010). Higher than normal temperatures can also result in smaller-sized hatchlings with reduced locomotor performance, increasing risk to predation as the hatchlings disperse from the nest and crawl to the water (Segura and Cajade, 2010; Maulany et al., 2012). Forecasted temperature increases caused by climate change could also elevate temperatures inside the nest above the lethal temperature threshold for eggs, potentially resulting in large-scale changes in egg survivorship and thus regional hatchling production (Pike, 2014). To predict the potential impacts of climate change on sea turtle populations, we must first understand the range of natural temperatures in which embryos develop and the temperature thresholds that reduce hatching success and alter hatchling phenotypes (Howard et al., 2014). This information can be used to identify those populations at most risk from climate change.

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Flatback turtles nest solely on Australian beaches (Limpus et al., 1993) and are listed as 'data deficient' on the IUCN Red List (Standards and Petitions Subcommittee, 1996). Studies on the thermal environment of developing embryos for the species have focused on southerly temperate populations (e.g. Limpus, 1971; Miller, 1982; Hewavisenthi and Parmenter, 2002) or those at intermediate latitudes (e.g. Blamires and Guinea, 2003), both of which nest in summer. In some flatback populations, egg survival is reduced when incubation temperatures exceed 32°C (Miller, 1982) whereas in others, egg survival is unaffected after reaching 35°C (Hewavisenthi and Parmenter, 2002), making it difficult to predict the potential impact of increased nest temperatures on hatchling production. The core flatback nesting population, which nests on tropical Crab Island and the surrounding beaches, is located further north than many of the populations that have been studied to date, and nesting occurs monthly with a peak during winter. We used a laboratory incubation experiment to determine whether the core nesting population is vulnerable to elevated nest temperatures, and whether the pivotal sex-determining temperature is similar to temperate nesting populations (29.3°C from Peak Island and Mon Repos in southeast Queensland; Limpus, 1971; Hewavisenthi and Parmenter, 2000). Our study will provide insight into the potential effects of climate change on reproduction of this data-deficient species.

MATERIALS AND METHODS
Field nest temperatures
We studied nests of the flatback turtle [*Natator depressus* (Garman 1880)] along a 10 km stretch of mainland beach located west of the mouth of the Jardine River, northeastern Gulf of Carpentaria, Australia (10°54′S, 142°11′E). The nesting season of this population lasts from August to January. During the week of 5–12 August 2013 we placed temperature dataloggers (Thermochron iButtons, Maxim Integrated Products, California, USA; factory-calibrated and accurate to ±0.5°C) inside nests at the bottom, middle and top of egg clutches during oviposition (N=10). To estimate the effects of metabolic heating on nest temperatures, we recorded sand temperatures at 30, 50 and 70 cm depth within 2 m of three monitored nests. Dataloggers recorded temperatures every 30 min during incubation and were retrieved after nests hatched. Predation by dingos (*Canis lupus dingo*), feral pigs (*Sus scrofa*) and goannas (*Varanus* sp.) resulted in some datalogger loss (Warren Strevens, personal communication), and thus final sample sizes vary among nest depths. We compared temperatures among the top, middle and bottom of egg clutches and between the middle of egg clutches and sand temperatures at comparable depth (50 cm) using repeated measures ANOVAs.

Effects of high-temperature incubation
We collected three clutches of eggs as they were laid on 12 August 2013 (N=181 eggs) and transported them to our Townsville laboratory for incubation experiments. Eggs were transported by car within a portable refrigerator maintained at 3–4°C (for 42 h until reaching our laboratory). Eggs were individually numbered with a pencil without regard to clutch origin and randomly placed in plastic incubation trays on a bed of moist vermiculite (N=12 eggs/container; 1:1 water: vermiculite by mass). Plastic wrap covering the containers prevented water loss during incubation.

The pivotal sex-determining temperature, which produces an equal ratio of male and female hatchlings, is 29.3°C for temperate flatback turtle populations nesting on Peak Island (Hewavisenthi and Parmenter, 2000) and Mon Repos (Limpus, 1971). Incubation temperatures of flatback nests generally increase throughout incubation, coinciding with the progression of summer (Limpus et al., 1993; Hewavisenthi and Parmenter, 2002) and metabolic heating during the final stages of embryonic development (Howard et al., 2014). We therefore used incubation treatments that began at 29.5°C and increased in temperature throughout embryonic development (Fig. 1). The control treatment (1) was maintained at 29.5°C throughout incubation (N=61 eggs, split between two incubators) and the other treatments increased by 1.5°C after the first 5 days of incubation and 0.5°C

every 5 days thereafter, until reaching a maximum of: (2) 31°C after 5 days (N=20 eggs); (3) 32°C after 15 days (N=20 eggs); (4) 33°C after 25 days (N=20 eggs); (5) 34°C after 35 days (N=20 eggs); or (6) 35°C after 45 days (N=16 eggs). To determine the effects of high temperatures both early and late in embryonic development, we maintained another treatment that (7) increased from 29.5 to 35°C over 45 days (N=24 eggs) and also experienced a sudden spike in temperature during days 10–12 of incubation, averaging >36°C for 48 h. Incubators remained within ±0.5°C of desired temperature (measured using four Thermochron iButtons placed inside each incubator), except the 34°C treatment, which reached 37–39°C during days 42–44 of incubation (2 days before hatchlings began pipping).

We recorded the fate of each egg (hatched, died). For hatchlings, we recorded the date of egg pipping (when the hatchling first broke the eggshell), emergence (when the hatchling fully exited the egg, both rounded to the nearest day) and 48–72 h after emergence we quantified body size (straight-line measurements of carapace and plastron length/width to 1 mm, and mass to 0.01 g) and visually assessed scute patterns for abnormalities. Hatchlings were euthanized using 0.1 ml of Valabarb (300 mg ml^{-1} pentobarbitone sodium), fixed using 10% buffered formalin and preserved in 70% ethanol. Two of our treatments experienced constant temperatures (29.5 and 31°C) during the mid-trimester sex-determining period, allowing us to determine how temperature influences hatchling sex ratio. We used histological techniques to sex hatchlings from these two treatments (Yntema and Mrosovsky, 1980). To compare hatching success, scute abnormalities, and sex ratios among temperature treatments, we used contingency table analyses with treatment as the independent variable. To compare incubation time (days) across treatments we used ANOVA, and to compare relative carapace length and body mass across treatments we used ANCOVA. We were unable to control for clutch of origin in our analysis. All means are presented ±s.e. and statistical significance accepted at $P<0.05$.

RESULTS
Natural nest temperatures
Nest depth averaged 67.9±2.85 cm (range 60–89 cm), with a mean clutch size of 54.3±2.4 eggs (range 42–68 eggs, N=10 clutches). Natural nest temperatures increased gradually throughout incubation, but did not differ significantly with regard to position within the egg clutch (Table 1, Fig. 2A). Temperature patterns during the incubation period revealed significant differences across
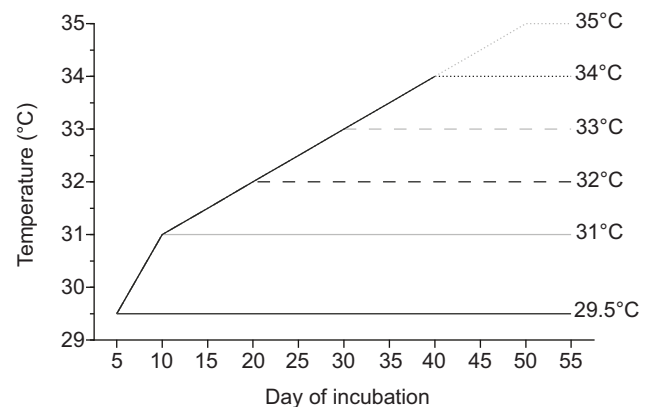

Fig. 1. Six temperature treatments used to determine the effects of high-temperature incubation on flatback sea turtle (*Natator depressus*) eggs. All treatments began at 29.5°C, and increased gradually to different maximum temperatures during incubation, except for a control, which was maintained at 29.5°C. For clarity, we refer to the temperature treatments according to the highest temperature reached. The 29.5°C and 31°C treatments both maintained constant temperatures during the middle third of incubation, when hatchling sex is determined, and we sexed these hatchlings to understand how these temperatures influence sex ratio. A seventh treatment, which is equivalent to the 35°C treatment but averaged >36°C during days 10–12 of incubation, is not shown.

time as well as a significant interaction between position within the nest and time (Table 1, Fig. 2A). The diel temperature change within nests was typically less than 1°C, averaging 0.6°C for the top of the nest ($N=7$), 0.3°C for the middle ($N=6$) and 0.2°C for the bottom ($N=10$). In terms of metabolic heating, temperatures within the middle of flatback turtle nests increased above that of sand temperatures at comparable depth after ~15 days of incubation, and this pattern continued until mean nest temperatures were ~2°C higher than sand temperatures near the end of incubation (Fig. 2B).

Effects of high-temperature incubation

Hatching success did not vary significantly among our six incubation treatments (Fisher's exact test, $P=0.076$); hatching success ranged from a low of 55% for the 34°C treatment to a high of 94% for the 35°C treatment (Fig. 3A). The eggs that experienced high temperatures both early and late in development also had a high hatching success of 83%. Incubation temperature significantly influenced the duration of embryonic development ($F_{5,137}=319.41$, $P<0.001$); eggs incubated at 29.5°C took significantly longer to hatch than eggs incubated at the higher-temperature treatments (Fig. 3B). We found no significant differences in the frequency of abnormalities among temperature treatments ($\chi^2=6.35$, d.f.=5, $P=0.27$; Fig. 3C); overall, scute abnormalities affected a low of 25% of hatchlings from the 33°C treatment to a high of 71% of hatchlings from the 31°C treatment (Fig. 3C). These values are within the ranges reported for other flatback turtle populations (Sim et al., 2014a,b).

Although egg size differed significantly among temperature treatments ($F_{5,165}=16.90$, $P<0.001$), egg size did not significantly influence hatching success ($F_{1,165}=1.72$, $P=0.19$; egg size×hatching success interaction: $F_{5,165}=0.56$, $P=0.73$; Fig. 4A). The relationship between egg mass and hatchling body mass differed significantly among temperature treatments ($F_{5,112}=3.37$, $P=0.007$); overall, larger eggs produced heavier hatchlings across temperature treatments (Fig. 4A). In addition, heavier hatchlings had significantly larger carapace lengths than did lighter hatchlings, and this pattern differed significantly among temperature treatments ($F_{5,116}=18.79$, $P<0.001$; Fig. 4B), probably as a result of initial differences in egg size among treatments.

Hatchling sex ratios

The sex ratio of hatchlings incubated at 29.5°C differed significantly from that produced at 31°C (Fisher's Exact Test, $P<0.001$). At 29.5°C, which we hypothesised would produce roughly equal sex
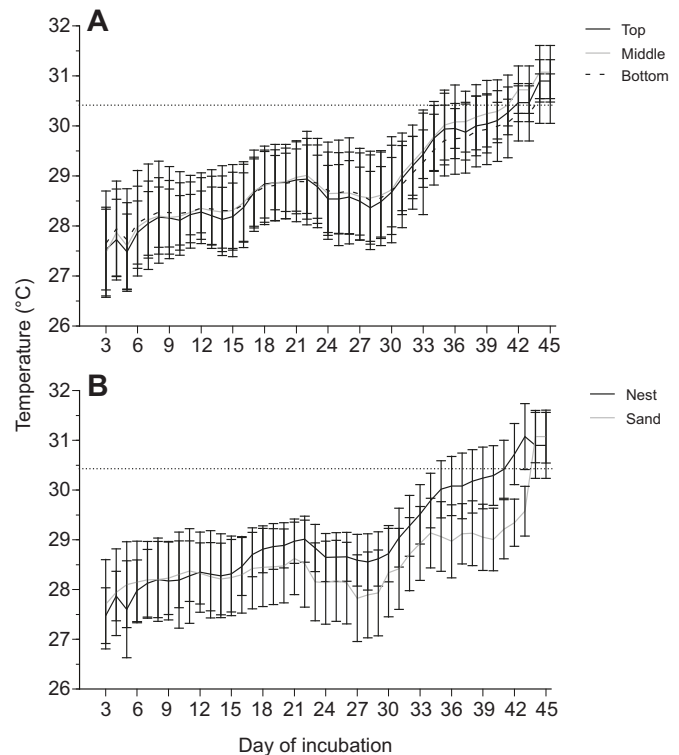

Fig. 2. Temperatures of flatback sea turtle (*Natator depressus*) nests recorded from August–October 2013 near the mouth of the Jardine River in the northeastern Gulf of Carpentaria, Australia. (A) Mean temperatures (±s.e.) at the top, middle and bottom of nests (partial data from $N=14$ nests). (B) Mean temperatures (±s.e.) in the middle of flatback nests ($N=10$) and sand temperatures ($N=2$) at comparable depth (sample sizes vary due to datalogger loss). Note evidence of metabolic heat production by the egg clutch, compared with sand temperatures.

ratios, only 19.6% of eggs produced female hatchlings ($N=10$ of 51 hatchlings); by contrast, incubation at 31°C resulted in 71.4% female hatchlings ($N=10$ of 14 hatchlings). The pivotal temperature at which an equal sex ratio is expected to be produced thus lies between these two temperatures; interpolation suggests ~30.4°C.

DISCUSSION

Elevated ambient temperatures resulting from contemporary climate change are likely to increase nest temperatures of many species. Female sea turtles lay eggs beneath sandy beaches in tropical and subtropical regions worldwide, which may be vulnerable to the effects of increased nest temperature (Pike, 2014). Thermal inertia of the sand is likely to limit nest warming to a few degrees (Fuentes et al., 2011; Fuentes and Porter, 2013), but this level of warming could still push nest temperatures beyond important biological thresholds (e.g. the pivotal sex-determining temperature or the temperature at which embryonic development ceases or cell death occurs), underscoring a need to understand how populations will respond. We found that the embryos of flatback turtle eggs from a tropical nesting site in the Gulf of Carpentaria are surprisingly resilient to high-temperature incubation. These eggs can withstand temperatures almost 4°C above those currently experienced in the field at more southerly rookeries (Limpus, 1971; Hewavisenthi and Parmenter, 2000) without reducing hatching success. The pivotal sex-determining temperature for this population is also among the highest known from any sea turtle species/population (Wibbels, 2003). The temperatures used in our incubation treatments are

Table 1. Results of repeated measures ANOVAs comparing flatback sea turtle (*Natator depressus*) nest temperatures within nests and between the middle of the nest and sand temperature at comparable depth

Comparison and variable	d.f.	F	P
Within nests			
Nest position	2	0.15	0.86
Time	41	520.95	**<0.0001**
Nest position×time	82	3.93	**<0.0001**
Error	738		
Nests versus sand			
Substrate type	1	1.54	0.24
Days	41	19.90	**<0.0001**
Substrate type × days	41	2.75	**<0.0001**
Error	451		

$N=10$ for nests at top, middle and bottom, respectively; $N=10$ for the middle of the nest; $N=2$ for sand temperature. P values in bold are significant.
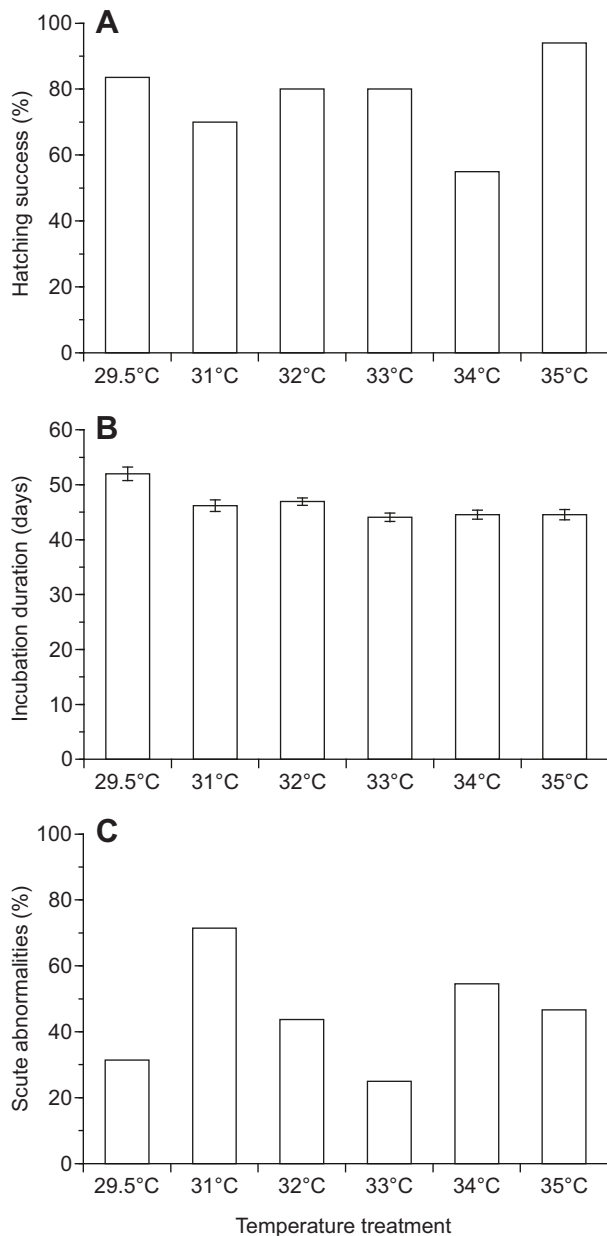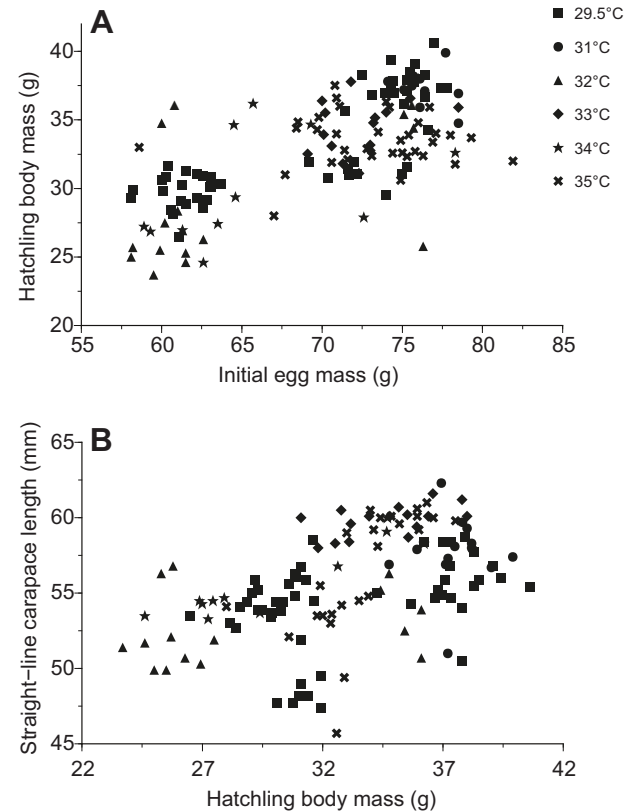
Fig. 4. Morphological characteristics of flatback sea turtle (*Natator depressus*) hatchlings. (A) Relationship between initial egg mass versus hatchling body mass. (B) Hatchling body mass versus hatchling straight-line carapace length, shown separately for six temperature treatments. Temperatures for each treatment are shown in Fig. 1.

Fig. 3. Effects of high-temperature incubation of flatback sea turtle (*Natator depressus*) eggs. (A) hatching success and (B) incubation duration of flatback sea turtle eggs. (C) Hatchling scute anomalies. Incubation treatments are shown in Fig. 1. Hatching success is the percentage of eggs hatching in each treatment, and incubation duration is the number of days (±s.e.) until hatchling emergence from the egg.

probably near or above the upper limit which most sea turtle eggs can tolerate and are among the highest recorded temperatures under which sea turtle eggs can successfully incubate and hatch with few obvious physical effects (Howard et al., 2014).

Exposure to elevated temperatures early during incubation often results in mortality of sea turtle eggs or abnormal embryonic development of hatchlings (reviewed by Howard et al., 2014). Surprisingly, flatback embryos exposed to maximum temperatures up to 38°C within 10 days of oviposition (averaging >36°C for 48 h) had high hatching success (83%). Although these extreme temperatures were reached relatively briefly, our findings suggest that embryos from this flatback turtle population are able to withstand higher temperature ranges than many other sea turtle

populations (Hewavisenthi and Parmenter, 2002; Howard et al., 2014). In addition, the amount of time that developing embryos are exposed to high temperatures is important for successful hatchling development (Valverde et al., 2010). Another reason for the high hatching success rates in our study could be that the overall mean temperature of even our most extreme treatment was below 33°C, which is within the range considered optimal for incubation (reviewed by Howard et al., 2014).

In field nests, incubation temperatures increased as the nesting season progressed and sand temperatures probably continue to increase throughout the summer nesting season (possibly by as much as 2–3°C), unless the summer monsoons arrive. Other populations of flatback turtles are known to experience increases in temperature during incubation of up to 7°C, which coincides with the progression of summer and includes effects of metabolic heating (Hewavisenthi and Parmenter, 2002). Flatback turtle populations generally experience the majority of nesting during winter, although in most populations some individuals nest during summer, when sand temperatures are likely to be warmest (e.g. along the Queensland coast south to Mon Repos; Limpus et al., 1983; Hewavisenthi and Parmenter, 2002).

Our hatchling morphology results are in contrast to findings from Peak Island, located ~1400 km south of our study area (Hewavisenthi and Parmenter, 2001). At Peak Island, hatchlings incubated at constant 26 and 29°C were larger than those produced at 32°C, but had lower energy reserves (Hewavisenthi and Parmenter, 2001). This pattern is similar to other sea turtle species, including olive ridleys (*Lepidochelys olivacea*), in which

high nest temperatures produce smaller hatchlings (Maulany et al., 2012). During longer incubation periods caused by low temperatures, more yolk material is converted to hatchling tissue, so that the emerging hatchlings are larger in body size but have smaller residual yolks (Booth, 2000). The fact that the hatchlings in our incubation treatments had similar relationships between egg mass and hatchling body mass, and hatchling body mass and carapace length, could be because the means of our incubation treatments were below 33°C, and thus overall fairly benign. It is also difficult to separate the effects of initial egg mass because we were unable to control for clutch-of-origin effects in our experiments. Morphological abnormalities such as scute deformities can arise when embryos are exposed to sub-optimal temperatures (Telemeco et al., 2013a; Howard et al., 2014). Although we did not find significant differences among temperature treatments in the frequency of occurrence of scute abnormalities, we found the incidence of abnormalities to be within the range of other studies (Fig. 3C; Sim et al., 2014a,b). Analysis of the internal bone structure of the carapaces of hatchlings with irregular scute patterns may provide insight into whether these abnormalities are detrimental to locomotor performance or function. High temperatures could also have other undesired effects on hatchling locomotor performance that we did not study. For example, temperatures >33°C at the neck of the nest chamber increase the chances of mortality in hatchling green turtles (Segura and Cajade, 2010), probably because muscle movement is inhibited, which can trap pre-emergent hatchlings inside the nest (Matsuzawa et al., 2002).

Our pivotal sex-determining data demonstrate that 29.5°C does not produce an equal sex ratio for flatback turtles on Cape York, as this temperature produced four times as many males as females. This result differs from previous studies on flatback pivotal temperatures, estimated at 29.3°C for Peak Island (Hewavisenthi and Parmenter, 2000) and Mon Repos (Limpus, 1971). We infer that the pivotal temperature is close to 30.4°C at our study site, one of the highest reported in any sea turtle population (Wibbels, 2003). This could be because flatback turtles nesting in northeastern Australia during summer are likely to experience high incubation temperatures. Given that the natural nest temperatures we recorded were below 30.4°C during the middle third of embryonic development, when sex is determined (Ackerman, 1997), most hatchlings from the nests we monitored early in the nesting season are likely to be male. Nearby Crab Island is also thought to produce a high proportion of male flatback hatchlings (Hewavisenthi and Parmenter, 2002), although Fog Bay (located further south, but still in the tropics) is more likely to be female-biased (Blamires and Guinea, 2001). The variation in pivotal sex-determining temperature, combined with different incubation temperatures experienced among populations would help to maintain a wider regional sex ratio close to parity.

Our study provides a number of key findings and poses additional questions about the thermal environment of flatback sea turtles. Notably, (1) flatback embryos on Cape York can survive mean temperatures almost 4°C above reported natural nest temperatures with little negative impact; (2) early in development, embryos can survive temperatures averaging >36°C for 48 h and ranging up to 39°C (briefly); (3) high-temperature incubation did not significantly influence hatchling body size; and (4) the pivotal sex-determining temperature (estimated to be ~30.4°C) of the Jardine River nesting population is ~0.9°C higher than temperate flatback nesting sites (Limpus, 1971; Hewavisenthi and Parmenter, 2000). In this region, green turtles (*Chelonia mydas*) could face the threat of near-complete feminisation of hatchlings by 2070 because climate change may push nest temperatures above the pivotal sex-determining temperature towards the point at which only females are produced (Fuentes et al., 2010; Fuentes and Porter, 2013). Our data for flatback turtles suggests that the Crab Island nesting rookery (<10 km straight-line distance from our study site) and surrounds mainly produces male hatchlings (at least early in the nesting season), and that climate change may push the sex ratio towards parity. This is an encouraging finding, although full protection of this species will need to focus on conserving habitats that are crucial to all aspects of the life cycle, in addition to minimizing additional anthropogenic effects on survival of all life stages.

Author contributions
R.H., I.B. and D.A.P. designed the study; R.H. and I.B. collected field data; R.H. performed laboratory experiments; R.H. and D.A.P. analyzed the data and wrote a draft manuscript, which I.B. edited.

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