

Assessing the Effects of Incubation Temperature on the Cognitive Ability of Hatchling

Loggerhead (*Caretta caretta*) Sea Turtles

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Sarah Milton, Department of Biological Sciences, and has been approved by all members of the supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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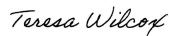
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Abstract

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Under the expected warmer temperatures due to climate change, sea turtle embryos may be subjected to thermal conditions detrimental to nest success and hatchling quality; one trait which may be negatively affected is cognitive ability. In this study, loggerhead sea turtle eggs were acquired from Boca Raton, FL and lab incubated under two female-producing temperatures: an “optimal” temperature of 31°C and a sublethal temperature of 33°C. Cognitive ability of post-hatchlings, assessed via associative learning and reversal was investigated using a y-maze. The sublethal temperature decreased incubation duration, hatch success, hatchling growth rates and produced smaller hatchlings with significantly more scute anomalies. Hot hatchlings performed worse on the reversal, taking longer to train, and thus hint at an effect of incubation temperature on cognitive flexibility in loggerhead turtles. With temperatures rising on

beaches in South Florida, this study provides evidence of further potential threats to hatchling quality and potentially even survival.

Dedication

This manuscript is dedicated to my incredible husband, Nicholas Serra, who always listens to my rants about sea turtles, pushes me to pursue my dreams, and has never stopped being my number one fan. I also dedicate this manuscript to my family, without whom I would not be where I am today.

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INTRODUCTION

Overview

Organisms are intrinsically connected to their environments through a variety of factors dependent on both species and location. These relationships can range from direct use of the immediate habitat, to more innate relationships which lend themselves to be explored in a physiological scope. Ectotherms are a unique group in which to explore the effects of the environment upon the individual organism due to their inability to warm themselves purely metabolically (Angilletta et al, 2002). Instead, they must rely upon the environment to regulate their body temperatures, with metabolic heat production only playing a minor role when compared to endotherms (Brown & Au, 2009). The close tie between temperature and the physiological and behavioral processes of ectotherms allows for a dynamic relationship to emerge in which many biological mechanisms may become greatly impacted by even minor fluctuations in body temperature (Angilletta et al., 2002). This comes as a result of ectotherms having only a narrow range of temperatures which permit optimal performance; temperatures outside of this range are deemed detrimental to function and performance is reduced (Huey & Stevenson, 1979). The optimal performance a species can attain is thus typically seen in the middle of a temperature-performance curve, wherein the optimal temperature lies, and as such may restrict the species to a particular thermal niche (Huey & Stevenson, 1979; Papastamatiou et al., 2015). As most physiological processes in ectotherms not only have optimal

temperatures, but also temperatures above and below which performance is compromised and at times, dramatically so, it comes as no surprise that body temperature impacts a broad range of factors ranging from immune function to habitat selection (Mondal & Rai, 2001; Harvey & Weatherhead, 2010).

The ectothermic sea turtle displays a multi-faceted relationship with temperature especially during the embryonic developmental stages of its life history. Temperature while in the nest has been extensively studied with respect to multifarious correlative factors such as hatchling success/survivorship, locomotor abilities, emergence, size (morphology), incubation duration and even sexual differentiation (Booth, 2017; Fisher et al., 2014; Kobayashi et al., 2018; Janzen & Paukstis, 1991). It has long been observed that extremes in warm temperatures negatively affect developing sea turtles with the highest temperatures being quite lethal to developing embryos and resulting in decreases in hatching success or even complete nest failure (Valverde et al., 2010). This is in line with the proposed thermal tolerance limits of sea turtles where mortality is both expected and observed when incubation temperatures reach or exceed the upper thermal tolerance limit (Milton & Lutz, 2003). Though I focus on warmer temperatures in the current study, temperatures dipping below the lower thermal tolerance limit, approximately 27 °C for loggerhead turtles, *Caretta caretta*, also compromise performance (Booth, 2017).

Many studies have already begun to implicate rising temperatures as a major driver of embryonic and hatchling mortality. Raine Island, the world's largest rookery for green sea turtles, provides interesting insight to this in a preliminary study conducted from 2016-2017 (Booth & Dunstan, 2018). This island experiences what the authors term early embryo death syndrome (EEDS) which was previously depicted as the inexplicable

death of embryos at very early developmental stages. Nests with the highest proportion of EEDS were found to also reach the highest incubation temperatures on the island.

Though preliminary, this is another sample of evidence in the mounting data which do not favor sea turtle population dynamics in the face of heightened incubation temperatures.

These heightened incubation temperatures have also been found to influence incubation duration, the length of time an embryo incubates for prior to hatching (Matsuzawa et al., 2002). This has been well reported in Minabe, Japan, where as incubation temperatures for nests at a hatchery increased from approximately 26 °C to 32 °C as the rainy season ended, incubation duration dropped from approximately 80 days to as little as 45 days. The higher incubation temperatures in this study also coincided with an increase in hatchling mortality during both pipping and emergence. This shortened incubation duration can be attributed to faster growth rates in developing embryos under temperatures nearing the critical maximum (Monsinjon et al., 2017). As incubation temperatures increase, embryonic growth rates also tend to increase and a positive correlation emerges; in Turkey and Libya, loggerhead embryonic growth rates peaked at a maximum incubation temperature of 32 °C (Monsinjon et al., 2017). Interestingly, though this particular study reported that the incubation duration did indeed become shorter as incubation temperatures became warmer, the incubation duration began to steadily grow again once temperatures rose above 32 °C.

Success during development and hatching is thus very dependent on the temperature experienced during incubation, though the exact temperatures at which this becomes problematic remain under investigation. Many papers published from studies at

different sites tend to cite a lethal temperature of either 33 °C or 35°C; these temperatures stem from two very pivotal papers from 1997 reviewed by Howard et al. (2014). In the analysis by Howard and colleagues of our current and past understanding of the thermal tolerances of sea turtle embryos, the authors discuss the disparity between these long-believed limits and what we now know to be occurring on a multitude of nesting beaches worldwide. An array of studies report nests with temperatures reaching or exceeding both 33 °C and 35 °C that are still capable of producing hatchlings, though with compromised success rates (Maulany et al., 2012; Read et al., 2013). In the studies conducted by Maulany and Read in Indonesia and Australia respectively, the three-day maximum temperature experienced by some loggerhead and olive ridley (*Lepidochelys olivacea*) nests exceeded 35 °C yet the nests did not fail completely in terms of hatching success. It is interesting to note that both studies also mention that increased temperatures affected the overall fitness of hatchlings through different performance measures such as crawl speed and self-righting time. Sim and colleagues found this same phenomenon occurring with loggerhead hatchlings in Queensland, Australia; a mean three day maximum temperature (T3DM) above 34 °C resulted in not only decreased emergence success, but also produced smaller sized hatchlings who performed worse on both swimming and crawling trials (Sim et al., 2015).

Our understanding of temperature's dynamic role during incubation has now shifted to better comprehend the mortality that follows a bout of hot incubation temperatures; both the length of time the temperature is high and when this increase occurs are both thought to be critical in determining if mortality is likely to occur (Maulany et al., 2012; Sim et al., 2015; Bladow & Milton, 2019). More work is needed to

fully comprehend at what point during development embryos are most at risk to extremely high temperatures, but it would appear that embryos have a higher tolerance as development progresses (Howard et al. 2014; Maulany et al., 2012). Though this is a plausible explanation, more developed embryos are also the ones most likely to experience hotter temperatures and T3DMs due both to metabolic heating and seasonal increases in temperature thus complicating this hypothesis (Bladow & Milton, 2019; Sim et al., 2015).

Temperature-linked hatchling mortality is consequently evolving into a major concern for sea turtle species as projected climate models indicate a warming trend in coming years (Laloë et al, 2017). Though much emphasis has been placed on understanding the effects of warming and climate change on future sex ratios due to turtles' temperature-dependent sex determination (TSD), less research has focused on the aspect of morbidity and mortality (Patrício et al., 2019; Maffucci et al., 2013) . Laloë and colleagues believe that this gap is quite problematic in our understanding of future sea turtle populations given that in some populations hatchling survival may drop before sex ratios are significantly skewed (Laloë et al, 2017). Though this may potentially be the case, there are instances where nests are completely feminized as a result of high incubation temperatures but still produce relatively high hatching success rates (Lolavar & Wyneken, 2015). As population-wide predictions regarding the relationship between survival and TSD are weighed, it is imperative to note that though populations may indeed begin to feminize, the effect may be less pronounced than what is believed due to decreases in survival rates at the feminizing temperatures (Boyle et al., 2014).

A potentially less lethal effect of varying incubation temperatures on embryological development can be found in embryonic energy consumption (Reid et al., 2009); embryos incubated at cool and warm temperatures have been shown to differ in their energy expenditure. In a study conducted by Reid and colleagues, cool embryos incubated at 27.6 °C had lower peak oxygen consumption than warmer, 31.8 °C, embryos. Though warm hatchlings would thus be expected to have the greatest total energy expenditure due to higher oxygen consumption rates, the opposite was actually true. Cooler hatchlings require a larger total energy expenditure than do warmer hatchlings with the difference arising due to the longer incubation period seen at cooler temperatures. The ramification of hotter temperatures during incubation is thus not merely amassing more time spent in the nest, but instead these temperatures have the ability to impinge a plethora of physiological traits.

Beyond developmental complications, both high and low temperature extremes have been shown to negatively affect those able to make it through development and out of the nest (Maulany et al., 2012; Read et al., 2013). This can be explained as a sublethal effect brought on by the reduced physical fitness or morphology of hot incubated hatchlings. Hatchling physical performance, a trait important for survivorship, is optimal at the middle range of the thermal tolerance limit with suboptimal performance seen in the upper and lower reaches (Burgess et al., 2006; Ischer et al., 2009; Fisher et al., 2014; Henaghan, 2018). It is important to note that high temperatures alone are not solely to blame for issues stemming from incubation temperature, but the lower reaches of the thermal tolerance limit can also pose a problem to developing embryos (Fisher et al., 2014; Burgess et al., 2006). Locomotor ability is typically assessed for performance and

includes variables such as swimming speed, crawling speed and(or) the righting response (Henaghan, 2018). Crawling speed and self-righting time decrease and increase respectively at higher temperatures considered to be sublethal; this may exemplify the effect that warmer temperatures could have on survivorship post emergence if these traits are critical for survival.

The morphology of hatchlings can also be impacted by temperature in certain fundamental ways (Kobayashi et al., 2018). One example can be found in the size of hatchlings and their yolk reserves under differing incubation temperatures as reported by Booth & Astill (2001). Eggs that were incubated at 26 °C had longer incubation periods producing not only larger hatchlings, but hatchlings with less residual yolk as compared to hatchlings incubated at 30 °C. When development occurs more slowly, more yolk is converted to tissue in the developing embryos and thus less yolk is available as an energy reserve to the hatchling once it emerges (Booth & Astill, 2001). Though this cache of energy may indeed be useful for the hatchling as it makes its trek out into the ocean, these “cold” hatchlings also tend to have significantly smaller carapacial dimensions but not overall mass (Booth, 2017; Sim et al., 2015; Staines et al., 2019). In a scenario where predation pressures are low, an increase in residual energy could prove useful in the traverse from coastal to pelagic waters and then foraging grounds (Cavallo et al., 2015). Larger hatchlings may have an advantage with respect to survival in cases where predation occurs by gape-limited predators; thus, if the frenzy period for hatchlings at a beach is riddled with a large number of predators, such as is common in coral reef ecosystems, a larger body size may be more favorable for survival. (Burgess et al., 2006; Booth & Evans, 2011). In Heron Island, this is the case for green sea turtle hatchlings

varying in size; hatchlings who were larger had a higher probability of safely crossing the fringing reef than their smaller counterparts (Gyuris, 2000).

Growth rates due to varying incubation temperatures not only differ during embryonic development, but extend into the hatchling life stage as well (Kobayashi et al., 2018). Loggerhead hatchling growth rates are significantly slower post-hatch when eggs are incubated at warmer temperatures, 31 °C, as compared to cooler temperatures, 27.5 °C, though this pattern has only been shown to persist in the short-term. Incubation temperatures may thus play a role in hatchling growth rates, especially when coupled with the initial date of food consumption and metabolite levels; in the study by Kobayashi and colleagues, initial food consumption was later in warm incubated hatchlings than in cool incubated hatchlings (Kobayashi et al., 2018). Levels of blood glucose were also significantly higher in hatchlings incubated at warmer temperatures and the authors postulated that this was the cause of not only a later initial date of food consumption, but also the slower hatchling growth rates experienced by the hot incubated turtles; if onset of feeding is cued by low blood glucose levels, colder incubated turtles would be induced to feed earlier and thus have higher growth rates than the warm incubated turtles. Slower growth rates for hatchlings post-emergence may be significant in regard to survival, especially when the faster growth rates experienced during development are considered (Maulany et al., 2012). Overall, warmer temperatures thus have the potential to increase embryonic growth rates producing smaller hatchlings who then experience slower growth rates once out of the nest. When predation is considered, this pattern could become problematic due to the predation risk, as described above, that small hatchlings face (Kobayashi et al., 2018). This increased risk can also be linked back

to temperatures effect on performance in that smaller, hot-incubated hatchlings are typically poorer swimmers and are unlikely to evade predators as effectively as larger hatchlings (Burgess et al., 2006). In the face of ongoing global warming, sea turtles are anticipated to retain the negative size trend associated with rising temperatures (Cavallo et al., 2015). Though this is what the models from Cavallo and colleagues predict (in conjunction with future climate models), they also forecast that the positive trend in residual energy stores seen with rising temperatures will be sustained. This complex interplay of energy stores and size muddles the answer to the question of what the exact effect of hotter temperatures will be on future dispersal and recruitment of sea turtles across many different nesting and coastal environments.

Morphological consequences of higher incubation temperatures are not just restricted to size, but also result in a prevalence of non-modal scute patterns (Zimm et al., 2017). A typical modal scute pattern for sea turtles features a set number of vertebral, costal, and marginal scutes. Deviations away from this pattern are termed non-modal and anomalies can consist of supernumerary, subnumerary and deformed scutes (Zimm et al., 2017). The exact cause of these anomalies is still under investigation but a recent study on DNA cytosine methylation in the loggerhead sea turtle hints at an epigenetic effect (Caracappa et al., 2016). One study in particular reported that as temperatures increased in loggerhead, flatback and green turtle nests, the frequency of scute anomalies also increased (Zimm et al., 2017). While intriguing, there is still a paucity of research on the potential relationship between scute patterns and temperature, though other work has focused on the life stages in which these non-modal scute patterns prevail (Sim et al., 2014). In a recent study on loggerhead and flatback hatchlings, two thirds of loggerhead

embryos which died during development (unhatched embryos) displayed a non-modal scute pattern; when compared to both emerged hatchlings and those in the nest, there was a significant difference in the incidence of non-modal scute patterns (Sim et al., 2014). The authors believe that their findings suggest embryos with non-modal scute patterns are also more susceptible to embryonic mortality and that this could potentially hint at other internal abnormalities producing low quality hatchlings. Though published studies relating temperature and scute anomalies are scarce, it seems that increased temperatures may indeed be a driver for a plethora of abnormalities arising in both embryos and hatchlings. Survivorship in sea turtle hatchlings must thus be viewed from more than a simple question of who does and does not make it out of the nest; the relationship between hatchling and post-hatch survival is complex and may include numerous interconnected factors we have yet to unearth.

Locomotor performance along with the other factors mentioned above such as sex determination and size have been the focus of numerous studies, but there is a paucity of research on other aspects of physiology that could also potentially impact hatchling survivorship. One such factor is the effect of incubation temperature on cognitive ability in sea turtle hatchlings. Cognition can be most simply defined as the processing of information; this requires that sensory information be attained, stored, and then retrieved for use in a behavioral decision (Rowe & Healy, 2014). The tasks of learning and decision making are thus mechanisms of cognition and are frequently investigated in cognitive studies (Shettleworth, 2010). Learning itself requires an organism to attain neuronal representations of novel information (Dukas, 2009).; this information is then stored and can be used in decision making or represented in behavior. Learning can thus

be examined by the quantification of neuronal activity, though this is not generally feasible for the animal behavioralist. Instead, this leaves scientists with the task of exploring learning through its effects on behavior, presenting challenges in experimental design and interpretation of findings. Investigating the consequences of learning on survival and fitness is one aspect of the study of cognition. Though not free of pitfalls, learning is of significance to our understanding of species given its important role in both ecological and evolutionary processes; learning can, for instance, allow for an animal to exploit its environment effectively in the face of changes (Dukas, 2009). This has been demonstrated in wild great tits where birds with superior cognitive ability, deemed problem-solvers, produced larger clutches than non-problem-solvers (Cole et al., 2012). The authors postulate that this was due to the solvers being able to better exploit their surroundings since foraging time was short and foraging area was also relatively small; this study thus exemplifies the possibility of a correlation between cognitive ability and fitness. A study on Australian magpies reached a similar conclusion in that birds who displayed increased cognitive performance were more likely to produce more hatched clutches and fledglings per year (Ashton et al., 2018). This study also showed a positive correlation between number of hatched clutches and foraging efficiency. Interestingly, it was recently discovered that bird species who tend to be more innovative, i.e. when problem solving they devise unconventional behaviors and solutions (Arbilly & Laland, 2017), are associated with a reduced risk of extinction in the face of habitat loss (Ducatez et al., 2020). The results of this study align with the cognitive buffer theory (CBT) whereby behavioral plasticity has the potential to increase survival rates and fitness. Innovation can be tied back to cognition in that it is used as an operational, reliable and

ecologically relevant measure of cognition (Lefebvre et al., 2004). If cognition can indeed influence traits linked directly to fitness, then studying cognition could inform scientists of the possible outcomes that variation in cognitive ability could have on organisms throughout their lifetime and what this may mean for the population at large.

A portion of the variation found in cognitive ability can be attributed to the differing environments organisms inhabit and the stressors they present (Odling-smee et al., 2008). There is a growing body of work across many taxa which elucidates the relationship between varying habitats, the abiotic and biotic factors they encompass and an organism's cognitive ability. In the threespine stickleback, fish who lived in either limnetic or benthic selective environments differed significantly in performance of a T-maze spatial learning task (Odling-smee et al., 2008). For zebra finches fed environmentally relevant sublethal levels of mercury, spatial cognitive performance was negatively impacted with the potential for an effect on fitness (Swaddle et al., 2017). Among insects, honeybees exposed to diesel exhaust exhibited decreased learning ability and even induced stress in the central nervous system (Reitmayer et al., 2019). Human cognition follows a similar trend as those discussed above in that cognitive performance can be impacted by a variety of environmental conditions such as temperature and hypoxia (Taylor et al., 2015) A prime example is found in groups of highlanders exposed to extreme cold temperatures for anywhere from 1-5 years; highlanders, compared to those living in plains regions, exhibit decreased cognitive ability alongside retarded color discrimination and reduced visual memory capacity (Gao et al., 2015).

These examples shed light on just a few ways in which the environment can impact cognitive function in both juvenile and adult organisms, but embryos are not

spared from the effects of their developmental surroundings either. The developmental period is actually quite sensitive to an array of environmental factors ranging from temperature to even sound (Amiel et al., 2014; Dayananda & Webb, 2017; Katsis et al., 2018). With respect to the latter, embryonic exposure to sound has been shown to affect song learning ability in the aforementioned member of the songbird group, the zebra finch (Katsis et al., 2018). Climatic effects are also of notable significance to developing embryos and are a popular area of ongoing research due to present-day climatic warming and future temperature predictions; an array of studies thus focus on the effects higher temperatures can have on cognition with both deleterious and beneficial consequences of these developmental conditions reported (Dayananda & Webb, 2017; Siviter et al., 2017a; Pouca et al., 2019; Amiel & Shine, 2012).

In cases where hot incubated individuals outperform their cooler counterparts, the outcome is believed to possibly allow organisms to compensate for other deleterious effects generated by climate change (Dayananda & Webb, 2017; Amiel & Shine, 2012). In sea turtles, the navigation of a novel environment by naïve hatchlings could arguably be one of the most pivotal life history events undertaken and cognition may aid with responding to the intricacies of life post-nest. If this is indeed the case, then one would predict that turtles with more adept cognitive function would also be more likely to survive; this could potentially extend far beyond the hatchling life stage if cognitive ability has the potential to influence factors such as foraging ability and reproductive success (Cole et al., 2012). The frenzy associated with synchronous hatching followed by safely traversing the beach to the surf is laden with obstacles hatchlings must overcome. These obstacles may include a variety of predators, uneven terrain, and/or artificial

sources of light presenting themselves within a short stretch of sand (Peterson et al, 2013; Triessnig et al., 2012; Kristen et al., 2006). If innate behaviors leave room for the influence of learning, then individuals have the ability to equip themselves with an extensive behavioral arsenal which can allow them to respond to a vast array of environmental intricacies once they reach the oceanic stage of their life history (Dukas, 2008; Though this ability to respond to novel information may then be related to fitness, careful consideration must be taken in interpretation of findings due to the inherent issues of studying learning through the lens of behavior; this is especially true when considering the cognition of such young individuals who may be susceptible to great change in cognitive ability throughout their early years.

Incubation temperatures have already been shown to affect cognitive function and personality in other reptiles such as bearded dragons and skinks, suggesting that the same may be true in sea turtles (Siviter et al., 2017a; Amiel & Shine, 2012). Though reptilian cognition is still an emerging field, recently there have been an influx of studies documenting the capacity of reptilian learning and its ability to be assessed similarly to other taxa. One such study in three-lined skinks, *Bassiana duperreyi*, reported that lizards were trained to a Y-maze and that learning ability differed among the two groups assessed (Amiel et al., 2013). Personality in the oviparous bearded dragon has been studied in terms of boldness as it has been linked to factors affecting fitness and can be placed under the broad umbrella of cognition (Siviter et al., 2017). Responding to novel objects requires decision making and it is this aspect the authors used to link cognition to the personality trait of boldness when presenting novel objects to bearded dragons (Greggor et al., 2015; Siviter et al., 2017). Lizards incubated under warmer temperatures

were initially bolder than those incubated at cooler temperatures though this effect did not continue into the long-term. If this same phenomenon is present in sea turtles, then there is a possibility that the climatic trend of warmer temperatures associated with global climate change could not only produce hatchlings with impaired locomotor ability, reduced size, and female bias, but also suboptimal cognitive ability (Fisher et al., 2014; Booth, 2017; Maulany et al., 2012). Sea turtles incubated at higher incubation temperatures have already been shown to have decreased levels of corticosterone, a glucocorticoid essential to responding to stress, which was also correlated with decreased terrestrial performance (Henaghan, 2018). As corticosterone is an integral hormone in the hypothalamus-pituitary-interrenal axis, this finding suggests that incubation temperatures may affect other aspects of brain function in sea turtle hatchlings. In line with previous work conducted on the squamata, there is the potential for impaired cognitive ability to be a driving force in the decrease seen in hatchling success and survivorship at sub-lethally warm incubation temperatures.

With respect to cognition, it is interesting to recall that warmer temperatures tend to produce smaller sized hatchlings (Wood et al., 2014). One could thus postulate that if body size is phenotypically plastic in regard to temperature during development, then brain size could also be a plastic trait under the influence of incubation temperature. This has been shown in the forebrain of a skink species which under different incubation temperature regimes showed not only differing cognitive ability, but also a range of telencephalon sizes (Amiel & Shine, 2012; Amiel et al., 2013; Clark et al. 2014; Amiel et al., 2017). This finding, when paired with the findings that the medial cortex is similar in learning function to that of the hippocampus in mammals (Lopez et al., 2003), could

suggest that this impairment of cognitive ability stems from changes in the size of the medial cortex. Interestingly, reptiles and amphibians with larger relative brain sizes are also more successful invaders after translocation which would imply that a larger brain, or a portion of it, may be beneficial in dealing with the novel challenges associated with a new environment and contribute to fitness (Amiel et al., 2012; Lefebvre & Sol, 2008). Brain size has already been shown to have significant effects on certain aspects of cognition for a guppy species (Buechel et al., 2018). Guppies of the same species with contrasting brain sizes performed differently in reversal-training tests in a study conducted by Buechel and colleagues; larger-brained guppies displayed more cognitive flexibility than their smaller-brained counterparts. Cognitive flexibility is synonymous to behavioral flexibility with the potential for reversal-learning to be implemented as an effective measure of this flexibility; this is particularly useful in depicting the capacity of an organism to problem-solve (Lea et al., 2020). The ability to unlearn a previously learned association in order to switch responses may be linked with the ability to forage opportunistically and to more effectively deal with a changing or novel environment (Bridgeman & Tattersall, 2019; Lea et al., 2020). Among reptiles, behavioral flexibility has been depicted in various training tasks; Day and colleagues showed that lizards who were active foragers performed better in a reversal-learning task than congenics who were sit and wait predators (Day et al., 1999). In the testudines specifically, tortoises have not only demonstrated the capacity to learn and perform a reversal, but they are capable of serial reversal learning (Bridgeman & Tattersall, 2019). Though animal cognition and brain anatomy are slowly becoming better understood within the scientific community,

cognition in sea turtles has been little studied and the effect of incubation temperatures on sea turtle cognitive ability and flexibility is still unknown

OBJECTIVES AND HYPOTHESES

The objective of this study was to explore whether the cognitive ability of sea turtle post-hatchlings is affected by the temperature of the nest during incubation. Incubation temperatures near and(or) exceeding the thermal tolerance limit of sea turtle species have been shown to have dramatic negative effects on sea turtle hatching success, performance, incubation duration, size, and sex ratios (Maulany et al., 2012; Matsuzawa et al., 2002; Booth, 2017; Janzen & Paukstis, 1991; Henaghan, 2018). Though these effects encompass issues, both sublethal and lethal, hatchlings will face with increasing frequency due to climate change, cognitive ability has not yet been assessed in any sea turtle species at the hatchling life stage. Thus, this project addresses a research gap which may become increasingly crucial to comprehend given future climatic predictions and the role of cognitive ability in fitness; the findings from this study shed light on another possible sublethal effect sea turtles may have to endure from an ever-warming environment.

Objectives

- 1. To determine if temperatures considered sublethal/lethal to developing embryos will negatively affect cognitive processes of loggerhead post-hatchlings.**

Hypothesis 1: Post-hatchlings incubated at temperatures near lethal will exhibit suboptimal cognitive ability and thus will take significantly longer to train in the initial round of y-maze training, as depicted in their learning curves, and learn the reverse training significantly slower than turtles incubated at cooler temperatures

MATERIALS AND METHODS

This study featured field egg collections, hatchling husbandry at FAU Marine Lab, and training of post-hatchlings at the FAU Marine Lab. All work was approved by the FAU Institutional Animal Care and Use Committee and was performed under Florida Sea Turtle Permit #19-053A.

Lab Incubation

To test cognitive ability, naïve loggerhead, *Caretta caretta*, post-hatchlings incubated at two incubation temperatures were subjected to a cognitive test which consisted of Y-maze target training and reverse training. Hatchlings were acquired by obtaining recently laid eggs from nesting mothers on beaches in Boca Raton. Nests were selected opportunistically with the help of Gumbo Limbo Nature Center's marine turtle specialists; only nests which were easily accessible by foot were chosen for this study. The morning after a clutch was deposited, within 10 hours of being laid, 4 eggs were carefully removed from each nest, placed in a sanitizable cooler with beach sand and then immediately transported back to Florida Atlantic University where the incubation chamber was located. To ensure that embryos remained intact, care was used to avoid greatly disturbing eggs while removing them from the nest and placing them in coolers for transportation. Eggs were lightly marked with a pencil to keep track of which clutch each egg was from during incubation and allowed us to distinguish hatchlings from different clutches when pipping began. Turtles could thus correctly be given a unique ID

based not only upon incubator, but on the specific nest as well. Upon arrival to FAU's incubation chamber, eggs were carefully dusted off, removing as much sand as possible in order to avoid contamination of sterilized sand (Lolavar & Wyneken, 2017).

Two separate incubation chambers are located at FAU and 32 eggs total were incubated per chamber, resulting in 64 eggs incubated throughout the duration of this study. Total egg numbers were chosen in the hopes of at least 21 hatchlings per temperature group reaching 4 weeks of post-hatchling age for training. Only four eggs total came from any specific clutch, two destined for each incubation chamber, as an effort to mitigate the effects of maternal bias. This partitioning of eggs was intended to allow us to better evaluate if differences in training times among the incubation groups could possibly be due to maternal effects instead of temperature alone. Though this was taken as a precautionary effort, there was still a small chance of separate clutches belonging to the same nesting female given that all eggs were collected in two rounds approximately two weeks apart. In each collection period 32 eggs were collected and divided between the two incubation chambers with 16 incubated at 31 °C and 16 incubated at 33 °C. By running two rounds of incubation spaced well enough apart, a safeguard was set so that incubation proceeded in a staggered manner and emergencies did not overlap or happen very close to one another. In this way, the first group of hatchlings emerged with ample time to mature to training age and become fully trained prior to the second group reaching training age. This was crucial for training as it has previously been shown that the training of post-hatchlings is time consuming, requiring many trials and consecutive training days (Young et al., 2012). With hatchlings only

being kept a total of 6 weeks, it was thus not feasible to train and reverse train all 64 hatchlings at approximately the same age at one time.

Following acquisition and transportation to FAU, eggs were placed in the incubation chamber in Styrofoam boxes which act as incubators (nest boxes). The boxes were sterilized with a Chlorhexidine solution and then filled with sterilized, autoclaved sand from a local beach before eggs were placed gently in the middle and covered in sand (Lolavar & Wyneken, 2017). Eight eggs were placed per nest box on approximately two inches of sand; prior to eggs being placed in the box, the sand was moistened with DI water in an attempt to mimic natural beach conditions. The chamber itself was set to one of two temperatures chosen for this study with nest boxes placed in the chamber equidistant from the heat source (light bulbs). This placement allowed for heat to be distributed uniformly to all eggs no matter the random box they were placed in. The two temperature regimes were established to mimic an optimal temperature and a very hot sublethal temperature as inferred from the thermal tolerance range for the species in Boca Raton. These temperatures were 31 °C and 33 °C respectively which represent temperatures commonly recorded in Boca Raton and are thus biologically relevant to the study. Temperature within the chamber was controlled using an Omega iSeries Temperature & Process Controller Model CNi323 and incandescent light bulbs placed in the chamber that acted as a heat source. Nest temperature was monitored using calibrated HOBO U22-001 temperature data loggers (accuracy \pm 0.2°C, resolution 0.02°C; Onset Computer Corp., Bourne, MA USA) to ensure that the temperature was constant throughout the incubation period and did not fluctuate significantly from the desired set point; temperature was recorded every 15 minutes. Moisture was monitored using HOBO

Micro Station data logger (H21-002) equipped with soil moisture sensors (Model S-SMC-M005; soil-specific accuracy $\pm 0.020 \text{ m}^3\text{m}^{-3}$, resolution $0.0007 \text{ m}^3\text{m}^{-3}$; Onset Computer Corp.) A humidifier was also placed at the back of the incubation chamber with a small fan directly behind it. The humidifier and fan were allowed to run 24/7 so that humidity levels were high ($>90\%$) and well distributed in the chamber and in turn, in the nest boxes. The sand in the nest boxes was also misted twice a day with DI water to maintain the high humidity levels needed by developing embryos.

Though these were the temperatures which we hoped to achieve for the sand in the nest boxes, the incubator was set to a constant temperature approximately 1.5 to 2 degrees warmer than the desired sand temperature. It has previously been observed that the sand in the Styrofoam nest boxes when placed in the chamber is significantly cooler than the air temperature in the incubation chamber (Tezak et al., 2018). This issue can be mitigated by increasing the chamber temperature by approximately 1.5 degrees and checking sand temperatures often. Though the temperature was initially set at 32.5°C and 34.5°C , the temperature in the incubator was not consistently at these temperatures during the experiment due to fluctuations in sand temperature found inside the incubator. When the sand temperature was found to significantly stray from the desired temperatures, modifications were promptly made to the incubator temperature; these minor alterations were still within the range of $1\text{-}2^\circ\text{C}$ higher than the desired sand temperature.

Prior to pipping, eggs from the same clutch and incubator were carefully moved into separate nest boxes so that only hatchlings from the same clutch would be found in any given nest box. When eggs began to pip, we no longer watered the nest boxes and

allowed hatchlings to fully emerge from the egg. If hatchlings were from the hotter incubator they were moved to the cooler incubator to prevent dehydration.

Approximately 3 days after hatching when the carapace straightened, hatchlings were removed from their box and were transported to Gumbo Limbo Nature Center. A unique ID which indicated both clutch and temperature treatment was prescribed to each turtle and depicted on the carapace using nail polish. Morphological measurements (carapace length, carapace width and carapace depth in mm) and mass (in g) were also taken on the turtles first day in the marine lab and every week thereafter. In addition to this, the presence or absence of a scute anomaly was also recorded upon intake. In cases where an anomaly was present, the scute(s) which resulted in the non-modal pattern were recorded and photographed. Hatchlings kept at the FAU marine lab were placed in a large tank with flowing sea water; small floating baskets, approximately 15 x 15 cm, with the turtle's unique ID were used to individually house turtles in the tanks. All turtles were cared for and fed strictly following the guidelines at the FAU Marine Lab for loggerhead hatchlings. Turtles began to be fed shrimp on their third day in the lab. Shrimp is fed in small bite-size pieces using toothpicks as follows: two pieces for the first two days of shrimp, four pieces on days three and four, 6 pieces on days five and six, and 8 pieces on day seven. After the seventh day of shrimp, 12 bite-size cubes of food are given daily to turtles for their first week of cubed food; the cubes are made in the marine lab using deboned fish, Mazuri™ Freshwater Turtle Pellets, calcium and mineral powder, freshwater, and gelatin. This diet was fed once daily in the morning and the amounts of food for subsequent weeks were calculated by taking the average weight of all turtles and feeding 9% of the average body weight in cubes.

Records of daily feeding were recorded along with weekly size and weight measurements for each turtle in this study. The weekly morphological and mass measurements were used to analyze the effect of incubation temperature on post-hatchling growth rates; for each turtle, the carapace length of the previous week “n-1” was subtracted from the carapace length of the desired week, “n”, and divided by 7 days to obtain the daily growth rate for week “n”. (NOT sure if using “” is what I need to do here.

Training

Turtles were trained at approximately 4 weeks post hatch since it has been previously shown by Young et al. (2012) that hatchlings are not food motivated to train prior to this age. In this project, we too found that hatchlings fed more consistently and exhibited higher levels of food motivation when approaching 4-5 weeks of age. Training post-hatchlings, though difficult, has been achieved in previous studies with the methods being quite time consuming and only a fraction of animals becoming trained. In order to train our post-hatchlings successfully, food was used as a positive motivator so that the food reward was associated with a specific cue. Hatchlings in captivity respond strongly to food and our goal was to shape the response to a visual cue and then reshape the response to the opposite visual cue using bite-sized portions of their daily diet. The specific visual cues used at the ends of the Y maze arms were a vertical black and white striped pattern and a black and white bullseye pattern (Fig. 1). These patterns were not presented to post-hatchlings prior to training and thus no turtles should have held a bias either for or against them.

Post-hatchlings were initially food deprived for 24 hours prior to the onset of training to ensure proper food motivation; 24 hours has been shown to be an appropriate length of time to deprive young sea turtles of food in behavioral studies (Kedzuz & Salmon, 2016). In order to maintain this motivation throughout multiple days of training, post-hatchlings were fed with half their daily feeding amounts after training if they were to train the following day; if training would not occur the next day, the normal daily feeding amount was given. Once a turtle completed either training phase, it was fed according to the FAU Marine Lab guidelines once more.

The training itself took place in a black plexiglass y-maze featuring a holding area with a clear plexiglass divider (Fig. 2). The ends of the y-maze arms were also constructed of clear plexiglass allowing the patterns to be adhered to the outside of the clear arm end and seen from inside the maze; patterns were printed out on white paper taped, using clear tape, to the clear plexiglass ends. The patterns were not permanently secured to the arms permitting us to switch the patterns from arm to arm when necessary; to prevent directional bias, the arm a pattern was on was randomized for each trial. The y-maze was placed inside of a black box which was lined with black mesh to prevent the turtles from viewing the experimenter during trials. This was done so that an association did not form between experimenter and food as opposed to target pattern and food during the trials. A small rectangle was cut in the front of the box and lined with mesh so that the experimenter could view the turtle as it swam down the maze in the opposite direction.

To begin training, each experimenter worked with two turtles at a time and placed them in a bucket of seawater used to house turtles between consecutive trials. All turtles were given 10 minutes of acclimation time in the bucket prior to being placed in the y-

maze for training. Following acclimation, a turtle was placed in the middle of the holding area of the maze facing the clear plexiglass divider. Since the maze was also a novel environment, another period of acclimation prior to the actual training trials was necessary before the first trial could begin; this was comprised of a 20-minute interval where post-hatchlings were placed into the holding area and allowed to familiarize themselves with the maze. To begin an actual trial, the clear plexiglass divider was removed, stopwatch started, and the turtle was allowed to swim in any direction. The pattern a turtle would be trained to, i.e. the pattern it received a food reward at, was chosen by a random number table which split the turtles into two training cohorts. Food rewards were given using a long wooden skewer so that training subjects could not see the experimenter. If the turtle swam to the “correct” pattern at the end of an arm, the stopwatch was stopped and the turtle was rewarded with a bite-sized piece of its daily cubed diet; food rewards were kept small so that turtles were food motivated for many trials per day. Turtles were only given the reward if they swam down the entire length of the arm and made contact with the pattern. For the first twenty trials only, if the turtle swam to the wrong arm of the maze it was assisted by gently placing it at the entrance of the correct arm, allowing to swim to the end and given a food reward; in this scenario, the stopwatch was stopped when the turtle first made contact with the incorrect pattern. The assistance phase of training helped shape the response of the turtle to training and began the association of food to pattern. Due to the variety in training trials a turtle could complete in one day, the 20 trial assistance phase was used to standardize assistance among the training subjects. Following these twenty trials, if the turtle swam down the incorrect arm it did not receive a food reward and it was considered a failed, versus a

successful, trial. Once a trial was completed, i.e. the turtle swam down an arm and either received/did not receive a food reward, the turtle was placed back into its bucket and the second turtle would begin its trial. In the event that a turtle did not swim out of the holding area, a two-minute cap per trial was enforced. Two minutes was the standardized time between trials in order to keep food motivation equal among training subjects. For each trial, the time to complete the trial, success or failure to choose the correct pattern and willingness to eat a food reward if presented one were recorded.

Turtles were considered done training for the day in one of three scenarios: the turtle reached the training criterion, the turtle no longer swam down the maze (floated in the holding area for two minutes), or it became satiated (no longer food motivated). Training turtles in succession not only allowed turtles to have time in between their trials, but allowed us to train multiple turtles per training day. Once one turtle was done training, it would be placed back into its individual basket and another turtle would take its place following the steps outlined above.

Trials were conducted in this manner in the expectation that post-hatchlings would become trained to only associate their training pattern with a food reward and exhibit a learned association to the cue by choosing to swim only in the direction which resulted in the presentation of food. A turtle was considered trained when it chose the correct pattern six out of seven trials in a row which we set as the criterion for learning in this study.; the seven trials were consecutive and thus only trials conducted in a given training day were considered (i.e. three correct trials the day prior would not count towards the 6/7 trials the following day). This process required turtles to be trained daily and a maximum of 80 trials was set as the cap for each training phase. Turtles who had

not yet learned to associate their target training pattern with food by the 80th trial were considered “not trained” and those who achieved training status prior to the 80-trial limit did not have to continue training any longer.

Once this first phase of training was achieved, a second phase of reverse training commenced for turtles that had achieved training status. In this second phase, turtles that succeeded in the first task were reverse trained to the opposite pattern. It is important to note that since turtles who reached the reversal phase had already gone through one training phase successfully, a 20-trial assistance phase was not employed. Once again, turtles were considered trained when they chose to swim to their new target training pattern six out of seven times in a row with the 80-trial limit still being imposed; time per trial, success/failure in choosing the target pattern, and if a turtle ate its reward were all once again recorded for future analysis. After having been trained and possibly reverse trained, post-hatchlings successfully completed training and were released into the Gulf stream by boat.

Statistical Analysis

Statistical analyses were carried out using the “R” program (R A Language and Environment for Statistical Computing, R Core Team 2012). Data was first tested for normality using a Shapiro Wilk’s test. For numerical data which was normal under the Shapiro Wilk’s test, a t-test was performed to analyze the effect of the two controlled incubation temperatures on mass and growth rates. Mass (in g) upon intake was compared between groups for initial mass and the average daily growth rates for each week turtles were housed at the marine lab, described previously, were used for the post-hatchling growth rates. For the categorical carapace pattern datum depicting the

absence/presence of a scute anomaly, Fisher's exact test was run to determine if there was an effect of incubation temperature on the modal scute pattern.

Training datum, both initial and reverse, was analyzed with a time-to-event analysis (TTE). This particular statistical method was employed due to its ability to consider both the training event, whether a turtle became trained or not, and the length of time it took to reach the event for analysis; most statistical methods would not be able to factor the length of time into the analysis and thus TTE was most fitting for this study. TTE was also optimal because of its inclusion of censored data which is when a subject does not experience the event of interest during the study time; for this study specifically, censored data refers to turtles who never reached the training criterion in the 80-trial limit. The TTE consists of constructing Kaplan Meir curves from the training data, i.e. the number of trials it took each turtle to reach the training criterion, and then performing a log-rank hypothesis test to compare median training time between curves; the log-rank test employs a chi-square test statistic derived from observed and expected differences in training time if there was no difference between groups. Once these analyses were complete, censored data was removed from both the initial and reverse training data and the time to event analyses were rerun.

Apart from this median based method, the initial and reverse training data was also analyzed using the restricted mean survival time (RMST) method in order to analyze the mean training trials among groups as opposed to the median training trials. The benefit to this method is that hazard ratios, and thus their assumptions, are not utilized and instead the area under the training curves up until a set time is assessed.

RESULTS

Overall nest success for *C. Caretta* hatchlings

Sand temperature in the sublethal incubation environment had a mean of 33.15 ± 0.5 °C while sand temperature in the cooler environment was 30.63 ± 0.59 °C. Hatch success for hotter “nests” was 37.5% and 50.0% for the first and second rounds of incubation from the two egg collections respectively. Combined, the sublethal nests produced a hatch success of 43.75% over both rounds. Cooler, more optimal, temperatures in the alternative incubator resulted in a hatch success of 100% and 43.75% for the first and second rounds respectively. Combined, the optimal temperature regime produced a hatch success of 71.88% over both rounds. Both temperatures resulted in hatch successes that were less than the mean hatch success for all loggerhead nests inventoried in Boca Raton for the 2019 nesting season (80%). Though this is the case for all nests inventoried, when looking at only nests in which eggs were collected from for this study, the overall 31°C hatch success for lab incubated eggs was higher than 9 of the 16 nests left *in situ* on Boca Raton.

Incubation Duration

Incubation duration for eggs incubated at 31°C ranged from 49-58 days with a mean of 52 days \pm 3 days. The incubation duration for eggs incubated at 33°C ranged from 43-46 days with a mean of 45 days \pm 1 day. Incubation duration significantly

differed between the two treatment groups with eggs incubated at 33°C hatching significantly faster than those incubated at 31°C ($t(14) = -7.00, p < .001$).

Temperature and Hatchling Morphology and Mass

Incubation temperatures significantly affected hatchling initial mass, the presence of modal/non-modal scute patterns and growth rates. Turtle mass upon hatching and intake at the FAU Marine Lab, for all hatchlings used in this study, ranged from 11.63 g – 19.60 g with cool turtles ranging from 13.87 - 19.6 g and hot turtles ranging from 11.63 – 19.20 g. Turtles incubated at 31°C were significantly heavier upon hatching with a mean mass of 16.83 g \pm 1.82 g while turtles incubated at 33° C had a mean mass of 15.15 g \pm 2.53 g ($t(21.49) = -2.15, p = 0.04$) (Fig.3). Cool turtles were also initially significantly larger in regard to carapace length ($t(21.89) = -2.28, p = 0.03$) (Fig. 4). Initial carapace length in cool turtles ranged from 39.70 mm to 45.40 mm with a mean of 43.05 mm \pm 1.62 mm while carapace length in hot turtles ranged from 36.50 mm to 44.00 mm with a mean of 41.49 mm \pm 2.20 mm. These hot incubated, lighter and smaller hatchlings also displayed significantly more non-modal scute patterns than their cooler incubated counterparts ($p = 0.04$) (Fig.5). Approximately half (42.9% of 14 hatchlings) of the hatchlings from the sublethal temperature featured a non-modal scute pattern typically consisting of one additional vertebral scute while only 9.5% of 21 hatchlings incubated under the more optimal temperature regime displayed a non-modal pattern.

Hatchling growth rates among the two incubation environments, based on straight carapace length measurements, were significantly different from one another after the first week for almost all weeks thereafter (Fig. 6); during this first week after intake, there was no significant difference in hatchling growth rates between the two groups ($t(30) = 2-0.07 p = 0.94$). For cool incubated turtles,

average weekly growth rates during the study period never decreased from the week prior but instead increased or remained the same. On the other hand, hot incubated turtles saw an increase in average weekly growth rates for all weeks except the fifth and final week; this last week saw a marked drop in the growth rate.

Overall, cool turtles grew the most rapidly over their time spent in the lab, in addition to starting larger on average at hatching. The minimum and maximum average growth rates during the study period were both higher in the cool incubated group than in the hot incubated group.

Maze Training (Initial)

For the first round of y maze training, a total of 32 turtles were properly food motivated and exposed to the training regime; of the 32 total turtles, 21 were cold incubated embryos and 11 were hot incubated embryos. From those 21 cool post-hatchlings, 17 (81%) reached the training criterion in ≤ 80 trials while the remaining 4 did not reach the training criterion by the 80-trial limit. Among those who did become trained, the range of trials to criterion was 8 – 80 trials (median=39 trials). All 11 hot incubated post-hatchlings reached the training criterion prior to reaching the 80-trial limit; in this group, the range of trials to criterion was 12 – 63 trials (median= 42 trials). Though the median for the cool group was less than the hot group, the first round of maze training showed no significant difference among the two post-hatchling groups using a time to event analysis (i.e. a log rank test comparing Kaplan Meir survival curves where the “event” is reaching the training criterion) $X^2(1, N=32) = 0.7, p=0.389$ (Fig. 7). If the censored data is removed, i.e. the four turtles who did not become trained, and the log rank test on the new Kaplan Meir curves is conducted, there is still no significant difference between the two groups $X^2(1, N=32) = 0.2, p=0.657$ (Fig. 8). Since there was

no censored data for hot incubated turtles, the median remains the same while the median for the cold group became 35 trials.

Comparing the survival curves based off of the average trials to become trained is an alternative to the median based log rank test of Kaplan Meir survival curves. The restricted mean survival time (RMST), in this case the restricted mean training time, for both the cool and hot incubated post-hatchlings was 39 trials, once again resulting in no significant difference between the two temperature groups (CI -13.966 - 13.308: $p=0.962$).

Maze Training (Reverse)

A total of 13 turtles, 6 cool incubated post-hatchlings and 7 hot incubated post-hatchlings, which had successfully reached the training criterion in the initial round of training continued to the next training phase, reverse training. Only 13 turtles were available for reverse training due to early release of post-hatchlings in accordance with the marine turtle permit. Of the 6 cool post-hatchlings, 5 reached the training criterion prior to the 80-trial limit with a range of 7-17 trials (median=13). Of the 7 hot post-hatchlings, 6 successfully reached the training criterion with a range of 7-64 trials (median=21). Incubation temperature did not significantly affect median training time as depicted in the Kaplan Meir plot of training curves $X^2(1, N=13) = 0.6$, ($p=0.4$)(Fig. 9). One turtle in each group did not reach the training criterion and thus created censored data. Running the log rank test without these two data points resulted in a dramatic decrease of the p value, from 0.4 to 0.06, though this was still not significant $X^2(1, N=11) = 3.4$, $p=0.06$ (Fig. 9).

Using the RMST method to analyze the curves instead, yields an RMST of 23 trials in the cool group and 35 trials in the hot group. Though the hot mean is substantially larger than the cool mean, the conclusion mirrors that of the log rank test: incubation temperature does not significantly affect mean training time in loggerhead post-hatchlings ($p=0.419$)

DISCUSSION

This study was the first to explore the effects of incubation temperature on the cognitive ability of loggerhead post-hatchlings in southeast Florida. To date, this has never been assessed for any sea turtle species from any particular region; these results thus fill an evident knowledge gap for the species as a whole while providing an effective experimental procedure for future work. Lab incubating hatchlings ensured that temperatures were constant and was intended to produce females only; this was part of an effort to avoid confounding results and focus solely on temperature's effect on cognition. The use of y-mazes has been proven effective in training an array of reptiles, including sea turtles, and were employed to target train post-hatchlings who were approximately four weeks old. This novel implementation of a y-maze revealed that in the initial round of training, post-hatchlings from both temperature groups did not perform significantly different from one another when both average and median training trials were assessed. Reverse training on the other hand shed light on a difference in training times with cooler incubated post-hatchlings reverse training in less trials on average than their warmer counterparts. This preliminary study is consistent with the finding that reptilian cognition has the potential to be impacted by temperatures experienced during the developmental period. Though previous work is primarily centered on the squamata, these findings corroborate the hypothesis that climatic change may impact learning ability in oviparous reptile species. Though nests *in situ* experience fluctuating temperatures and an array of

other environmental factors, this study provides a necessary foundational groundwork for studying cognition in sea turtle post-hatchlings in the face of a warming climate.

Hatch Success and Incubation Duration

Hatching success for eggs incubated under a constant temperature of 31°C was 43.75% while hatching success for eggs incubated under a constant temperature of 33°C was 71.88%. These differing percentages are consistent with the trend of declines in both hatching success seen under hotter incubation temperatures (Sim et al., 2015). For all sea turtle species, temperatures nearing/exceeding the thermal maxima during development are associated with declines in hatching success and emergence success (Fisher et al., 2014). This pattern emerges due to development being disrupted and ceasing at temperatures considered extreme for embryonic development (Telemeco et al., 2013). Declines in hatching success have already been shown in southeast Florida as the nesting season progresses and are associated with rising temperatures during this later part of the season (Henaghan, 2018; Lolavar & Wyneken, 2015). A study on lab incubated eggs has also demonstrated the effect of incubation temperature on both hatch and emergence success (Fisher et al., 2014). Fisher and colleagues reported decreases in both hatch and emergence success at both cold and hot temperature extremes. For hot temperatures specifically, hatch success was lowest at 32°C with only 33.3% hatch success observed. In the present study, overall hatch success at 33°C was approximately 10% higher than the 33% seen in Fisher's study; though the reason for such a difference is unknown, it is possible that experimental factors such as major differences in incubation protocol between both studies and location of the populations are responsible. For almost all eggs incubated at 33°C, no signs of development were witnessed upon dissection; this suggests

eggs were either unfertilized or died within the first couple days of being placed in the incubator. Hatch success at 31°C in my study was expected to be higher but is likely caused by changes to the incubation environment during the second round of incubation. During this round, a major hurricane was expected to hit Boca Raton, FL; as a precaution, eggs were watered in excess to offset a potential day when the lab would not be accessible. The precautionary measures may thus have caused the low hatch success experienced by cool-incubated eggs in round 2 though this is purely speculative.

Incubation duration between the 31°C and 33°C incubation groups was significantly different with hot-incubated turtles hatching approximately one week earlier than cold-incubated turtles (means of 45 days vs 52 days respectively). It has long been observed that incubation duration shortens with increasing temperature due to the increase in the embryonic growth rates associated with these hot temperatures. The observed difference between the two groups was thus expected and incubation durations under 50 days have previously been reported for hot nests (Matsuzawa et al., 2002). In their work at a Japanese hatchery, Matsuzawa and colleagues reported a minimum incubation duration of 46 days for four nests with a mean incubation temperature of approximately 31.5°C -32°C. Average sand temperature in our hot incubator was 33.15°C, approximately one degree higher than that reported by Matsuzawa, and the minimum incubation duration was 3 days shorter at 43 days. Comparing this to the work conducted by Lolavar and Wyneken in Boca Raton, Florida, one again sees a minimum incubation duration of 46 days reported though no average incubation temperature is provided (Lolavar and Wyneken, 2015). Comparisons between temperature and incubation duration between these studies may be tempting to form since it would appear

that one degree of change reduces incubation duration by at least one full day. A previous study, also conducted in Florida, demonstrated that an average incubation temperature difference of approximately one degree Celsius could result in incubation durations up to roughly two days apart (Milton et al., 1997); though intriguing, it is important to note that this work was conducted with both field incubated nests and those placed in a hatchery which featured two distinct sand compositions. Careful consideration must thus be given for differences resulting from the procedures of lab incubation, work in hatcheries, and nests left *in situ*. A duration of 46 days is similar to a duration of 43 days but results of this kind which stem from a range of temperatures typically include both temperatures above and below the average of 33°C used in this study. It would thus be interesting to incubate eggs in the lab under fluctuating temperature regimes representative of natural beach conditions; conducting a lab incubation in this manner would still isolate the effect of temperature on aspects of sea turtle development but would do so in a more biologically relevant way.

Hatchling Morphology: Mass, Scute Pattern, and Growth Rates

Upon turtles hatching and straightening their carapace, they were transported to FAU where an initial mass measurement was taken. Turtles incubated at 33°C weighed significantly less on average upon hatching than those incubated at 31°C though the range of weights observed for both groups was similar; these turtles were also significantly smaller with respect to carapace length. It is commonly stated that larger hatchlings are produced at cooler temperatures due to the longer incubation period associated with cooler temperatures (Booth & Astill, 2001; Maulany et al., 2012). The longer an embryo develops within an egg, the more yolk can be converted to hatchling tissue; if

development occurs rapidly, as is the case under hot temperatures, the resulting hatchling will instead have a larger residual yolk sac. Though a variety of studies do indeed report larger hatchlings, it is important to note that larger is commonly referring to size dimensions of the carapace (Maulany et al., 2012; Sim et al., 2015; Glen et al., 2003). Body mass is typically reported to not be affected by temperature with the belief that the larger yolk associated with smaller sized hatchlings compensates for the difference in carapace size; i.e. a turtle with a large carapace body size and smaller residual yolk will weigh relatively the same as a turtle with a smaller carapace size and larger residual yolk (Sim et al., 2015; Ischer et al., 2009). In natural loggerhead nests, Reece and colleagues also found a negative correlation between temperature and carapace length but not mass (Reece et al., 2001). Interestingly, they found the opposite to be true for carapace length in experimental nests at a hatchery and postulated that differing water levels may have been the cause of the large warm hatchlings produced. These results demonstrate that manipulation of nests can produce results atypical of what may naturally occur on a beach. As stated above, we found incubation temperature to significantly affect mass at hatching; this is uncommon and may be a result of a constant high incubation temperature, no temperature fluctuations throughout incubation, or slight differences in sand water content among incubators. Though humidity was kept high, optimal sand moisture was difficult to consistently achieve at 33 C. Measures were taken to improve water content in these nest boxes as it was lower than desired on multiple occasions. Though the moisture levels were similar, the slight differences observed may have impacted hatchling mass and warrant mentioning (Tezak et al., 2020) . Factors not considered in the present study were egg mass and maternal origin though some studies

report an effect of both variables on hatchling size (Booth et al., 2012; Tezak et al., 2020). Future work should consider adding this step upon egg collection as the split clutch design is optimal for comparisons within a clutch.

Another morphological consequence of altering temperature during development was the prevalence of a modal or non-modal scute pattern. Scute patterns were found to be affected by temperature with hot hatchlings displaying significantly more non-modal scute patterns than cool hatchlings. A modal scute pattern can be thought of as the expected pattern of scutes on the carapace of a turtle; in loggerheads specifically, the modal pattern consists of 5 vertebral scutes, 5-6 costals per side, 11-12 marginals per side, 1 nuchal, and 2 supracaudal scutes. A non-modal pattern is a deviation from this modal pattern and could include either additional scutes or the absence of scutes (supernumerary and subnumerary). For hatchlings incubated in sand that was 33°C, the non-modal pattern prevailed with an additional vertebral scute seen in 6 of 13 hot turtles. In the cool incubated group, only 2 of 21 turtles displayed this supernumerary pattern. Non-modal patterns have been observed in other loggerhead hatchlings and flatback and green hatchlings as well (Zimm et al., 2017). Of particular interest in the study of Zimm and colleagues is the finding that as nest temperatures rose, so did the frequency of scute anomalies. It has been hypothesized that epigenetic effects may be the driving force behind the presence of scute anomalies due to reduced levels of DNA cytosine methylation (Caracappa et al., 2016). These findings corroborate the hypothesis that environmental factors have the ability to influence scute patterns but also bring new questions to light. If DNA cytosine methylation is in fact a critical component affecting scute pattern, will abnormalities present in hatchlings be passed on to future generations?

Caracappa and colleagues argue that if this is the case, there is a potential for the adaptive capacity of the species to be affected. These hypotheses warrant further research as a higher incidence of scute anomalies are being seen in turtles on warming beaches (Wyneken, pers.comm). In painted turtles (*Chrysemmys picta*), the frequency of hatchlings displaying a scute anomaly was positively correlated with the time spent above 34.2°C during the incubation period (Telemeco et al., 2013). 34°C is thus thought to be the thermal maximum for the fundamental developmental niche of embryos; once embryos spent more than 60 hours at or above this maximum, significant abnormal scute development was apparent. In respect to the aforementioned question of heritability induced by Caracappa's work is the finding that painted turtles observed nesting with scute deformities did not have a greater risk of producing hatchlings with deformities when compared to normal turtles (Telemeco et al., 2013). This bolsters the argument of environmental change being responsible for inducing scute anomalies/deformities. Considering that the eggs were incubated in the lab under similar conditions, it is my belief that the greater incidence of a non-modal scute pattern is a result of increased temperatures during incubation.

These issues in scute development can also be related back to the low hatching success in hot incubated embryos witnessed in the present work. In a 2013 study, two thirds of loggerhead embryos that experienced mortality while in the egg had a non-modal scute pattern (Sim et al., 2014). The non-modal pattern was likely not the reason for development ceasing but instead signals the susceptibility of these hatchlings to other possible complications and mortality. With approximately half of all hot incubated hatchlings displaying a non-modal scute pattern in this study, a low hatch success at

33°C, a temperature near the thermal maxima, is not surprising. A recent investigation of the non-modal scute pattern across various life stages of loggerhead turtles revealed that turtles with a modal pattern may have a greater chance of survival (Maffucci et al., 2020). Out of all hatchlings examined in that study, 20.5% exhibited a major non-modal scute pattern with the supernumerary pattern of 6 vertebral scutes being the most prevalent; this too was the pattern most common in the present study. The non-modal hatchlings were also significantly lighter than their modal counterparts but no differences in dimensions of the carapace were found. Interestingly, temperature and frequency of the modal pattern were not related, and the authors believe this is because nest temperatures were relatively optimal. The percentage of turtles examined with the non-modal pattern decreased in early juveniles to 12.1% and again to 4.8% in late juveniles/adults. These results led the authors to suggest that non-modal turtles are not only less likely to survive to adulthood, but that selection against these individuals may not be happening as soon as they hit the ocean; if this were the case, one would expect a much lower percentage of early juveniles to have the non-modal pattern. As Sim and colleagues noted with respect to hatchlings, the authors of this 2020 paper also believe that the scute irregularities themselves are not directly to blame for mortality; instead, they argue these anomalies are physical representations of stressors impacting embryonic development. Other effects may be occurring in tandem but are not as easily distinguishable upon visual inspection (i.e. effects on internal organ systems). With the non-modal pattern present in many of the turtles in the present study, one must consider if there are other systems under epigenetic control which are influenced by high incubation temperatures. Further research is critical

in this area as warm temperatures become more common across many beaches, including southeast Florida.

Growth rates among hatchling turtles differed between the cool and hot incubated groups for almost all weeks examined. In the first week spent at the lab, growth rates did not differ between temperature groups but this is likely due to the feeding regimen at the marine lab; turtles are not fed for the first 3 days at the lab and are then fed small amounts of shrimp for 6 days prior to being fed their normal diet. Growth rates increased in both groups during the 5-week period turtles were housed at the marine lab, doubling from week 1 to week 5 in the cool incubated hatchlings and increasing approximately 60% in the hotter group. Differences in hatchling growth rates from natural loggerhead nests along the eastern coast of the United States have previously been reported (Stokes et al., 2006). Nests in that study were not solely from Florida but spanned 8 sites along the east coast to North Carolina. As latitude increased, hatchling size also increased with growth rates being faster in northern populations relative to growth rates found in Florida. Early season growth rates were also faster than late season growth rates, a phenomenon to which one can tie incubation temperatures. The northern nests in the study featured cooler temperatures with longer incubation durations while nests from Florida were warmer with shorter durations (Stokes et al., 2006). Combining seasonal and spatial results, one could postulate that the differences seen in hatchling growth rates may be driven by incubation temperatures; turtles were fed and housed in facilities which standardizes the food available to hatchlings, limits competition, and produces more favorable conditions which eliminates much of the variability present in the open ocean. Kobayashi and colleagues investigated hatchling growth rates experimentally with lab

incubations and found a short-term difference between cool and warm incubated hatchlings (Kobayashi et al., 2018). For the first 3 weeks, cool incubated hatchlings had a faster growth rate than warm hatchlings though this did not extend into the long term. At week 4, cool incubated hatchlings displayed a faster growth rate than warm hatchlings; the authors describe this as a reversal allowing the warm hatchlings to catch up and argue that incubation temperatures are responsible for the differences observed. My findings are consistent with this belief that hot temperatures may cause slow hatchling growth rates though we did not see a reversal in the 5 weeks hatchlings were raised. At week 5, there is actually the largest difference between growth rates indicating that a reversal was not likely to occur in the following weeks; this is further supported by a slight drop in the average growth rate of hot hatchlings in week 5. Kobayashi and colleagues incubated eggs at 27.5°C and 31°C for their cool and warm temperatures respectively. The hot/cold comparisons between the present study and Kobayashi's are thus less direct with the present cooler temperature of 31°C being Kobayashi's "hot" temperature. Keeping this in mind, it would appear that as temperatures increases from cool male producing temperatures to more lethal temperatures, hatchling growth rate decreases. The reasoning behind this is unknown, though these findings suggest that males would display faster hatchling growth rates than females in the short-term. The results thus confer a possible advantage to males but the adaptive significance of this warrants further questioning.

The explanation for why these growth rates differ may not be clear, but there is a potential for growth rates and multiple morphological factors examined to impact hatchling survival. As discussed earlier, cooler incubation temperatures produce not only

larger hatchlings, but hatchlings who, at least in the short term, grow faster. Size can be relevant to survival in the context of gape-limited predators which can only feed upon objects of certain sizes. In sea turtles, hatchling size has been shown significant in this regard with larger hatchlings having the advantage in predator dense waters such as coral reef systems (Gyuris, 2000). Gape limited predation is often used to back a bigger is better theory in which larger hatchlings hold the advantage though there is limited actual information on this for sea turtle hatchlings (Burgess et al., 2006; Booth & Evans, 2011; Stokes et al., 2006). In the common snapping turtle (*Chelydra serpentina*), survival was dependent on size with larger hatchlings having a greater survival probability from nest to river (Janzen, 1993). Beyond the advantage of eluding predators with a narrow gape, larger hatchlings also tend to produce more thrust per stroke (Booth & Evans, 2011). This increase in thrust may then result in hatchling swim speed increasing, allowing a hatchling to cross areas with potential predators faster and thus boosting survival. Bigger does indeed seem better for hatchlings in predator rich waters where a large body size and fast swim speeds may be crucial for a life stage which does not show evidence of active predator avoidance (Gyuris, 1994). With hot incubated hatchling growth rates never reaching or exceeding cool incubated growth rates per week in the first 5 weeks, there was no evidence of a method by which these already small turtles could catch up during this time frame.

Initial Maze Training

This is the first study to assess the effects of incubation temperature on the cognitive ability of loggerhead post-hatchlings. Using a y-maze has been successful in training loggerhead sea turtle hatchlings to colored light and has also been effective in

assessing cognition in various other reptile species (Young et al., 2012; Fritsches, 2012; Bridgeman & Tattersall, 2019; Amiel et al., 2013). Though the use of a y-maze to train loggerhead post-hatchlings is thus not novel, to my knowledge the implementation of such a maze to investigate cognition has not been reported for any sea turtle species. Training an animal once in a bifurcated maze can allow investigators to assess basic cognitive function through mechanisms such as associative learning and concept learning (Czaczkes, 2018; Yilmaz et al., 2017; Giurfa et al., 1999). I used the former learning type in this study since sea turtles are known to be able to distinguish some patterns and colors while also being highly food motivated (Bartol, 1990; Young et al., 2012; pers.obs.). Incubation temperature has already been shown to affect the learning ability of other reptiles with either hot or cold temperatures being advantageous depending on the species (Amiel et al., 2013; Siviter et al., 2017; Dayanada & Webb, 2017). Given the multifarious negative effects of hot incubation temperatures on sea turtle embryonic development and hatchling quality, I hypothesized that hotter incubation temperatures would produce hatchlings with decreased cognitive ability.

In the initial round of training, there was no significant difference in median training time between cold (median=39 trials) and hot incubated hatchlings (median=42 trials). Median training time for 31°C turtles was lower than that of 33°C turtles but only by 3 trials, a number much smaller than the standard deviation in either group. It is important to note that these results are obtained from a survival analysis which allows the user to keep subjects who don't reach a criterion by a set time (in this case the criterion is becoming trained and the set time to reach the criterion is 80 trials). If turtles who failed to reach the training criterion are removed from the assessment (n=4), the training curves

indicate a greater difference between groups with the cool median training time now 7 trials less than the hot median training time (Fig.7). Upon visual inspection of the Kaplan Meir data, the curves for each group seem quite distinct from one another; the difference remains insignificant, though now by a more modest amount. These results indicate that temperature does not significantly affect basic cognitive function in the loggerhead sea turtle. This may be accurate, but the small sample size of the study has the potential to shroud any possible effect of temperature on learning. It is also plausible that the patterns used in training were too complex or visually similar for the loggerhead post-hatchlings to differentiate. Turtles may have seen the patterns as merely black and white lines at the beginning of the maze and arbitrarily chosen a pattern; this is especially true for the first day of training when the process was novel, and turtles were potentially less comfortable with the setup. In order to acclimate turtles to the maze environment prior to just the first day of training, turtles could be fed their daily diet once in the maze prior to beginning training the following week; training subjects may then feel more comfortable with the training environment and show increased levels of motivation, though this is purely speculative. Other issues arose in the maze structure itself which at times seemed to distract post-hatchlings with drain plugs found on the side walls. Turtles would find the plugs while in a training trial, abandon swimming towards a pattern, and continuously bite at the small plug. These events added trials to a turtle's total and may have affected their training ability overall. Future training of post-hatchlings must ensure that the maze used has no potential distractions and if possible, an opaque black material for the inside walls; this lining would prevent turtles from seeing their reflection, another cause of distraction in a subset of turtles trained.

Though these results do not indicate a significant effect of temperature on cognitive ability in this first round of training, it is my belief that they do preliminarily hint at this phenomenon being plausible. A larger sample size could bolster results and corroborate findings in other reptiles like that of Dayananda and Webb in velvet geckos (*Amalosia lesueurii*) (Dayananda & Webb, 2017). The authors incubated velvet gecko eggs and investigated the effect of temperature on the learning ability of 64 hatchlings. Cool hatchlings not only learned to find shelter more quickly, but the fast learners were also associated with higher survival rates up to approximately eight months following release. These findings reveal the possibility that incubation temperatures and subsequent differences in cognitive ability may influence future survival and possibly fitness. In fact, geckos are not the only reptiles which display lasting effects of temperature on learning ability (Siviter et al., 2017a). In 2017, Siviter and colleagues demonstrated that social cognitive ability was influenced by incubation temperature in adult bearded dragons (*Pogona vitticeps*) (Siviter et al., 2017a). As hatchlings, these bearded dragons were used to assess the effect of temperature on boldness and had thus already been experimentally manipulated (Siviter et al., 2017b). The hatchlings were then trained as sexually mature adults of approximately one year of age; analyses revealed that cold incubated hatchlings from the first study who had initially been less bold in the short term, now performed significantly better than hot incubated lizards on a social cognitive task as adults (Siviter et al., 2017b; Siviter et al., 2017a). Beyond confirming that effects on cognitive ability may not be fleeting, this study shows that simply training and testing an individual may not give an adequate representation of the effects of incubation temperature on cognition. Extending beyond reptiles, fish also demonstrate the need for multiple training rounds

(Buechel et al., 2018). In guppies with differing brain sizes (*Poecilia reticulata*), an initial color discrimination task revealed no difference in cognitive ability between groups. Upon reverse training the guppies, a significant difference emerged with larger brained guppies performing significantly better, switching their response faster and making fewer mistakes. These studies depict not only the importance of testing subjects over a long period of time, but assessing multiple levels of cognitive ability.

Reverse Maze Training

One such method for increasing the difficulty of a cognitive task is to test for cognitive flexibility. This aspect of cognition is termed flexible because it requires the participating subject to go beyond a previously learned behavior and modify their response accordingly. This aspect of cognition requires problem solving and thus takes the act of associative learning to a higher, more complex level (Lea et al., 2020). Though there are multiple definitions for behavioral flexibility, we chose to view it in the scope of problem solving and reversals since turtles had already experienced one basic learning task; a reversal would thus elevate the difficulty of the task while allowing the experimental setup of a y-maze and patterns used to remain the same. With limited work available on turtle training at the hatchling stage and the difficulties associated with training turtles on even a simple task, assessing cognitive flexibility through a reversal is logistically feasible. This proved true in that we found reversals to be effective with the majority of turtles reaching the training criterion and doing so much faster than in the original training trials. In this reversal training, turtles were required to switch their previously learned response and were only rewarded at the pattern opposite to what was used in round 1 (i.e. if a turtle trained to a striped pattern in round 1, it would only be

given a reward with the bullseye pattern in round 2). Cold incubated post-hatchlings performed better on the reversal, training faster than warm incubated post-hatchlings, though the difference was not significant. The median training time was 13 trials for cool post-hatchlings and 21 trials for hot post-hatchlings. Upon removing two untrained turtles from the dataset, the new medians became 12 trials and 19.5 trials respectively. The training curves now excluding censored data look starkly different (Fig.9), and the lower p-value at 0.06 is now significant under a significance level of 0.1. Given the very small sample size of the reverse training cohorts, I believe these results are promising and hint at a possible effect of incubation temperature on both cognitive flexibility and complexity in loggerhead sea turtles. Due to low hatching success and early release of some turtles, the sample size did not meet the requirements of the conducted power analysis for either training round. A small sample size is thus worth mentioning as problematic in this study and future work should aim at resolving this issue. Training more turtles during the reversal phase could have allowed for differences between the temperature groups to emerge and be detected in the analyses. Unfortunately, this is a risk associated with incubating embryos at sublethal/lethal temperatures for the entire incubation duration. Moving forward, studies may benefit from keeping temperatures in an optimal range until later stages of development in order to boost hatching success rates. This is especially relevant when working with an endangered species like sea turtles where the collection of more eggs may not be feasible.

As noted above, it is possible for differences in learning ability to become apparent in a reversal as opposed to in simpler, more basic cognitive tasks (Buechel et al., 2018). In the field of reptilian cognition in particular, Leal and Powell echo this belief

with their work on *Anolis evermanni*; a lizard's performance on either a motor task or associative learning did not predict capability of a successful reversal (Leal & Powell, 2012). Differences in the neural components used in simple associative learning versus reversal learning may be the cause for differences in cognitive ability emerging when the two learning types are investigated (Izquierdo et al., 2017; Ghahremani et al., 2020). In humans, it has been shown that the lateral orbitofrontal cortex (OFC) is more active in the reversal learning process when compared to the more simple process of acquisition (initial training) (Ghahremani et al., 2010). This finding is not exclusive to humans in that mice with OFC lesions learn to perform reversals more slowly than mice without lesions in this region of the brain (Izquierdo et al., 2013). Levels of critical neurotransmitters also affect reversal learning with dopamine and serotonin at the forefront of much of the research in this field (Izquierdo et al., 2017). With neural regions and neurochemical differences associated with changes in reversal learning, one may hypothesize that intraspecific differences in cognitive flexibility may arise when these components are altered. In line with this, if the environment is capable of altering levels of neurotransmitters and(or) brain anatomy, one would expect differing levels of cognitive flexibility to arise within species with environmental variability present during embryonic development. Work conducted by Dias and colleagues on leopard geckos (*Eublepharis macularius*), are in line with this hypothesis; the researchers found that incubation temperature affected levels of dopamine in a region of the basal forebrain of the geckos (Dias et al. 2007). In further work conducted by Amiel and colleagues, skinks incubated under hot and cold temperature regimes not only displayed differing levels of cognitive ability, but disparities in facets of the cortical forebrain (Amiel et al., 2017). The authors

postulate that the observed differences in telencephalon size and neuronal densities may be the drivers behind the temperature induced differences in cognitive ability between groups. These studies depict the potential for the environment, namely temperature, to affect critical components of cognition in reptiles. Such effects may also be possible in sea turtle embryonic development where, as shown in the present study, high incubation temperatures induced scute anomalies; it has also been noted that deformities are prevalent when nest temperatures are high in southeast Florida (Wyneken pers. comm.). With temperatures expected to continue their gradual rise, future research in sea turtle cognition would benefit from an investigation of brain size and levels of neurotransmitters in hatchlings from a range of incubation temperatures.

The reversal results hint at a possible effect of temperature on cognitive flexibility and it is thus important to note the consequences of this flexibility. The term itself is at times defined in the context of allowing an organism to better face a changing environment (Audet & Lefebvre, 2017; Izquierdo et al., 2017; Lea et al., 2020). Defined as such, individuals who are cognitively flexible will be better equipped to handle and adapt to changes in their environment. As temperatures rise and bring with them fluctuating environmental conditions, it may be imperative for species to be ready to adequately respond to disturbances in their surroundings; one such challenge may come in the form of food availability. Behavioral flexibility can have implications on foraging strategy and efficiency, a concept demonstrated in the Northern pike (*Esox lucius*). Though it may seem intuitive to believe that behavioral flexibility would boost foraging performance, the opposite was true for this top predator; behavioral flexibility was inversely related to foraging performance (Pintor et al., 2014). Though this relationship

was observed, the predation experiments were run with only stickleback available as prey. Flexibility is likely to increase diet breadth and the study did not account for this in their predation trials. Though speculative, one is inclined to believe that under circumstances where prey availability suddenly becomes scarce, behaviorally flexible individuals would be better suited to their environment. In line with this, in a study of congeneric lizards, the active foraging species performed better on a reversal task than the species employing a sit-and-wait strategy (Day et al., 1999). Research by Claudio and colleagues (2018) supports the findings above in two subspecies of honeybees. Here, reversal learning was once again implemented to assess the cognitive flexibility of two specialist and generalist bee species (Claudio et al., 2018). Caucasian honey bees (*Apis mellifera caucasica*), generalists, performed significantly better on a reversal than the specialist Syrian honey bees (*Apis mellifera syriaca*); no differences were found between subspecies in initial associative learning. Loggerhead sea turtles feed on a wide variety of benthic organisms and are thus considered generalists (Plotkin et al., 1993). Plotkin and colleagues note that the diversity in prey consumed by loggerheads demonstrates their ability to be versatile in their foraging behavior. If loggerhead sea turtles do rely on a generalist strategy, it is conceivable that behavioral flexibility would be beneficial in foraging. To date, this hypothesis remains largely unaddressed, but these preliminary results on reversal learning in loggerhead post-hatchlings bring new questions to light. It would be interesting to assess whether loggerhead turtles of multiple age classes are effective foragers and display high levels of behavioral flexibility beyond the hatchling life stage. Under the belief that flexibility is a plastic trait, incubation temperature's effect on cognition could have potential effects on fitness. In line with this, the act of escaping

predators may also benefit from behavioral flexibility (Evans et al., 2019). A recent review of escape behavior points out that escape is not purely instinctive, but can also rely on stored information and decision-making; organisms thus benefit from displaying flexibility in aspects of threat evaluation and escape action (Evans et al., 2019). The potential consequences of temperature impacting cognitive ability are thus relevant to survival, especially if differences in cognitive ability persist until adulthood or can be passed down to offspring. Siviter and colleagues work discussed earlier on bearded dragons, shows that temperature induced effects on cognition in reptiles can be long-lasting (Siviter et al., 2017). With respect to the hereditary nature of cognition and flexibility, there is evidence to support that both general cognitive ability and cognitive flexibility are heritable in other taxa (Croston et al., 2015; Ferguson et al., 2001). In a recent review of this heritability, examples including zebra finches, humans, and non-human primates report fairly high heritability scores for general cognitive ability (Croston et al., 2015). Supplemental to this is the finding that honey bees demonstrate a heritable component of flexibility (Ferguson et al., 2001). Reptilian cognition on the other hand is still widely understudied, making predictions regarding heritability more complicated. One study in particular does shed light on this issue, revealing little genetic effect on a spatial learning task in delicate skinks (*Lampropholis delicata*) (Reut et al., 2020). Female lizards and their adult offspring were subjected to a y-maze task; the performance of the wild-caught mothers did not predict or affect performance of captive-raised adult offspring. These findings contradict the studies listed above and raise further questions regarding the heritability of cognition in sea turtles. Unfortunately, training an

adult female loggerhead and her hatchlings is logistically extremely difficult, if not impossible, at this time and would also not account for paternal influence.

Relevance: Climate Change

With climatic warming projected to continue in coming years, it is crucial for scientists to assess the impact of hotter temperatures on sea turtle population dynamics and viability. This is notably relevant for hatchlings who are highly impacted by their surrounding environment; temperature alone is known to influence a variety of developmental traits ranging from hatchling size to sex (Booth & Astill, 2001; Janzen & Paukstis, 1991). In recent years, hotter temperatures on nesting beaches have become increasingly common with beaches like Boca Raton, FL displaying hot temperatures throughout the season (Lolavar & Wyneken, 2015). The results from the present study corroborate previous findings of the effect of hot incubation temperatures on sea turtle development and hatchling quality. Beyond this, my results suggest that incubation temperature has the potential to affect cognitive ability and flexibility in loggerhead sea turtles. This study is the first of its kind to assess cognition in hatchlings of any sea turtle species and thus fills a previous knowledge gap.

Little is known on the role cognitive ability plays in the life history of sea turtles though work in other taxa indicate potential effects on foraging, survival and ultimately fitness (Pintor et al., 2014; Ducatez et al., 2020; Ashton et al., 2018). Collectively, temperatures effect on learning ability and brain size, demonstrated in other reptile species, highly favors the hypothesis that sea turtles are likely to be experiencing similar effects while completing embryonic development (Amiel et al., 2017). In recent years, the effects of climate change are being explored to better understand how the future of

sea turtles will be impacted by this unprecedented change (Laloe et al., 2017; Montero et al., 2018). In a study on the effects of climate change on hatchling mortality and emergence success, Laloe and colleagues report challenging future scenarios for loggerheads on the western coast of Africa (Laloe et al., 2017). They predict hatching success and emergence will decrease as temperatures increase in the 21st century. Interestingly, TSD is predicted to initially buffer temperature effects, producing an increase in relative nest numbers; as temperatures exceed the thermal tolerance limits of embryos, mortality increases and a decline in nest numbers is predicted. TSD may thus not be strong enough to mitigate relatively fast warming in short time frames as is expected under climate models. The ability of TSD to alter sex ratios is expected to assuage warming to some extent, but hatchling survival is likely to be impacted prior to imbalanced sex ratios becoming problematic.

Under warming conditions like those described in by Laloe and colleagues, the quality of hatchlings produced thus becomes markedly critical for the species. As hatching success declines and hatchling production drops, is it imperative for hatchlings who do complete embryonic development and emergence to have a high chance of survival. Unfortunately, as this study describes, hot temperatures expected under climatic warming negatively impact an assortment of morphological and ecologically relevant traits in loggerhead hatchlings. The findings from this study which suggest a negative correlation between temperature and behavioral flexibility are not promising for current and future sea turtle populations, notably those in tropical climates. In order to fully assess the effect of warming on the viability of future sea turtle populations, it is necessary for research to focus on multiple effects of temperature on hatchling quality,

survival and ultimately fitness. In the present study, we aimed to investigate traits not commonly studied in sea turtles, learning and behavioral flexibility. Future research efforts will benefit from taking an interdisciplinary approach to conservation-based studies. Doing so can give us a more complete and robust assessment on challenges sea turtles are likely to face under climatic variability. In turn, this has implications for both scientists and management efforts, supporting the formulation of better hypotheses and conservation practices for sea turtles in a warming world.

FIGURES

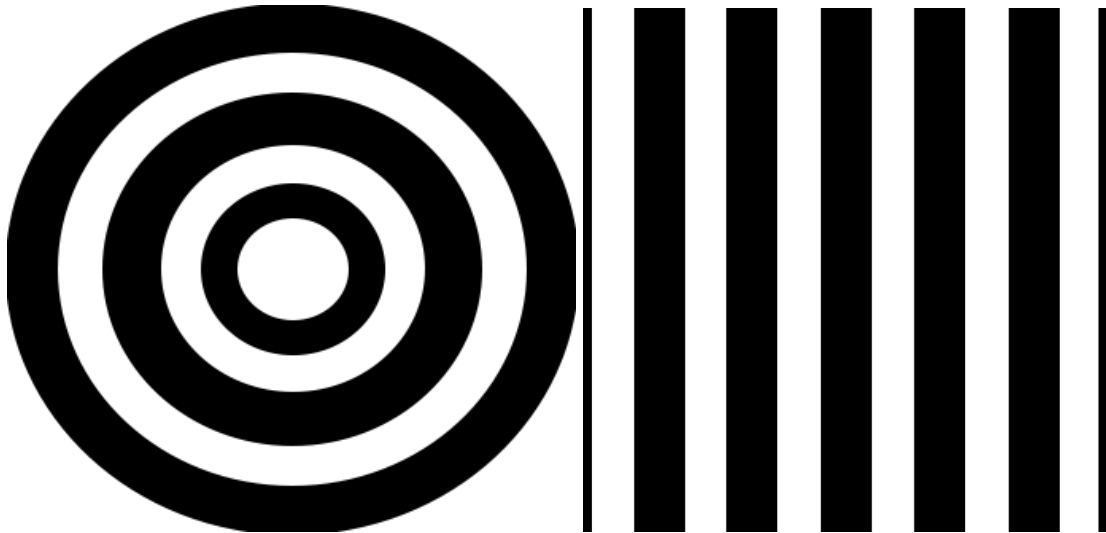


Figure 1. Training patterns placed at the ends of the y-maze.

Bullseye (R) and Stripes (L).

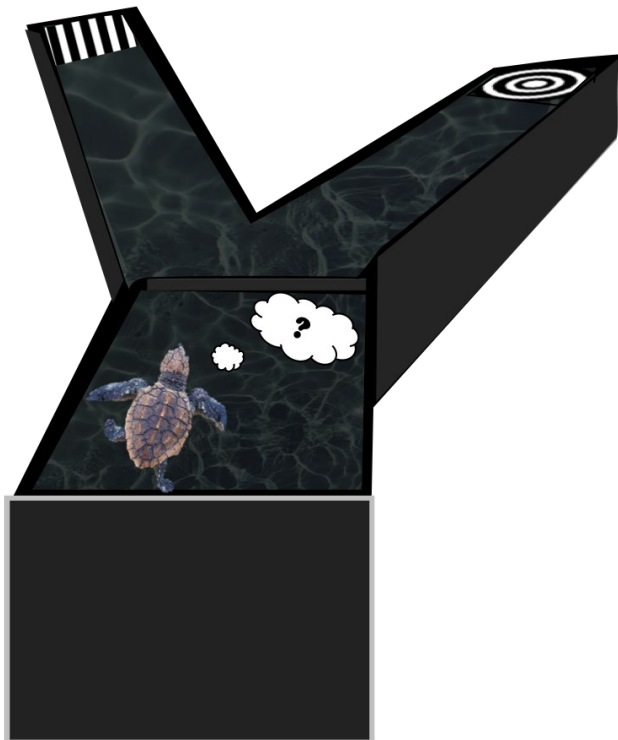


Figure 2. Representation of turtle in Y-maze for a training trial.

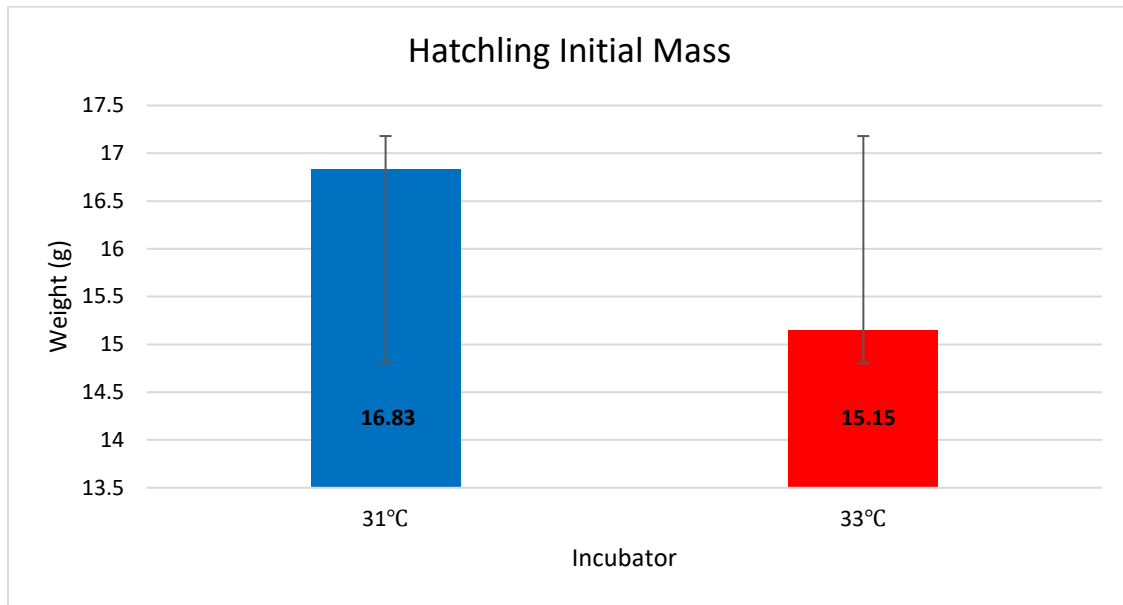


Figure 3. Effect of Incubation Temperature on Hatchling Initial Mass.

Eggs incubated at a constant temperature of 33° C produced significantly lighter hatchlings (n=14) than those incubated at 31° C (n=22) ($t(21.49) = -2.15, p = 0.04$). Solid lines extending above the box depict standard deviation.

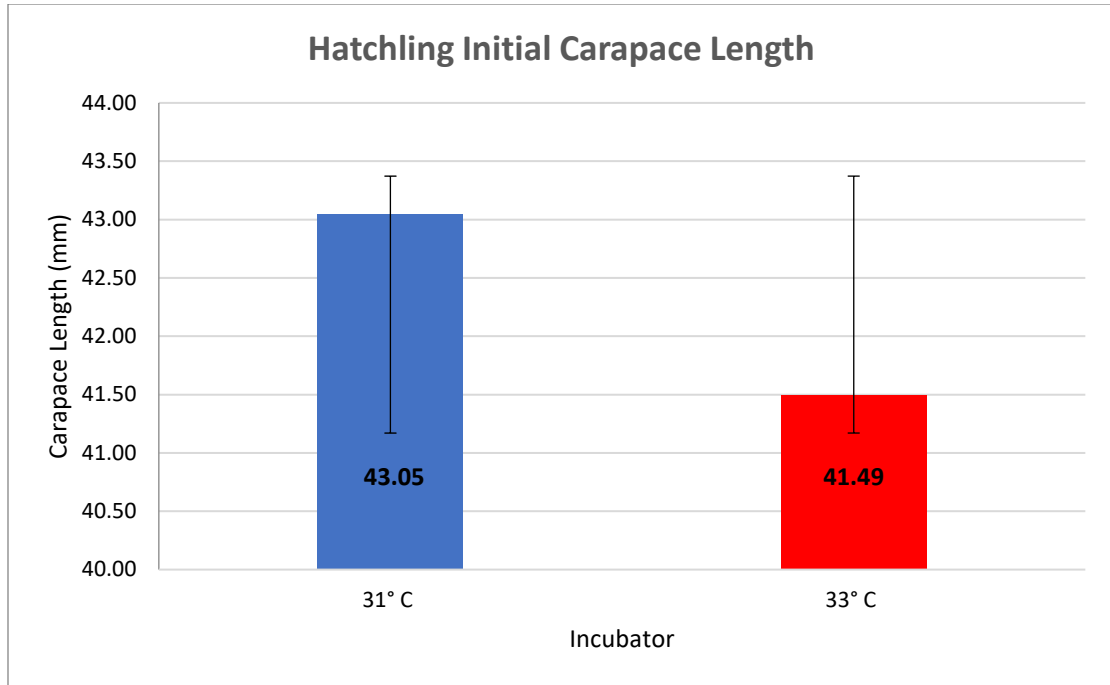


Figure 4. Effect of Incubation Temperature on Hatchling Initial Carapace Length.

Eggs incubated at a constant temperature of 33° C produced significantly smaller hatchlings (n=14) than those incubated at 31° C (n=22) ($t(21.89) = -2.28, p=0.03$). Solid lines extending above the box depict standard deviation.

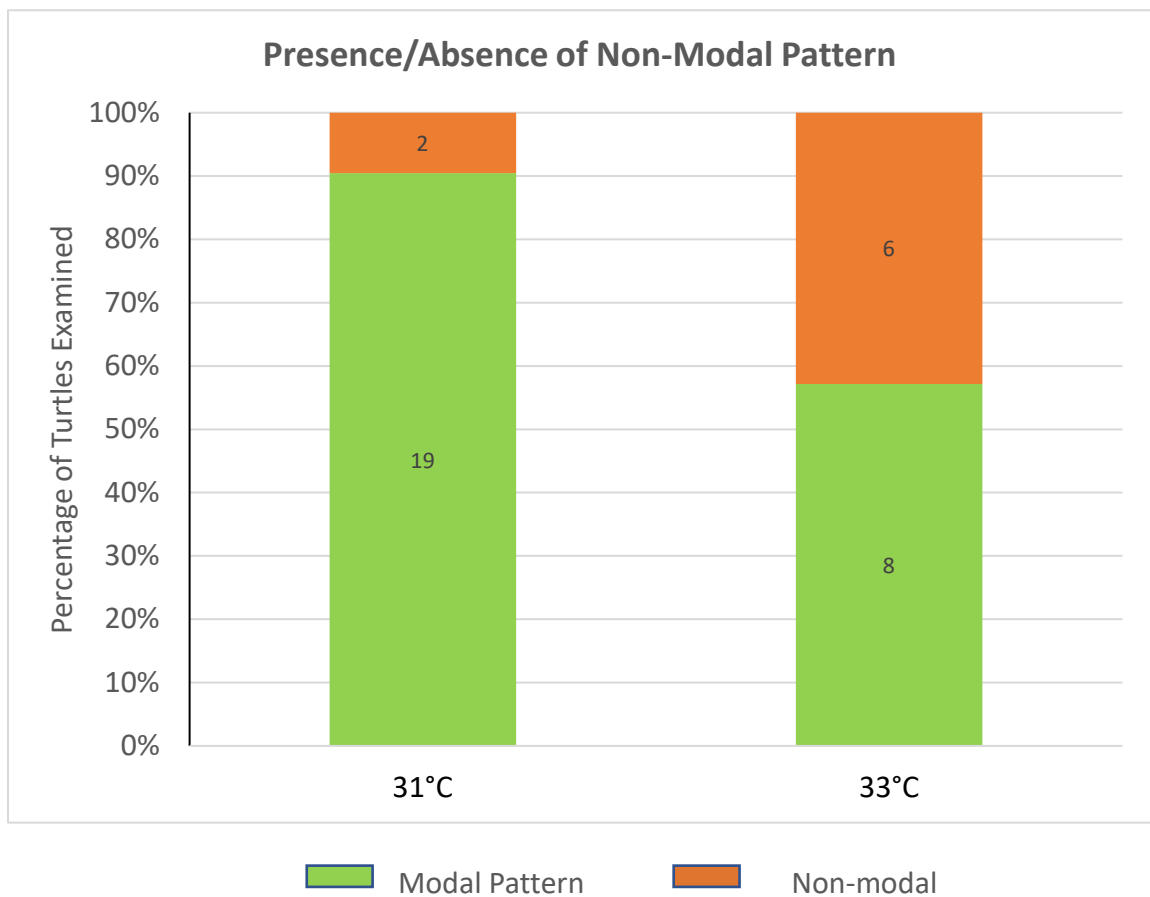


Figure 5. Effect of incubation temperature on the presence of a non-modal scute pattern. Eggs incubated at a constant temperature of 33° C produced significantly more hatchlings with a non-modal scute pattern compared to those incubated at 31° C ($p=0.04$). Numbers located inside the colored bars represent the amount of hatchlings either with or without a scute abnormality in that group.

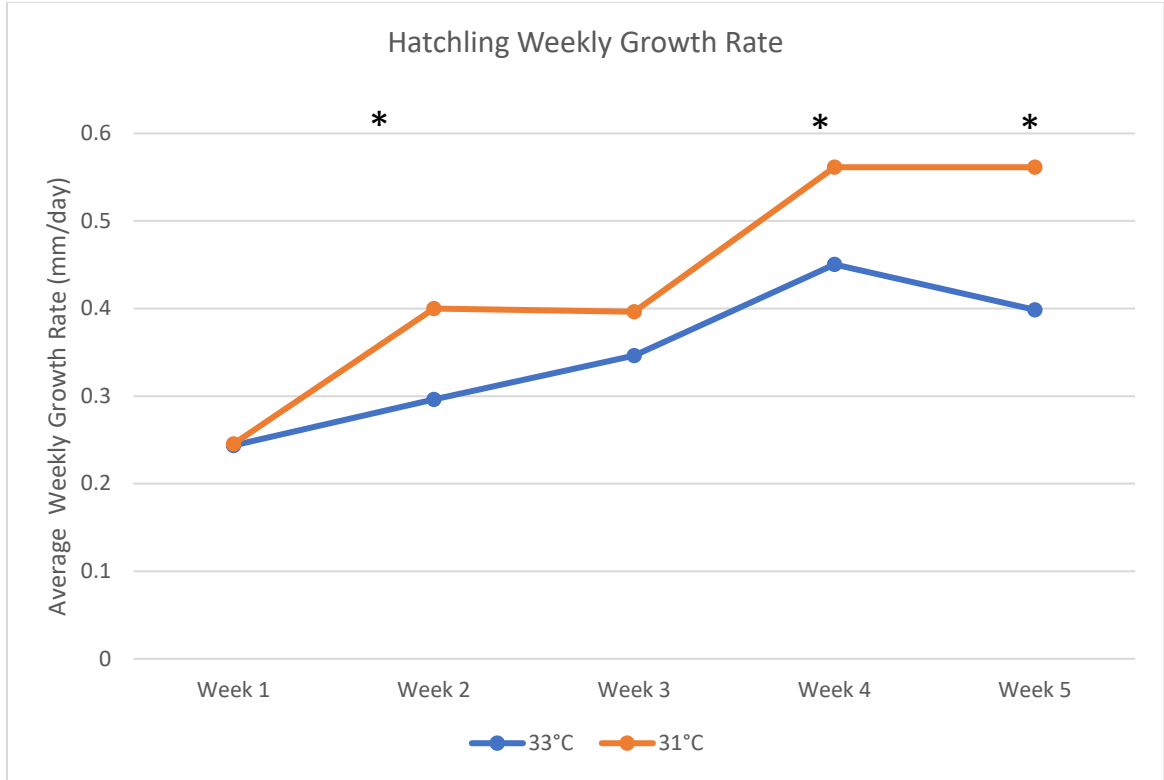


Figure 6. Effect of Incubation Temperature on Hatchling Weekly Growth Rates.

Asterisks denote a significant difference ($p < 0.05$) among the average weekly growth rates between the two incubation environments.

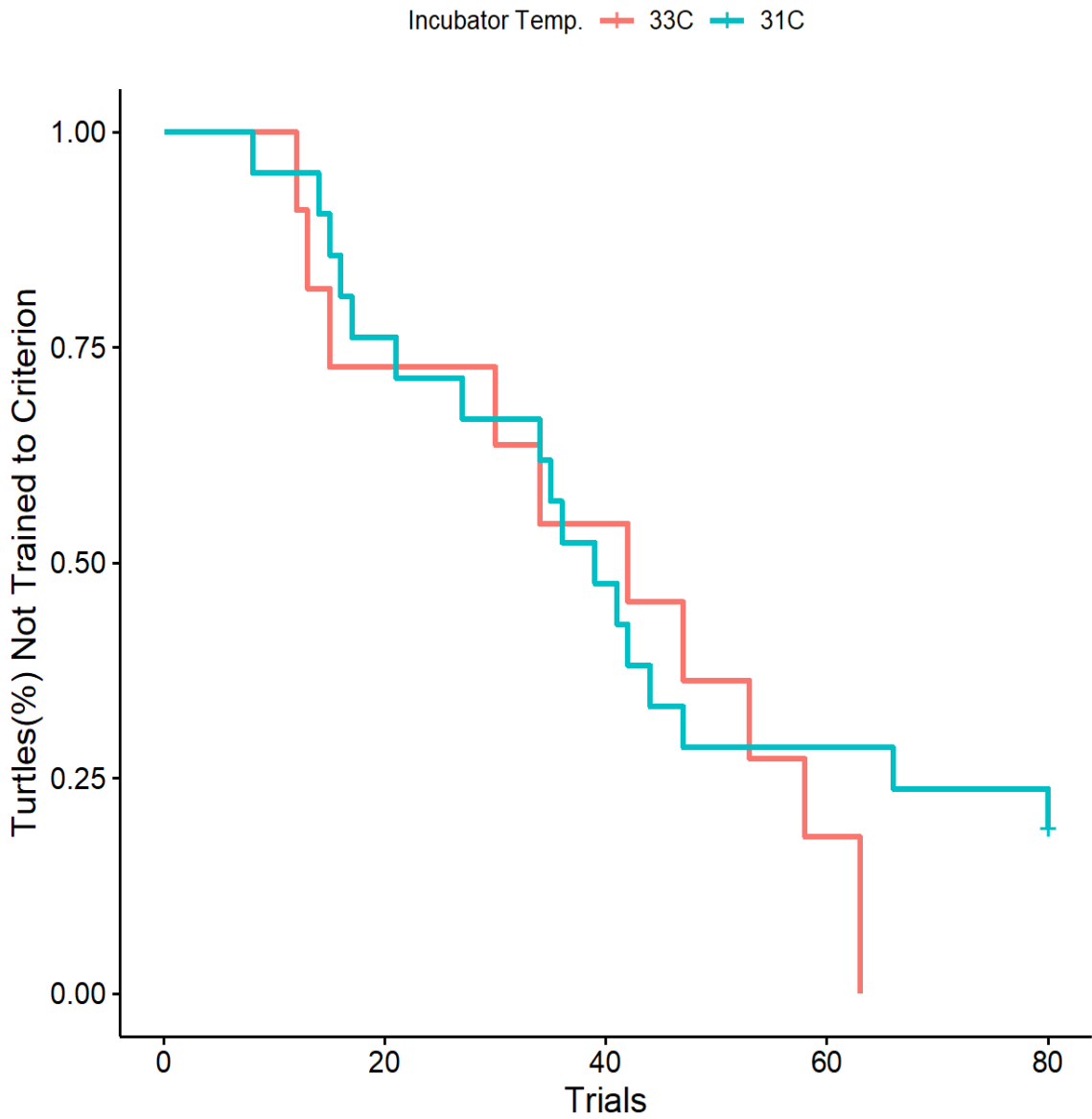


Figure 7. Kaplan Meir plot of training curves for the 33° C and 31° C post-hatchling incubation groups

Incubation temperature did not significantly affect median training time between groups ($p=0.39$). Each step down the plot indicates a turtle has become trained and left the pool of turtles who had not yet reached the training criterion. A horizontal dashed line at the 80th trial denotes censored data.

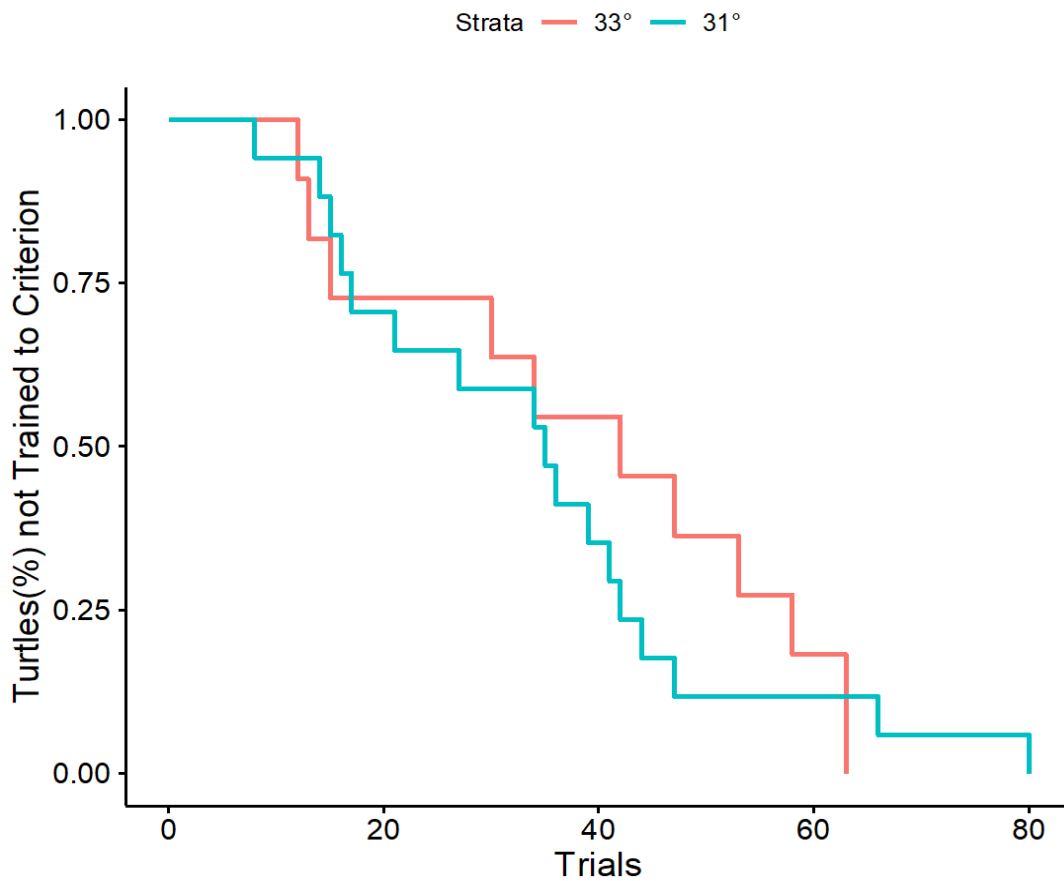


Figure 8. Kaplan Meir plot of training curves for the 33° C and 31° C post-hatchling incubation groups excluding censored data.

Incubation temperature did not significantly affect median training time between groups ($p=0.66$).

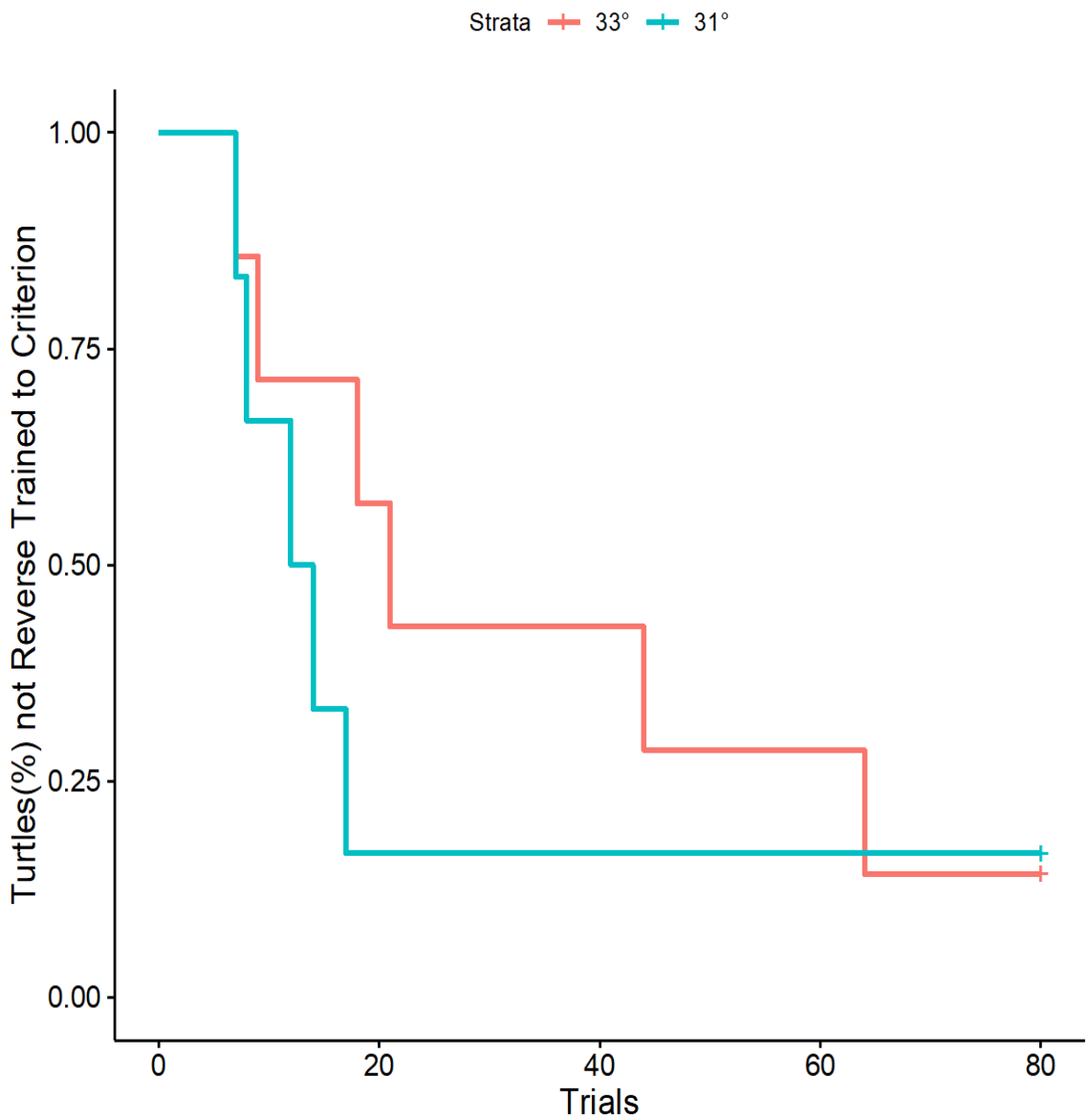


Figure 9. Kaplan Meir plot of reverse training curves for the 33° C and 31° C post-hatchling incubation groups.

Incubation temperature did not significantly affect median training time between groups ($p=0.4$). Each step down the plot indicates a turtle has become trained and left the pool of

turtles who had not yet reached the training criterion. A horizontal dashed line at the 80th trial denotes censored data

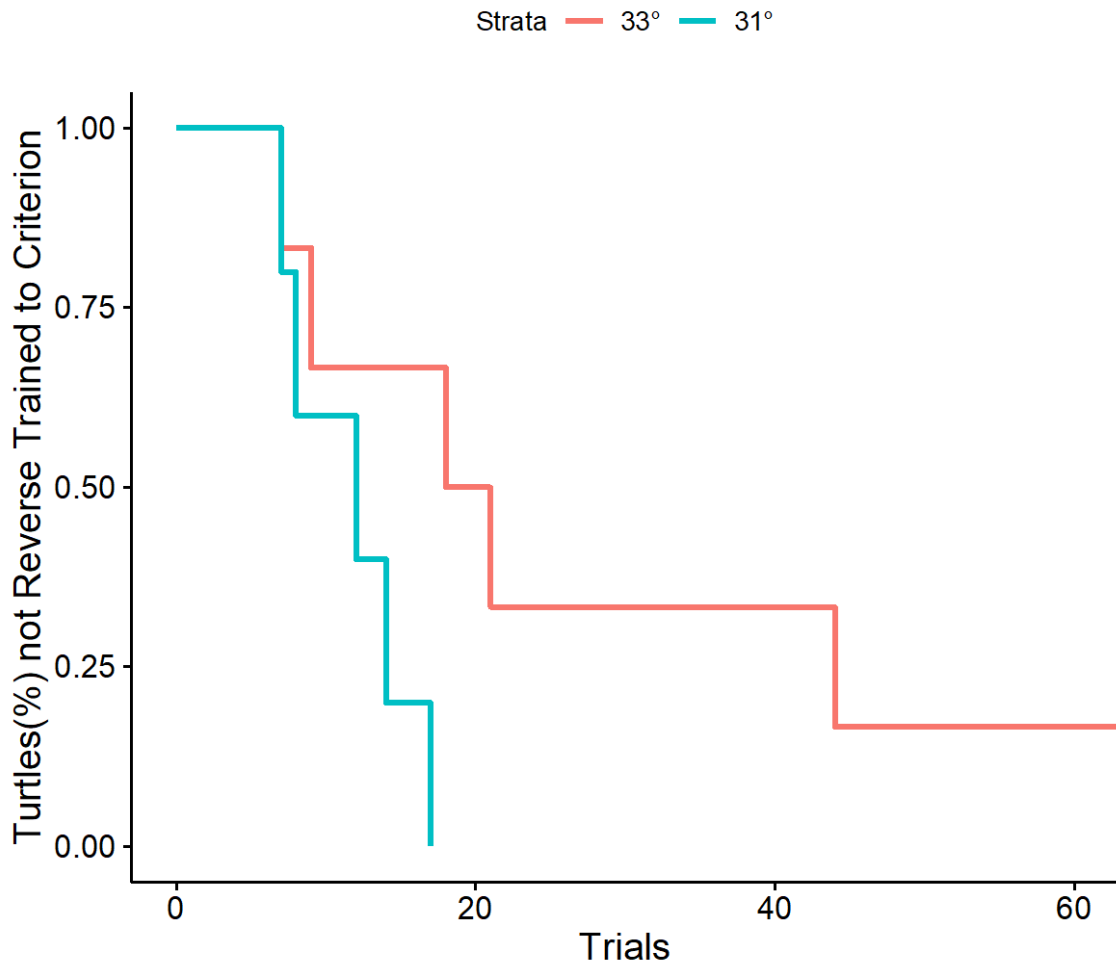


Figure 10. Kaplan Meir plot of reverse training curves for the 33° C and 31° C post-hatchling incubation groups excluding censored data.

Incubation temperature did not significantly affect median training time between groups ($p=0.06$).

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