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Effects of incubation temperature on hatchling performance and phenotype in loggerhead sea turtle *Caretta caretta*

Alejandro Usategui-Martín[1,*], Ana Liria-Loza[1], Jeffrey D. Miller[2], María Medina-Suárez[3], Saray Jiménez-Bordón[4], Valentín Pérez-Mellado[5], Daniel Montero[1]

[1]University of Las Palmas de Gran Canaria, EcoAqua University Institute, Telde, Las Palmas 35214, Spain
[2]Biological Research and Education Consultants, Missoula, Montana 55801, USA
[3]NGO Cabo Verde Natura 2000, Sal Rei 5110, Cape Verde
[4]NGO Asociación para el Desarrollo Sostenible y Conservación de la Biodiversidad, Agüimes, Las Palmas 35118, Spain
[5]University of Salamanca, Department of Animal Biology, Salamanca 37007, Spain

ABSTRACT: Under natural conditions sea turtle eggs are subjected to a changing thermal environment, but little is known about the effect of these temperature fluctuations during incubation on the performance and phenotype of hatchlings. The aim of this study was to determine how incubation temperature pattern (increasing or stable) and incubation temperature regime (low or high) affect incubation and hatching duration, hatching and emergence success, hatchling phenotype (carapace length and weight) and self-righting interval at hatching. Loggerhead sea turtle *Caretta caretta* (Linnaeus, 1758) clutches were collected at different beaches on the Cape Verde archipelago and divided among incubators with different temperature regimes and patterns. Minimum straight carapace length and weight of all individuals were measured at hatching. In addition, the hatching duration and the time interval required for each hatchling to self-right were recorded. Results showed that incubation temperature regimes influenced all studied parameters more than the increasing temperature pattern. Low incubation temperature regimes, both in the increasing and stable pattern, increased the incubation time, produced bigger hatchlings and caused a slower righting response compared to the high temperature regimes. An optimal range of incubation temperatures was determined by assessing the most favorable values for hatchlings, although some differences were found in the higher temperatures of this optimal range between different rookeries. This means that turtle incubation in the laboratory should, as far as possible, follow the natural incubation temperature fluctuation of the studied rookery.

KEY WORDS: Loggerhead sea turtle · *Caretta caretta* · Incubation temperature regime · Incubation temperature pattern · Hatchling performance · Hatchling phenotype · North Atlantic

1. INTRODUCTION

The northeastern Atlantic subpopulation of loggerhead sea turtles *Caretta caretta* (Linnaeus, 1758), is considered Endangered based on its small area of occupancy and continuing decline in habitat area (Casale & Marco 2015). Anthropogenic threats at sea (bycatch, marine litter, pollution) and habitat destruction on nesting beaches and in feeding areas have added new pressures to the already high levels of natural predation on nesting beaches and at sea in the turtles' early life stages (Heithaus 2013).

*Corresponding author: ausategui@gmail.com

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Hatchling phenotype and performance are influenced by a combination of maternal phenotype and fitness during egg formation (Hewavisenthi & Parmenter 2001, Glen et al. 2003, Andrews 2004). Furthermore, several environmental factors during the incubation period, such as hydric properties of the substrate (Reece et al. 2002) and nest temperature (Booth et al. 2004) also contribute to hatchling performance, growth rate and size, and therefore the amount of residual yolk (Reece et al. 2002, Booth et al. 2004, Booth 2006, Burgess et al. 2006). Sea turtles exhibit temperature-dependent sex determination (TSD) (Mrosovsky 1994, Mrosovsky et al. 2002, Wibbels 2003). For the loggerhead turtle, equal numbers of males and females are produced around 29°C, known as the pivotal temperature (PT). Different proportions of both sexes are produced at 2 to 3°C around the PT, known as the transitional range of temperature (TRT) (Mrosovsky 1994). Incubation temperature outside the TRT results in 100% males at lower temperatures, and 100% females at higher temperatures (Mrosovsky 1994, Godfrey & Mrosovsky 1997). Higher temperatures are also known to accelerate embryonic development, decrease the incubation period and reduce the amount of yolk transformed into tissues (Booth & Astill 2001). Eggs that incubated at temperatures lower than 23°C or greater than 33°C for extended periods do not hatch (Miller 1997), while the optimal incubation temperature range, at which embryonic growth is maximal, is between 31.5 and 32°C (Monsinjon et al. 2017). Consequently, it has been suggested that female hatchlings have shorter incubation times (Stokes et al. 2006), higher residual yolk (Booth et al. 2004, Burgess et al. 2006) and, to some extent, are smaller than males (Booth & Astill 2001, Reece et al. 2002, Booth 2006). While temperature is the main factor affecting embryonic development, moisture is also important, as increased moisture reduces the temperature of the nest environment. Therefore, an increase in substrate moisture will increase the incubation time and the length and weight of the hatchlings (Sifuentes-Romero et al. 2018).

Extreme low and high incubation temperatures decrease hatching success (Fisher et al. 2014, Booth 2017) and increase self-righting times (Read et al. 2013, Fisher et al. 2014, Wood et al. 2014). In addition, the incubation temperature affects hatchling locomotor performance and post-hatching growth (Booth 2006). For example, a hatchling from an egg incubated at a lower temperature would have poorer swimming performance (Booth et al. 2004, Booth 2006) compared to a hatchling coming from a warmer nest. In addition, the hatchlings incubated at lower temperatures have a slower stroke rate which they are able to sustain for a longer period of time (Burgess 2006), and exhibit a better crawling performance (Ischer et al. 2009).

Studies on sex determination and hatchling performance have been done under controlled conditions at stable temperatures (Booth et al. 2004, Mrosovsky et al. 2009, Fisher et al. 2014). However, constant incubation temperature is rare under natural conditions, which typically oscillate over the course of incubation (Packard & Packard 1988, Plummer et al. 1994, Shine et al. 1997).

The aim of this study was to determine how temperature pattern (i.e. increasing or stable) and temperature regime (i.e. low or high) during the incubation period affect incubation and hatching duration, hatching and emergence success, hatchling phenotype (i.e. carapace length and total mass) and self-righting time in hatchlings of the loggerhead sea turtle.

2. MATERIALS AND METHODS

2.1. Egg collection and transportation

Eggs used in the experiment were collected from beaches of the 'Reserva natural das Tartarugas', southeast Boa Vista Island, Cape Verde (Fig. 1A), and translocated to the laboratories of ECOAQUA Institute of Las Palmas de Gran Canaria University, Canary Islands, Spain (Fig. 1B).

Forty-eight eggs were collected from each of 4 different clutches on 2 September 2008, and the same number again on 3 August 2009, by staff and volunteers from the NGO Cabo Verde Natura 2000, making a total of 384 eggs, 192 per year. Turtle eggs were collected directly from the cloaca, avoiding any contact with the sand, to prevent the introduction of any kind of pathogen (e.g. bacteria, fungi) to the translocation site (Canary Island). Eggs not used in this study were incubated in the hatchery of the NGO Cabo Verde Natura 2000, close to the nesting area. All eggs were placed into the same isothermal plastic container (24 × 35 × 19 cm) to maintain a stable temperature and to avoid temperature fluctuations during their transport, as fluctuations could induce embryo mortality during the first hours after laying. Containers were filled with vermiculite at −150 kPa hydric potential, maintaining oviposition order arranged in columns and rows. Different nests were separated by plastic grids. The containers were

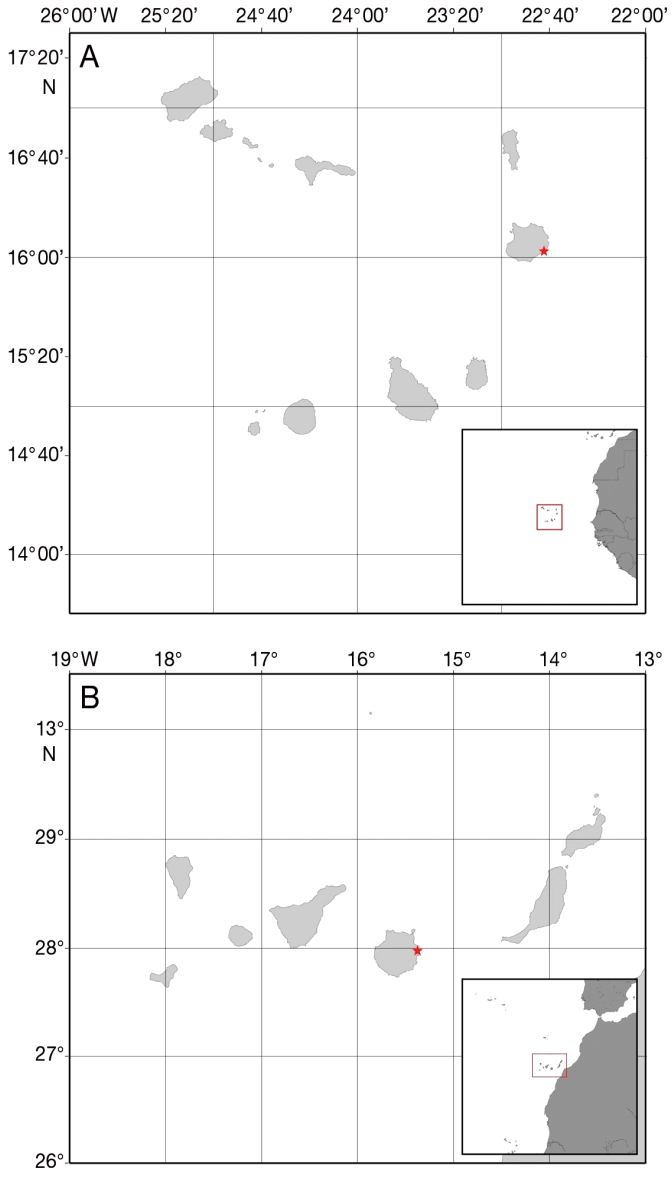

Fig.1. (A) Republic of Cape Verde, western Africa, with the location of the archipelago (red square in inset) and the 'Reserva natural das Tartarugas' (red star). (B) Canary Islands, western Africa, with the location of the archipelago (red square in inset) and ECOAQUA institute (red star), (source: SEATURTLE.ORG Inc. Maptool, 2002, www.seaturtle.org/maptool/ [accessed 21 June 2018])

transported by a four-wheel drive vehicle to Boa Vista airport and then taken to Gran Canaria Island, Spain, by plane. Finally, the containers were transported by car to the ECOAQUA laboratories. The entire process required less than 24 h and followed long-distance nest translocation protocols established by Abella et al. (2007) and L. F. López-Jurado (pers. comm.) under CITES permits (ES-DE-00008/08I; ES-DE-00005/09I).

2.2. Treatments

Each group of 48 eggs was divided into 6 plastic containers (3 l) filled with vermiculite (at −150 kPa hydric potential) so that 8 eggs from each group were placed in each container. Three containers from each group were placed in Medilow® incubators at different temperature patterns and regimes, with a total of 96 eggs per treatment. In the first year, incubators were set to a stable temperature pattern: incubator 1 to 27.0 ± 0.5°C (stable low, SL) and incubator 2 at 31.0 ± 0.5°C (stable high, SH), where the ± values simply show the slight variation possible in the incubators. In the second year, incubators were set to a gradually increasing temperature pattern of +0.5°C every 2 weeks: incubator 3 increased from 26.5 to 28.5°C (increase low, IL), incubator 4 increased from 30.5 to 32.0°C (increase high, IH). Twenty-four hours after the containers were placed into the incubators, viable eggs were identified by the development of a white area on the uppermost surface of each egg (Miller 1985). One group was excluded from the study because it did not show any sign of developing this white area.

The incubation time was defined as the period between oviposition and 3 d after a hatchling had fully emerged from the egg (Godfrey & Mrosovsky 1997). Hatching duration was defined as the time between the pipping and when the hatchling had fully emerged from the egg (Gutzke et al. 1984). Hatching success was the number of neonates that emerged fully from the egg. Emergence success was the number of neonates that survived 3 d after hatching. To determine incubation and hatching duration, incubators were checked daily at 9:00, 12:00, 15:30, 19:00 and 23:00 h. The incubators were controlled according to the standardized animal welfare protocols of the facilities, so access to the facilities was restricted at night. Hatching time was calculated in hours and then transformed into decimal days.

The minimum straight carapace length (SCLmin) was measured after emergence from the anterior point at midline (nuchal scute) to the posterior notch at midline between the supracaudals using calipers to the nearest 0.1 mm (Bolten 1999), and the weight was recorded using a precision balance (bs3000a, MOBBA) to 0.1 g.

Self-righting time was determined after emergence by placing the hatchling in supine position and recording the time it took to right itself. The test was repeated 3 times per hatchling. Maximum time allowed was 60 s, after which the hatchling was manually turned over. Hatchlings from the first year were

used to compare self-righting times between high and low incubation temperature conditions (i.e. SH vs. SL); hatchlings from high temperatures were used to compare the effect of incubation pattern on self-righting times (i.e. SH vs. IH).

All hatchlings used in this study were released after a head-start program as part of the project 'Enlargement of the reproductive habitat of the loggerhead sea turtle in the Macaronesia region'.

2.3. Statistical analysis

All statistical analyses were conducted using R version 3.1.2 (R Development Core Team 2014). Incubation time violated the assumption of homoscedasticity, so it was analyzed using a Welch's ANOVA with temperature pattern/regime as a factor and a Games-Howell post hoc test. Hatching duration was analyzed using ANOVA with temperature pattern/regime as a factor, followed by Tukey's post hoc test. The percentages of hatching success were converted to binary data and analyzed using a logistic regression test, with temperature pattern/regime as a factor. Gamma GLM was fitted to analyze the effect of the temperature pattern/regime over hatchling size (SCLmin) and the weight, because of lack variance homoscedasticity of both variables. One-way ANOVA was used to analyze self-righting time with temperature regime and temperature pattern as separate factors. Results were considered significant at $p \leq 0.05$.

3. RESULTS

3.1. Incubation and hatching duration

Incubation time was significantly affected by incubation temperature, both pattern and regime ($F = 9714$, $p < 0.05$). The Games-Howell test determined that eggs incubated at high temperature presented shorter incubation times ($p < 0.05$) than eggs incubated at the lower temperature. Eggs incubated at stable temperature had shorter incubation times (mean ± SD) than eggs incubated at increasing temperatures ($p < 0.05$), within both temperature regimes (SH X = 51.1 ± 0.22 d, IH X = 51.7 ± 0.44 d, SL X = 67.5 ± 1.18 d and IL X = 71.2 ± 1.30 d) (Fig. 2).

Hatching duration was significantly modified by both the pattern and regime of the incubation temperature ($F = 12.36$, $p < 0.05$). Tukey's HSD test determined that hatchlings incubated at low temperatures spent significantly ($p < 0.05$) less time in the hatching process than the ones incubated at high temperatures, independent of the incubation pattern (SL X = 1.2 ± 0.52 d, IL X = 1.0 ± 0.61 d, SH X = 1.5 ± 0.57 d and IH X = 1.3 ± 0.52 d) (Fig. 3).

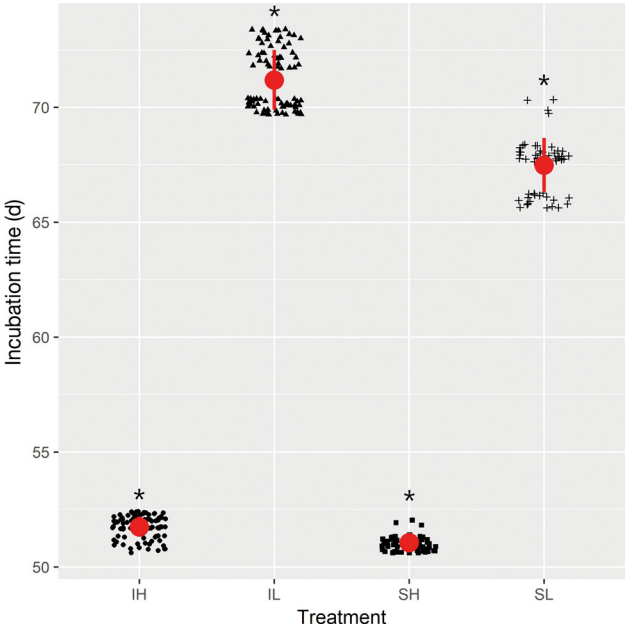

Fig. 2. Incubation time distribution across the 4 thermal incubation treatments, all showing significant differences (*) ($F = 9714$, $p < 0.05$) in incubation times. Red dots and red lines represent mean and SD. Small black dots are hatchlings incubated at stable-high temperature (SH), triangles are hatchlings incubated at stable-low temperature (SL), squares are hatchlings incubated at increasing-high temperature (IH), and the crosses represent hatchlings incubated at increasing-low temperature (IL).

3.2. Hatching and emergence success

Hatching and emergence success were almost the same because only 2 hatchlings out of 336 died during emergence.

Hatching success was not affected by either the high temperature regime or low temperature regime ($Z = -1.010$, $p > 0.05$) (90.40 and 84.52%, respectively), but it was significantly improved by the increasing temperature pattern ($Z = 2.903$, $p > 0.05$) (increasing vs. stable pattern: 94.27 vs. 78.47%, respectively). When comparing the 4 experimental protocols, hatching success was higher for the IH treatment ($Z = 3.61$, $p < 0.05$) and for the IL treatment ($Z = 2.83$, $p < 0.05$), 96.88 and 91.66% respectively, compared to the other treatments.

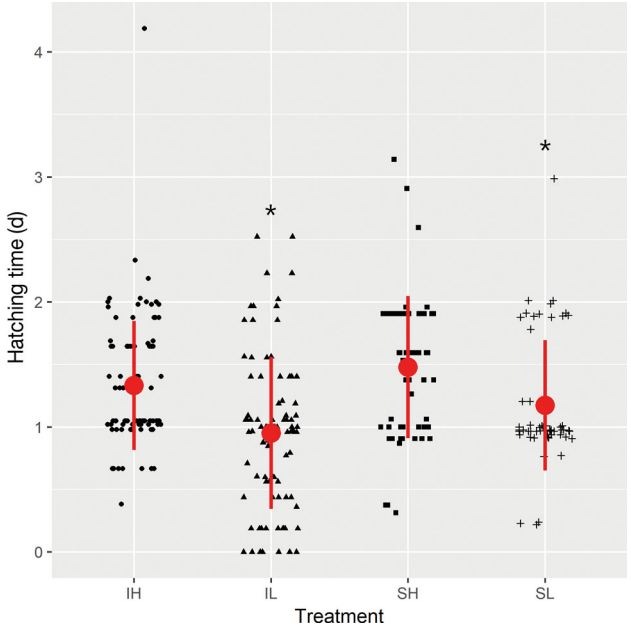

Fig. 3. Hatching duration across the 4 thermal incubation treatments. Hatchlings incubated at low temperatures, both increasing and stable, spent significantly ($F = 12.36$, $p < 0.05$) less time in the hatching process than those incubated at both increasing and stable high temperature treatments. See Fig. 2 for definition of symbols and abbreviations

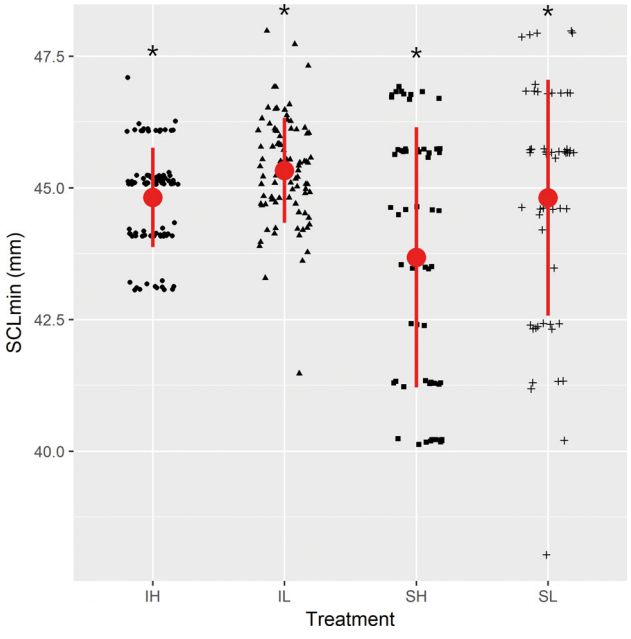

Fig. 4. Minimum straight carapace length (SCLmin) across the 4 thermal incubation treatments. Hatchlings incubated at low temperatures were significantly ($t = -3.65$, $p < 0.05$) larger than those incubated at high temperatures. Hatchlings incubated at increasing temperatures hatched significantly ($t = -4.13$, $p < 0.05$) larger than hatchlings incubated at a stable temperature. See Fig. 2 for definition of symbols and abbreviations

3.3. Hatchling phenotype

Increasing temperatures produced significantly larger hatchlings than stable temperatures ($t = -4.13$, $p < 0.05$) (mean ± SD: 45.1 ± 1.0 mm and 44.2 ± 2.42 mm, respectively). Low temperature incubation resulted in larger individuals than high temperature incubation ($t = -3.65$, $p < 0.05$) (45.1 ± 1.60 mm and 44.4 ± 1.78 mm) (Fig. 4).

The same effect was found respective to hatchling weight, where increasing temperatures resulted in an increase in hatchling weight ($t = -4.93$, $p < 0.05$) compared to hatchlings from eggs incubated at stable temperatures (19.4 ± 1.52 and 17.0 ± 2.92 g, respectively). In addition, low temperatures produced heavier hatchlings than high temperatures ($t = -2.41$, $p < 0.05$) (18.9 ± 2.27 and 17.7 ± 2.23 g, respectively) (Fig. 5).

3.4. Self-righting response

The self-righting response of hatchlings was affected by the temperature regime ($F = 104.9$, $p < 0.05$). Hatchlings incubated at low temperatures took longer to turn over than hatchlings incubated at high

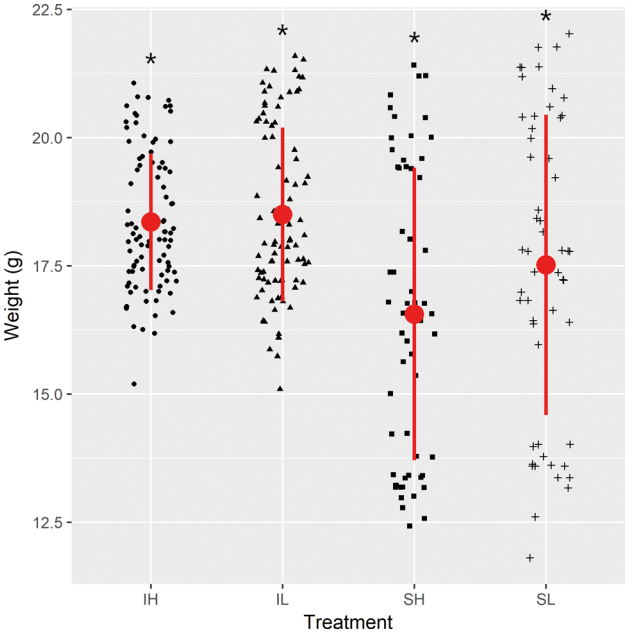

Fig. 5. Weight across the 4 thermal incubation treatments. Hatchlings incubated at low temperatures were significantly ($t = -2.41$, $p < 0.05$) heavier than those incubated at high temperatures. Hatchlings incubated at variable temperatures hatched significantly ($t = -4.93$, $p < 0.05$) heavier than hatchlings incubated at a stable temperature. See Fig. 2 for definition of symbols and abbreviations

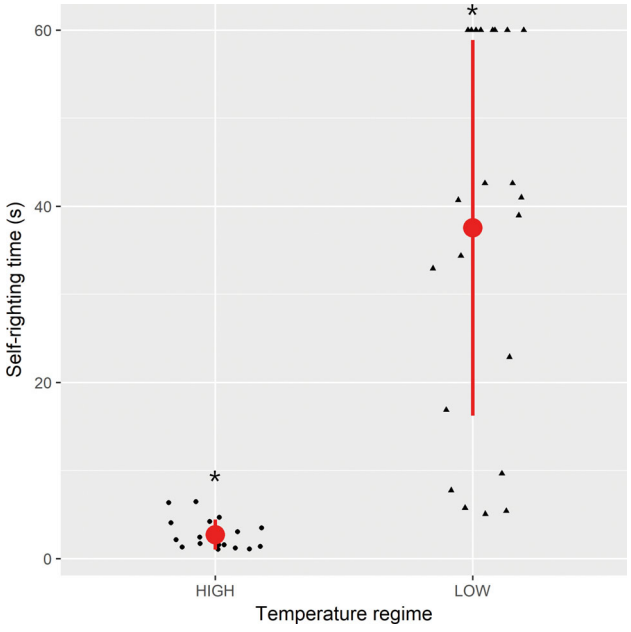

Fig. 6. Self-righting time at high and low temperature incubation regimes. Hatchlings incubated at low temperatures took significantly ($F = 104.9$, $p < 0.05$) less time to right themselves than those incubated at high temperatures. See Fig. 2 for definition of symbols

temperatures (37.5 ± 21.32 s and 2.7 ± 1.72 s, respectively) (Fig. 6). The incubation temperature pattern had no effect on the interval of the self-righting response ($F = 35$, $p > 0.05$).

4. DISCUSSION

Eggs of sea turtles have been incubated under controlled conditions in conservation programs (Plotkin 2007) and scientific studies, for example studies of sex ratio (Booth et al. 2004, Mrosovsky et al. 2009), hatchling fitness (Fisher et al. 2014) and embryonic development (Miller 1985). However, incubation temperature in wild nests typically oscillates over the course of incubation (Packard & Packard 1988, Plummer et al. 1994, Shine et al. 1997). Fisher et al. (2014) and Booth (2017) reported lower hatching success of loggerhead turtle eggs incubated at extreme low (50–60% at 27°C) and high incubation temperatures (<50% at 31°C), whereas maximum hatching success (69.2%), occurred at 29°C. Results obtained in the present study differ from Fisher et al. (2014), as greater hatching success (81.94%) occurred at 31°C than at 27°C (75%). This apparent discrepancy could result from latitudinal differences between the loggerhead population used by Fisher et al. (2014) and the population of the present study. Fisher et al. (2014) used eggs from the North Carolina rookery, which is the northern-most breeding colony for loggerhead turtles in the North Atlantic (Bowen & Karl 2007), whereas in the present study we obtained eggs from the Cape Verde colony which has a climate ranging from tropical dry to semi-desert (Duarte & Romeiras 2009). Laloë et al. (2017) studied the same Cape Verdean population under natural conditions, reporting a higher emergence success at higher mean incubation temperatures (82.6% at 28.5°C and 81.6% at 32.2°C) than found by Fisher et al. (2014). This supports our theory that the latitudinal and genetic differences between the Florida and Cape Verde populations (Shamblin et al. 2014) could provide the latter with a higher thermal tolerance in which at least the upper, but possibly also the lower, thermal tolerance limit is higher than for other populations.

Translocation and handling issues can affect hatching success (Limpus et al. 1979). Values obtained by Fisher et al. (2014) were lower (<70%) than those obtained in the present study (>75%), while the translocation distance in Fisher et al. (2014) was shorter. However, hatching success values >75% have been reported by several authors (Marcovaldi & Laurent 1996, Mrosovsky et al. 2002, Wood et al. 2014), using similar long-distance egg translocation and hatchling handling protocol as used in the present study.

Significant ‘“”differences in hatching success were found in relation to the temperature pattern, where increasing temperatures produce almost 16% more hatchlings than stable temperatures. Strong maternal effects on egg quality have been described by Booth et al. (2013). In the present study, eggs incubated at different temperature patterns were collected in 2 different years (i.e. laid by different females), therefore the maternal origin cannot be completely discarded as a factor leading to differences between the sampled groups. Further investigations are needed to elucidate whether incubation temperature pattern or maternal origin (or both) produces the differences in hatching success.

Incubation temperature has an inverse relation with incubation time (Booth & Astill 2001, Stokes et al. 2006, Booth 2017) but only within a certain range of temperature, because very high incubation temperatures produced longer incubations (Monsinjon et al. 2017). However, the effect of the temperature pattern on incubation time under increasing temperature conditions has not been studied sufficiently. We cannot form a conclusive hypothesis based on the results of the present study, thus we consider our suggestions on this topic to be speculative.

In our study, hatching duration was longer for those animals incubated at high temperatures (and hence with shorter incubation periods). Ewert (1979, 1985) considered hatching duration, including the internalization of the residual yolk and the unfolding of the carapace and the plastron, to be controlled by the metabolic rate of hatchlings (and thus affected by temperature). Ligon et al. (2009) reported that hatchlings incubated at higher temperatures had higher metabolic rates, suggesting that hatchlings with faster metabolic rates would complete the hatching process sooner than those with slower metabolic rates. However, results from the present study indicate the opposite, an apparent discrepancy that could be due to size differences of the neonates. Larger hatchlings could emerge from the egg faster, breaking the entire eggshell, while smaller ones emerged in stages: first the pipping, followed by the emergence of the head, then one flipper, and finally the other flipper and the rest of the body. Eggs incubated at lower temperatures (and hence with longer incubation periods) produce hatchlings that are larger than those produced at higher temperatures (Booth & Astill 2001, Reece et al. 2002, Booth 2006) because embryos are able to transform more yolk into tissues (Booth et al. 2013, Read et al. 2013). All the aspects mentioned above are also affected by nest moisture (Sifuentes-Romero et al. 2018), but it was not taken into account in the present study because the moisture levels were the same in all the eggs.

Metabolic heat produced by the embryos during development (Packard & Packard 1988, Plummer et al. 1994, Shine et al. 1997) reaches its highest values toward the end of the incubation period. The growth rate of sea turtle embryos follows a similar pattern, being slow at the beginning and increasing exponentially during the incubation period (Ackerman 1981, Booth & Astill 2001). In the present study, hatchlings produced at stable temperatures were smaller (44.2 cm SCLmin) than the ones produced at increasing temperatures (45.1 cm SCLmin), probably because the increasing temperature may have influenced the exponential growth phase at the end of the incubation (Ackerman 1981, Booth & Astill, 2001). While incubation temperature has a strong effect on early stages of embryonic development, moisture has a higher impact on later stages (Sifuentes-Romero et al. 2018). In our study, we maintained the same moisture levels in all the treatments, so the only factor affecting embryonic development—besides genetic or maternal effect—was temperature (Andrews 2004, Glen et al. 2003). Hatchling size has been widely reported to be affected by incubation temperature, but few studies deal with the relationship between weight and temperature (Read et al. 2013, Fisher et al. 2014, Horne et al. 2014, Booth 2017). Hatchlings incubated at low temperatures have more body mass and less residual yolk, while the ones incubated at high temperatures have less body mass and more residual yolk (Booth 2017). Consistent with this, in the present study eggs incubated at low temperatures produced heavier hatchlings (18.93 ± 2.28 g) than eggs incubated at high temperatures (17.67 ± 2.23 g).

Although self-righting time is not an exact fitness estimator for sea turtles, the ability of a hatchling to turn itself over is essential to survival during their race to the sea after emerging from the nest (Fisher et al. 2014) and has been used in previous studies as a proxy of hatchling fitness (Booth et al. 2013, Read et al. 2013, Wood et al. 2014). Self-righting times increase at extreme low and high temperatures in both sea and freshwater turtles (Read et al. 2013, Fisher et al. 2014, Wood et al. 2014). Booth (2017) suggested an optimal range of incubation temperatures of 28°C to 32°C that leads to shorter self-righting times. In the present study, the mean self-righting time of hatchlings incubated at 27°C was 37.5 s while Fisher et al. (2014) present a mean self-righting time at that same incubation temperature of ~30 s. However, at 31°C hatchlings took an average of 2.72 s to turn themselves over, which is faster than the values reported previously (~60 s; Fisher et al. 2014). The slight difference at low temperatures and the large difference at high temperatures may result from the genetic differences between populations. The optimal temperature range for hatchlings from Cape Verde seems to be shifted to higher temperatures than for the North Carolina rookery. More studies are needed to find the optimal range for each colony, rookery or location to be able to develop more efficient hatchery programs. Incubation temperature patterns did not affect this useful ability (i.e. self-righting). This result is the same as described for smooth soft-shell turtles *Apalone mutica* (Ashmore & Janzen 2003).

In summary, low incubation temperatures produced larger hatchlings and extend the incubation time, in line with previous studies that report that more yolk is transformed into tissues at lower temperatures. Smaller hatchlings are produced at stable temperatures because the stable temperature pattern does not stimulate embryonic growth during the exponential growth at later incubation stages. Incubation temperature regimes have a greater influence on hatchlings than temperature patterns, indicating that incubation temperature patterns of natural nests

should be imitated in laboratory studies. Even if the temperature regime is the main modulator of hatchling performance, the temperature pattern also has an important influence on hatching success.

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